DAPP1: a dual adaptor for phosphotyrosine and 3-phosphoinositides

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We have identified a novel 280 amino acid protein which contains a putative myristoylation site at its N-terminus followed by an Src homology (SH2) domain and a pleckstrin homology (PH) domain at its C-terminus. It has been termed **d**ual **a**daptor for **p**hosphotyrosine and 3-**p**hosphoinositides (DAPP1). DAPP1 is widely expressed and exhibits high-affinity interactions with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, but not with other phospholipids tested. These observations predict that DAPP1 will interact with both tyrosine phosphorylated proteins and 3-phosphoinositides and may therefore play a role in regulating the location and/or activity of such proteins(s) in response to agonists that elevate PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2.

Key words: DAPP, PI 3-kinase, phosphoinositide, PH domain, SH2 domain

INTRODUCTION

Stimulation of cells with insulin and growth factors activates several signal transduction pathways that regulate many physiological processes [1]. The first steps of these signalling pathways involves the activation of receptor tyrosine kinases, followed by recruitment to the membrane of intracellular polypeptides with key signalling properties [2]. These include members of the phosphoinositide 3-kinase family (PI 3-kinases) which phosphorylate 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 [3]. Proteins that possess a certain type of pleckstrin homology (PH) domain are able to interact specifically with PtdIns(3,4,5)P_3 [4] and its immediate breakdown product, PtdIns(3,4)P_2, which is also thought to be a signalling lipid [3]. Although the molecular basis by which only certain PH domains are able to interact with PtdIns(3,4,5)P_3 has not been established definitively, recent work has identified four conserved residues required for high-affinity binding of PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 which lie at the N-terminal region of the PH domain in a K-X_{8-12}-R/K-X-R-Hyd motif, where X is any amino acid and Hyd is a hydrophobic amino acid [4]. Proteins possessing such PH domains include the serine/threonine protein kinases, protein kinase B (PKB) [5] and 3-phosphoinositide-dependent protein kinase-1 (PDK1) [6], the Bruton's tyrosine kinase BTK [7], the Rho/Rac GTP exchange factor VAV [8] and the ADP-ribosylating factor GTP exchange factor GRP1 [9]. Interaction of PtdIns(3,4,5)P_3 with PKB, BTK and VAV not only causes their translocation to the membrane of cells, but also induces a conformational change which results in them becoming phosphorylated and hence activated by specific upstream protein kinases.

This paper describes the identification and initial characterization of a widely expressed novel protein termed DAPP1 (**d**ual **a**daptor for **p**hosphotyrosine and 3-**p**hosphoinositides) which contains a PH domain that interacts specifically with PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 as well as a phosphotyrosine-binding Src homology (SH2) domain. This protein is likely to play an important role in triggering signal transduction pathways that lie downstream from receptor tyrosine kinases and PI 3-kinase.

MATERIALS AND METHODS

Materials

sn-1-Stearoyl-2-arachidonoyl D- and L-isomers of PtdIns(3,4,5)P_3 and *sn*-2-stearoyl-3-arachidonoyl D- and L-isomers of PtdIns(3,4,5)P_3 were synthesized as described previously [10]. diC_{16}-PtdIns(3,4,5)P_3 and diC_{16}-PtdIns(3,4)P_2 and all other synthetic phosphoinositides were from Echelon, phosphatidic acid was from Calbiochem, and other phospholipids were from Avanti Polar Lipids. Hybond-Cextra was from Amersham Pharmacia, Advantage Taq cDNA PCR kit, Marathon-Ready human placenta and mouse lung cDNA libraries, Multiple Tissue Northern Blot, and human cDNA Multiple Tissue cDNA panel were from Clontech. Human Universal cDNA Library was purchased from Strategene, and pCR 2.1Topo vector from InVitrogen. PKB [11] and PDK1 [12] were expressed as fusion proteins with glutathione S-transferase (GST) in 293 cells. PDK1 [13] was also expressed in insect cells with a His tag.

General methods and buffers

Restriction enzyme digests, DNA ligations and other recombinant DNA procedures were performed using standard protocols. All DNA constructs were verified by DNA sequencing.

Abbreviations used: BTK, Bruton's tyrosine kinase; DAPP1, **d**ual **a**daptor for **p**hosphotyrosine and 3-**p**hosphoinositides; hDAPP1, human DAPP1; mDAPP1, mouse DAPP1; EST, expressed sequence tag; GST, glutathione S-transferase; PKB, protein kinase B; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI 3-kinase, phosphoinositide 3-kinase; PH, pleckstrin homology; RACE, rapid amplification of cDNA ends; SH2, Src homology.

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The human and mouse DNA and protein sequences have been submitted to the National Center for Biotechnology Information database and appear under the accession numbers AF163254 and AF163255 respectively.

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Table 1 EST clones in the NCBI database derived from several tissues that encode fragments of DAPP1

Accession number	Species	Tissue
W58743	Human	Heart
AA459342, AA251658*,AA767425, AA459123*, AA934575,AA761790, AA809680, AA766027, AA761771, AA806320, AI206889	Human	Germinal centre B cells
AA149488, AI148081, AI128099, AI095529, AA149861, AA149868, AA150362, AI138611, AA151606	Human	Uterus
R14729*	Human	Brain
AA064488*	Mouse	Testis
AI596724	Mouse	Skin
F14802	Pig	Intestine

* ESTs whose sequence we have verified.

The following buffers were used. 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 50 ml; Roche). Buffer B: 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 10 mM 2-mercaptoethanol and 0.27 M sucrose.

Cloning of DAPP1

A partial human DAPP1 (hDAPP1) sequence encoding the C-terminal 156 residues of DAPP1 was obtained from DNA sequencing of the expressed sequence tag (EST) clones listed in Table 1. The 5′-end of the DAPP1 cDNA was obtained by two independent procedures. Firstly, by carrying out a 5′-rapid amplification of cDNA ends (RACE) using the Marathon-Ready human placenta cDNA library as a template, and an antisense primer derived from DAPP1 (5′-AACGACCGAGA-TCGGATCGTGCC-3′) and an adaptor primer, AP1, provided with the library. The PCR product was cloned into pCR 2.1Topo vector (InVitrogen). Three independent clones possessed identical sequences, and all had the same in-frame stop codon 5′ to the predicted initiating ATG codon. We were also able to amplify the predicted full-length DAPP1 by PCR using primers specific to the 5′ (5′-CACAGAGCGAGAAGGTGTCAGGAGC-3′) and 3′ (5′-CAAGGAGATGGCAACATCATGG-3′) untranslated regions of DAPP1. Secondly, we screened a Stratagene Human Universal cDNA Library with a DNA probe corresponding to the C-terminal 156 residues of DAPP1 and isolated a full-length DAPP1 clone identical in sequence with that obtained from 5′-RACE, which also had the same stop codon 5′ to the predicted initiating ATG codon. A partial mouse DAPP1 (mDAPP1) sequence encoding the C-terminal 160 residues was obtained by sequencing of the EST 515361 (accession number AA064488). The 5′-end of the mDAPP1 cDNA was obtained by carrying out a 5′-RACE PCR reaction using the Marathon-Ready mouse placenta cDNA library as a template, and nested primers derived from EST 515361 [5′-TGTTCTCGAGCAGA-TGGCCAGGTCCAGC-3′ and 5′-TCTGAAGTCTGCCACA-GATCAGACACC-3′] and the adaptor primer, AP1, provided with the library.

Expression of DAPP1 as a GST-fusion protein in 293 cells

DNA constructs expressing full-length human DAPP1 (residues 2–280), the isolated SH2 domain of DAPP1 (hDAPP1-ΔPH residues 2–166), or the isolated PH domain of DAPP1 (hDAPP1-ΔSH2 residues 125–280) with the FLAG epitope tag (DYKDDDDK) at the N-terminus were obtained by a standard PCR-based approach using Marathon-Ready human placenta cDNA as template. The PCR products were designed to incorporate *Kpn*1 restriction sites at the termini and were cloned into the eukaryotic expression vector pEBG-2T [14], which codes for the expression of these constructs with an N-terminal GST tag. The construct encoding the isolated mouse PH domain (mDAPP1-ΔSH2 residues 125–280) was also prepared as above, except that EST515361 was used as the template for the PCR with *Spe*1 restriction sites designed for the termini. PH domain mutants were generated by site-directed mutagenesis carried out using the QuikChange Kit (Strategene) according to the manufacturer's instructions.

For the expression of GST-fusion proteins in 293 cells, twenty 10 cm-diameter dishes of 293 cells were cultured and each dish was transfected with 10 μg of the indicated pEBG-2T DAPP1 construct, using a modified calcium phosphate method [15]. The cells were lysed 36 h post-transfection in 1 ml of ice-cold buffer A, the lysates were pooled, centrifuged at 4 °C for 10 min at 13000 ***g*** and the supernatant was incubated for 60 min on a rotating platform with 1 ml of glutathione–Sepharose previously equilibrated in buffer A. The suspension was centrifuged for 1 min at 3000 ***g***, the resin beads were washed three times with 10 ml of buffer A containing 0.5 M NaCl, and then a further ten times with 10 ml of buffer B. The protein was eluted from the resin at ambient temperature by incubation with 1.0 ml of buffer B containing 20 mM glutathione, and the beads were removed by centifugation through a 0.44 μm filter. The eluate was divided into aliquots, snap frozen in liquid nitrogen and stored at −80 °C.

Analysis of tissue distribution of DAPP1

A kit consisting of single-stranded cDNA isolated from different human tissues (Multiple Tissue cDNA panel) was obtained from Clontech. These were used as template to obtain the full-length hDAPP1 by PCR using the sense primer 5′-CACAGAGCGA-GAAGGTGTCAGGAGC-3′ and the antisense primer 5′-CA-AGGAGATGGCAACATCATGG-3′. The following PCR conditions were employed. The initial denaturing step was carried out at 94 °C for 1 min followed by 30–38 cycles of 94 °C for 0.5 min and 68 °C for 4 min using Advantage Taq (Clontech). Control reactions to PCR glyceraldehyde 3-phosphate dehydrogenase were carried out in parallel using the primers provided by Clontech.

Protein–lipid overlay

To assess the phospholipid-binding properties of each wild-type and mutant DAPP1, a protein–lipid overlay was performed using the GST fusion protein [16,17]. Lipid solution (1 μl) containing 3 pmol of phospholipids dissolved in a mixture of chloroform/methanol/water (1 : 2 : 0.8) was spotted onto 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 [10 mM Tris/HCl, pH 8.0, 150 mM NaCl and 0.1 % (v/v) Tween-20] for 1 h. The membrane was then incubated overnight at 4 °C in the same solution containing 0.5 μg/ml of the indicated GST–DAPP1 fusion protein. The membrane was

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Human DAPP 1   MGRAELLEGKMSTQDPSDLWSRSDGEAELLQDLGWYHGNLTRHAAEALLLSNGCDGSYLLRDSNETTGLYSLSVRAKDSVKHFHVEYTGYSFKFGFNEF
Mouse DAPP 1   .......G.N.......E..G.A..GTD........................R..........Q............................... Y

Human DAPP 101 SSLKDFVKHFANQPLIGSETGTLMVLKHPYPRKVEEPSIYESVRVHTAMQTGRTEDDLVPTAPSLGTKEGYLTKQGGLVKTWKTRWFTLHRNELKYFK
Mouse DAPP 101 .................................E....C................N...............................Q........

Human DAPP 198 DQMSPEPIRILDLTECSAVQFDYSQERVNCFCLVFPFRTFYLCAKTGVEADEWIKILRWKLSQIRKQLNQGEGTIRSRSFIFK  280
Mouse DAPP 198 ...................................................................K.....D...D.V........  280
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Figure 1 Amino acid sequence of human and mouse DAPP1

The amino acid residues corresponding to the SH2 domain are indicated by a solid line and those of the PH domain by a broken line.

SH2 DOMAIN ALIGNMENT

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hDAPP1            33  LGWYHGNLTRHAAEALLLSNG    CDGSYLLRDSNETTGLYSLSVR       AKD S
Src              149  EEWYFGKITRRESERLLLNAE  NPRGTFLVRESETTKGAYCLSVSDFDNAKG L
PLC-γ1           666  KEWYHASLTRAQAEHMLMRVP   RDGAFLVRKRNEPN SYAISFR       AEG K
PI3-KINASE p85α  622  KTWNVGSSNRNKAENLLRGK    RDGTFLVRESSKQ GCYACSVV       VDG E
PTP-2C             4  RRWFHPNITGVEAENLLLTRG   VDGSFLARPSKSNPGDFTLSVR       RNG A
APS              415  YPWFHGTLSRVKAAQLVLAGGPRNHGLFVIRQSETRPGEYVLTFN        FQG
Ras GAP          179  NQWYHGKLDRTIAEERLRQAG   KSGSYLIRESDRRPGSFVLSFL       SQMNV

                      βA      αA                βB              βC

hDAPP1                SVKHFHVEYTG YSFKFGFNE FSSLKDFVKHFANQPLIGSE  TGTLMVLKHPY  129
Src                   NVKHYKIRKLDSGGFYITSRTQFNSLQQLVAYYSKHADG        LCHRLTTV  247
PLC-γ1                KIKHCRVQQEG QTVMLGNSE FDSLVDLISYYEKHPLY      RKMKLRYPI  756
PI3-KINASE p85α       EVKHCVINKTATGYGFAEPYNLYSSLKELVLHYQHTSLVQHN DSLNVTLAYPV  718
PTP-2C                AVTHIKIQNTGDYYDLYGGEK FATLAELVQYYMEHHGQLKEKNGDVIELKYPL  102
APS                   KAKHLRLSLNGHGQCHVQHLW FQSVLDMLRHFHTHPIPLESGGSADITLRSYV  515
Ras GAP               VVNHFRIIAMCGDYYIGGRR  FSSLSDLIGYYSHVSCLLK       GEKLLYPV  272

                      βD    βD'   βE   βF      αB                         βG
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PH DOMAIN ALIGNMENT

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                            *            *  * *
hDAPP1        167  KEGYLTKQG   GLVKT   WKTRWFTLHRN   ELKYFKD  QMS   PEPIRILDLTECSAVQFDYSQE
GRP1          267  REGWLLKLG   GRVKT   WKRRWFILTDN   CLYYFEY  TTD   KEPRGIIPLENLSIREVEDPR
PDK1          453  ENNLILKMGPVDKRKGLFARRRQLLLTEGP  HLYYVDP   VN   KVLKGEIPWSQELRPEAK
PLC-δ1         24  KGSQLLKVK    SSSW   RRERFYKLQEDC  KTIWQES  RKVMRSPESQLFSIEDIQEVRMGHRTEGLEK
PKB             8  KEGWLHKRG   EYIKT   WRPRYFLLKNDGTFIGYKERPQDVDQRESPLNNFSVAQCQLMKTERPRP
APS            25  MVADDAAAG   SGGSAQ  WQKCRLLLRRAVAEERFRLEFFVPPKASRPKVSIPLSAIIEVRTTM
Pleckstrin      7  REGYLVKKG   SVFNT   WKPMWVVLLED   GIEFYKK  KSD   NSPKGMIPLKGSTLTSPCQDGKRQ
p21 RAS-GAP   468  KKGYLLKKG    KGKR   WKNLYFILEGSDAQLIYFES  EKR  ATKPKGLIDLSVCSVYYVVHDSLFG

                   β1                  β2            β3             β4   β5

hDAPP1                      RVNCFCLVFPFR                             TFYLCAKTGVEADEWIKILRWKLSQIRK  263
GRP1                        KPNCFELYNPSHKGQVIKACKTEADGRVVEGNHVVYRISAPSPEEKEEWMKSIKASISRDPF  384
PDK1                        NFKTFFVHTPNR                             TYYLMDPSGNAHKWCRKIQEVWRQRYQ  548
PLC-δ1             FARDVPEDRCFSIVFKDQ                             RNTLDLIAPSPADAQHWVQGLRKIIHHSGS  134
PKB                         RPNTFIRCLQWTTVIE                         RTFHVETPEEREEWATAIQTVADGLKR  112
APS                PLEMPEKDNTFVLKVENG                              AEYILETIDSLQKHSWVADIQGCVDPGDS  133
Pleckstrin            DGKRKFVFKITTTKQ                              QDHFFQAAFLEERDAWVRDINKAICIEG  105
p21 RAS-GAP                 RPNCFQIVVQHFSEE                        HYIFYFAGETPEQAEDWMKGLQAFCNLRKS  472

                               β6                                  β7         α1
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Figure 2 Alignment of the SH2 and PH domains of DAPP1 with other proteins possessing these domains

The alignment of the SH2 domains was carried out using the Clustal W program. NCBI accession numbers: Src, P12931; PLC-γ1, P19174; PI 3-kinase p85α subunit, P27986; PTP-2C, Q06124; APS, BAA 22514; Ras GAP, P20936. Alignment of PH domains was carried out using the Clustal W program with slight adjustments carried out by eye. NCBI accession numbers: Grp1, AF001871; PDK1, AA 017995; PKB, S33364; pleckstrin, P08567; Ras p21 GAP, P20936. The four key residues thought to be required for high-affinity interaction with PtdIns(3,4,5)P_3 [4] are indicated with asterisks. Numbering is based on the sequences in the database. The conserved Arg residue found in all SH2 domains and the conserved Trp residue found in all PH domains are shaded in black, and homologous residues found in these domains are boxed. Previous structural studies of SH2 domains [23] and of PH domains [24] indicate that the conserved residues of these domains fold into the indicated secondary α-helix (indicated by solid bars) and β-strands (indicated by hatched bars).

washed six times over 30 min in TBST and then incubated for 1 h with a 1/1000 dilution of anti-GST monoclonal antibody (Sigma). The membrane was washed as before, then incubated for 1 h with a 1/5000 dilution of anti-mouse–horseradish peroxidase conjugate (Pierce). Finally, the membrane was washed as above and the GST-fusion protein bound to the membrane, by virtue of its interaction with phospholipid, was detected by enhanced chemiluminescence.

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Surface plasmon resonance measurements of DAPP1–lipid interaction

Kinetic analyses of the interaction between DAPP1 and the polyphosphoinositides were made using a surface plasmon resonance based assay described previously [18], 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 to 0.27 M sucrose to reduce the bulk refractive index changes associated with the addition of PDK1 storage solutions. 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 (BiaCore AB, Uppsala, Sweden) for several sensorgrams at different protein concentrations. DAPP1 binding did not fit well to this model due to the slow dissociation of the protein from the surface. Therefore the affinities of binding of DAPP1 to polyphosphoinositides are likely to be overestimated by this method but are given as apparent equilibrium dissociation constants for comparative purposes.

RESULTS

DAPP1 is a novel SH2 domain- and PH domain-containing protein

The National Center for Biotechnology Information (NCBI) EST database was interrogated with the DNA sequence encoding the N-terminal region of the PH domain of human PKBα (residues 8–33). This search revealed several EST sequences (see Table 1) encoding the partial sequence of a novel PH domain-containing protein most homologous to the PH domains of PKB, PDK1 and other PH domains known to interact with PtdIns(3,4,5)P_3. Full-length clones were then isolated from human and mouse cDNA libraries as described in the Materials and methods section. The open reading frame encoded a protein of 280 amino acids with a predicted molecular mass of 32 kDa, and the human and mouse sequences were 93 % identical (Figure 1). A stop codon immediately 5′ to the predicted initiating ATG codon in the human sequence indicated that the protein sequence was full length. Both the human and mouse proteins start with a Met-Gly sequence (Figure 1), which indicates that after cleavage of the initiating methionine, DAPP1 may be myristoylated at the N-terminal glycine residue [19]. Analysis of the sequence revealed the presence of an SH2 domain at the N-terminus of the protein and a PH domain at its C-terminus (Figure 2). The former contains the conserved residues present in all known SH2 domains, including the invariant Arg residue (Arg-61, Figure 2A). The PH domain contains the conserved residues found in all PH domains, as well as the invariant Trp residue (Trp-250, Figure 2B). Furthermore, the PH domain also contains the motif K-X_{8-12}-R/K-X-R-Hyd (Figure 2B), found in all the PH domains that interact with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 with high affinity [4]. Because of these features this protein was termed DAPP1, for **d**ual **a**daptor for **p**hosphotyrosine and 3-**p**hosphoinositides.

Tissue distribution of DAPP1 mRNA

We have identified 25 EST clones in the NCBI database derived from several tissues that encode fragments of DAPP1 (Table 1). This indicates that DAPP1 is a widely expressed protein. The tissue distribution of DAPP1 mRNA was also investigated by PCR using cDNA derived from a variety of tissues (Figure 3). This analysis demonstrated that the highest levels of DAPP1 mRNA expression were observed in placenta and lung, with brain, heart, kidney, liver, pancreas and skeletal muscle possessing lower levels of DAPP1 mRNA. We were also able to detect DAPP1 mRNA by reverse-transcriptase PCR from Hela, PC12, Rat-2, Swiss 3T3 and HEK 293 cell extracts (results not shown). Northern blot analysis of human tissues revealed that DAPP1 was detected as a 2.7 kb transcript in all tissues examined (heart, brain, placenta, lung, liver, kidney and pancreas), with the highest levels observed in placenta and lung (results not shown). DAPP1, like PKB and PDK1 [12], appears to be ubiquitously expressed. In contrast, APS, another adaptor protein which contains a PH and SH2 domain [20–22] (see Discussion section), is expressed to a much higher level in skeletal muscle, heart and adipose than in other tissues.

Interaction of DAPP1 with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2

The full-length hDAPP1, as well as the isolated SH2 domain (GST–hDAPP1-ΔPH) and the isolated PH domain (GST–

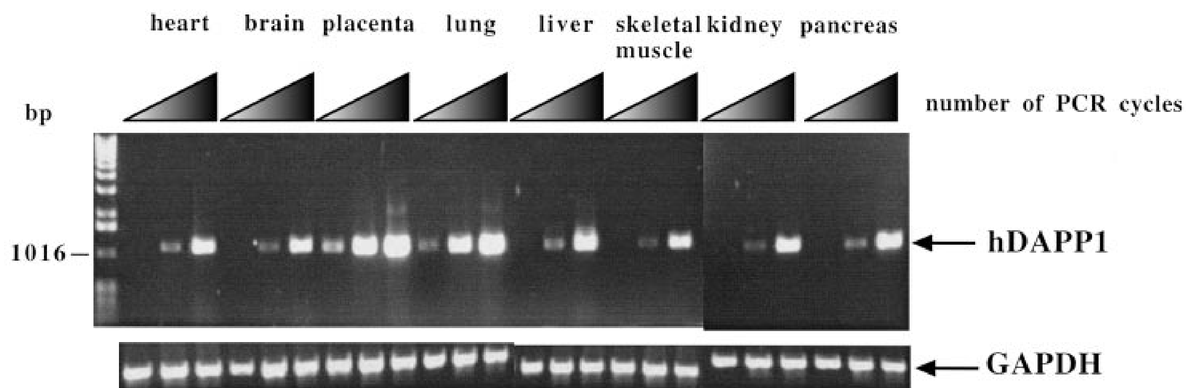

Figure 3 Tissue distribution of hDAPP1 mRNA expression

Full-length DAPP1 was amplified by PCR from a cDNA panel derived from the indicated human tissues (see Materials and methods section). After 30, 34 and 38 cycles of PCR an aliquot of the reaction mixture was removed and analysed by electrophoresis on agarose gel. The PCR product identified as DAPP1 was verified from each tissue by DNA sequencing and a Southern blot. As a control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified by PCR from the same cDNA panel. Identical results with those shown were obtained in three separate experiments.

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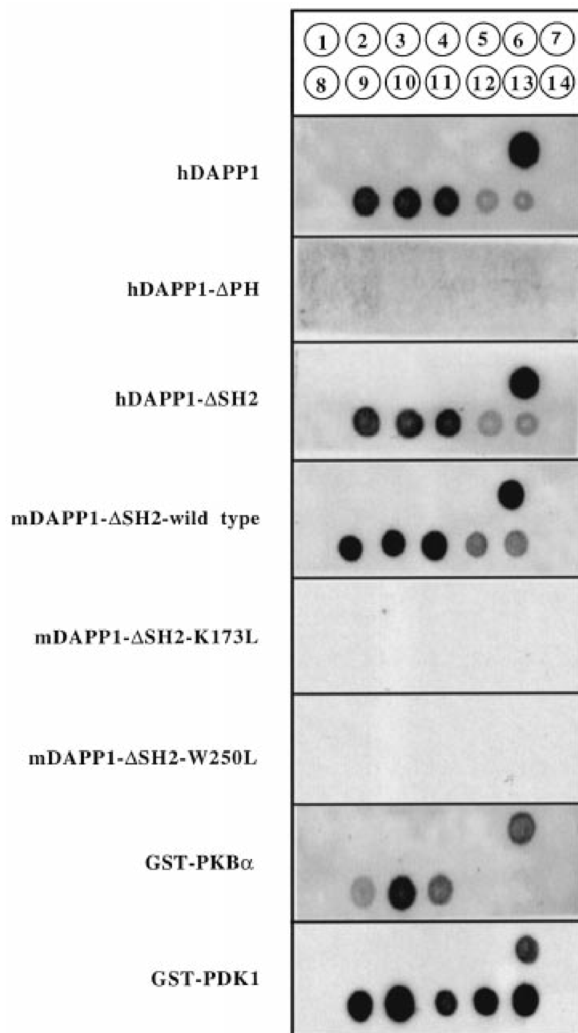

Figure 4 Comparison of the phospholipid-binding properties of DAPP1 with PDK1 and PKB

The ability of the following GST fusion proteins of hDAPP1 or mDAPP1, as well as PDK1 and PKB, to bind a variety of phospholipids (see below) was analysed using a protein–lipid overlay. The indicated phospholipids (3 pmol) were spotted on to a nitrocellulose membrane which was 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 experiment of three is shown. The lipids are identified by the positions of encircled numbers at the top of the Figure. 1, Phosphatidic acid; 2, phosphatidylinositol; 3, PtdIns3*P*; 4, PtdIns4*P*; 5, PtdIns5*P*; 6, *sn*-1,2-dipalmitoyl PtdIns(3,4)P_2; 7, PtdIns(3,5)P_2; 8, PtdIns(4,5)P_2; 9, *sn*-1,2-dipalmitoyl PtdIns(3,4,5)P_3; 10, *sn*-1-stearoyl, 2-arachidonoyl D-PtdIns(3,4,5,)P_3; 11, *sn*-2-stearoyl, 3-arachidonoyl D-PtdIns(3,4,5,)P_3; 12, *sn*-1-stearoyl, 2-arachidonoyl L-PtdIns(3,4,5,)P_3; 13, *sn*-2-stearoyl, 3-arachidonoyl L-PtdIns(3,4,5,)P_3; 14, buffer.

hDAPP1-ΔSH2), were expressed in human 293 cells as fusions to GST. After purification from cell lysates by affinity chromatography on glutathione–Sepharose, single Coomassie Blue-staining bands were observed that migrated with the expected molecular masses for each protein on SDS/PAGE (results not shown).

We studied the interaction between these proteins and phospholipids using a protein–lipid overlay assay [16,17]. Phospholipids, including phosphoinositides, were spotted on to a nitrocellulose membrane and incubated with the indicated DAPP1–GST fusion protein and with GST–PDK1 or GST–PKBα as controls. The membranes were then washed and immunoblotted using a GST antibody to detect the GST fusion proteins bound to the membrane by virtue of their interaction with lipid. The wild-type GST–DAPP1, GST–DAPP1-ΔSH2, GST–PDK1 and GST–PKBα, interacted with *sn*-1-stearoyl, 2-arachidonoyl D-PtdIns(3,4,5)P_3, *sn*-2-stearoyl, 3-arachidonoyl D-PtdIns(3,4,5)P_3, *sn*-1,2-dipalmitoyl D-PtdIns(3,4,5)P_3 [C_{16}-PtdIns(3,4,5)P_3] and *sn*-1,2-dipalmitoyl PtdIns(3,4)P_2 [C_{16}-PtdIns(3,4)P_2], but not with PtdIns3*P*, PtdIns(4,5)P_2, PtdIns4*P*, PtdIns5*P* and PtdIns(3,5)P_2 (Figure 4), nor with phosphatidylserine or the zwitterionic phospholipids, phosphatidylcholine and phosphatidylethanolamine (results not shown). The binding of DAPP1 to PtdIns(3,4,5)P_3 appeared to be relatively stereospecific, as DAPP1 interacted only weakly in the assay with the non-physiological L enantiomers of *sn*-1-stearoyl, 2-arachidonoyl L-PtdIns(3,4,5)P_3 and *sn*-2-stearoyl, 3-arachidonoyl D-PtdIns-(3,4,5)P_3. The interaction of DAPP1 with lipids was mediated by the PH domain because GST–DAPP1-ΔPH did not interact with any lipid tested (Figure 4). This also demonstrates that the SH2 domain of DAPP1 is incapable of interacting with 3-phosphoinositides. Furthermore, mutation of a conserved lysine residue (Lys-173) or the invariant tryptophan (Trp-250) in the isolated PH domain of mDAPP1 abolished its interaction with 3-phosphoinositides (Figure 4).

We have also measured the kinetics of binding of full-length DAPP1 to a supported lipid monolayer containing a low mole fraction of phosphoinositide using a modification of the surface plasmon resonance based assay described previously [18]. Using this assay, GST–hDAPP1 and GST–hDAPP1-ΔSH2 bound to 0.1 mole percent of *sn*-1-stearoyl, 2-arachidonoyl D-PtdIns(3,4,5)P_3 with an apparent equilibrium dissociation constant (K_d) of 3 nM, compared with 60 nM for His-PDK1 measured in parallel experiments. DAPP1 interacted far less strongly with its stereoisomer *sn*-1-stearoyl, 2-arachidonoyl L-PtdIns(3,4,5)P_3, with an apparent K_d of 160 nM compared with 330 nM for His-PDK1. Both His-PDK1 and GST–hDAPP1 interacted weakly with the diC_{16}-PtdIns(3,4,5)P_3/PtdIns(3,4)P_2. However, the magnitude of the binding was too small to give reliable estimates of the apparent affinities of PDK1 or DAPP1 for these lipids. Nevertheless, the selectivities for these lipids were revealed by the protein–lipid overlay assay (Figure 4). Therefore, as observed previously with PKB and PDK1, DAPP1 interacts with highest affinity with the physiological PtdIns(3,4,5)P_3 enantiomer.

DISCUSSION

To our knowledge only one other protein, termed APS, is known which, like DAPP1, is composed solely of an SH2 domain and a PH domain. APS appears to associate with activated B-cell receptors [20], growth factor receptors [21] and insulin receptors [22] and becomes tyrosine phosphorylated in response to agonists that activate these receptors. However, in contrast to DAPP1, the PH domain of APS is located N-terminal to the SH2 domain. Moreover, APS is unlikely to interact with PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 with high affinity and specificity because it lacks three out of the four conserved residues in the consensus 3-phosphoinositide-binding motif lying in the N-terminal region of the PH domain (see Figure 2B). All proteins that bind with high affinity to PtdIns(3,4,5)P_3, such as DAPP1, PKB, human PDK1, GRP1 and BTK, possess this motif [4]. Plant PDK1, although possessing a PH domain very similar in sequence to that of human PDK1, lacks two of the four residues. This may explain why plant PDK1 fails to interact with PtdIns(3,4,5)P_3 with high

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affinity or specificity [17]. Also, the SH2 domain of APS is no more similar to the SH2 domain of DAPP1 than other SH2 domain-containing proteins shown in Figure 2A. These observations indicate that APS is likely to possess distinct cellular functions to those of DAPP1.

DAPP1 could potentially be recruited to the cell membrane by three mechanisms. Firstly, it possesses a putative myristoylation site at the N-terminus which could facilitate the interaction of DAPP1 with the lipid bilayer. Secondly, DAPP1 possesses an SH2 domain which could interact with phosphotyrosine residues on membrane-associated proteins such as activated tyrosine kinase receptors. Thirdly, DAPP1 contains a PH domain which exhibits a high-affinity interaction with the PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 second messengers produced at the cell membrane following the activation of PI 3-kinases. It is likely that DAPP1 functions as an adaptor to recruit other proteins to the plasma membrane in response to extracellular signals. Future work will focus on identifying the proteins with which DAPP1 interacts and the roles of the putative myristoylation site, SH2 and PH domains in the function of DAPP1.

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