Purification and cloning of phosphatidylinositol transfer proteins from *Dictyostelium discoideum*: homologues of both mammalian PITPs and *Saccharomyces cerevisiae* Sec14p are found in the same cell

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Soluble phosphatidylinositol transfer proteins (PITPs) have important roles in lipid-mediated signalling as well as in membrane traffic. Two PITPs (α and β) have been cloned from mammalian cells, which are unrelated in sequence to yeast PITP (the product of the *SEC14* gene). However, all three PITPs can perform interchangeably to reconstitute function in mammalian cells. We have now purified the major PITP from the cytoplasm of *Dictyostelium discoideum* and cloned the gene. This protein, DdPITP1, is homologous with mammalian PITPα and PITPβ. We have also cloned a second gene (DdPITP2) related in sequence to DdPITP1. In addition, an independently cloned cDNA encodes a relative of the *SEC14* family of yeast PITPs. DdPITP1, DdPITP2 and DdSec14 proteins were all able to mediate the transfer of PtdIns from one membrane compartment to another; they thus exhibited the hallmark of PITPs. Secondly, all three PITPs were able to rescue phospholipase C-mediated phosphoinositide hydrolysis in PITP-depleted HL60 cells, indicating that all three PITPs were capable of stimulating phosphoinositide synthesis. The identification of PITPs related to both mammalian PITPs and yeast Sec14p in a single organism will provide a unique opportunity to examine the functions of this class of protein with genetic approaches.

Key words: Golgi localization, phosphoinositides, phospholipase C.

INTRODUCTION

Phosphatidylinositol transfer proteins (PITPs) are defined by their ability to mediate the exchange of PtdIns or phosphatidylcholine (PtdCho) monomers between membranes *in vitro* [1]. In mammalian cells, two soluble PITP isoforms have been described, PITPα and PITPβ, which are 77% identical. In addition, the PITP domain has also been identified in the RdgB/Nir family, which are large proteins with putative membrane-spanning regions [2–4]. In *Drosophila*, RdgB functions together with rhodopsin, G_q-related G-proteins and a phospholipase Cβ (PLCβ)-related enzyme (NorpA) in the *Drosophila* visual signal transduction system and was identified from a gene that causes retinal degeneration when mutated [5]. In mice, the *vibrator* mutation causes neurodegeneration via decreased expression of PITPα [6].

In mammalian cells, PITP is believed to couple phosphoinositide synthesis to signal transduction reactions and to membrane traffic [7]. PtdIns is the major inositol lipid that is synthesized at the endoplasmic reticulum, and it can be phosphorylated at the 3, 4 and 5 positions by multiple kinases to yield a variety of minor phosphorylated species including PtdIns3*P*, PtdIns4*P*, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. These phosphorylated derivatives of PtdIns can bind to target proteins and alter their activities. For example, PtdIns3*P* exerts its effects through FYVE domains (a FYVE domain is a zinc-binding domain known to bind PtdIns3*P*, that was originally found in early endosomal antigen 1), and PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 can bind to pleckstrin homology domains [8]. Phosphoinositides are essential in several aspects of membrane traffic [9,10], and PITP has been found to be required. PITP is required for the exocytosis of secretory granules [11,12] and budding of secretory vesicles from the trans-Golgi network [13,14]. Many phosphorylated inositol lipids are synthesized on demand, directly from PtdIns, at different sites in the cell. The greatest demand for PtdIns comes from PLC-mediated hydrolysis of PtdIns(4,5)P_2, which can occur in multiple compartments including the plasma membrane and the nucleus. Thus PLC-mediated hydrolysis of PtdIns(4,5)P_2 is dependent on the availability of PITP, whatever the mode of activation of the PLC [15–18]. PtdIns(4,5)P_2 is also used as a substrate by phosphoinositide 3-kinase; again, PITP has been identified as a requirement for this signalling pathway [19].

PITPs have also been identified in yeasts, but their sequences are dissimilar to those of mammalian proteins. The best-characterized protein, encoded by *SEC14* of *Saccharomyces cerevisiae*, is essential and is required for protein secretion from the Golgi complex [20]. Analysis of bypass mutants has led to the suggestion that Sec14p is required for maintenance of a critical pool of diacylglycerol [21,22]. Moreover, Sec14p can also fa-

Abbreviations used: GFP, green fluorescent protein; PITP, phosphatidylinositol transfer protein; PLC, phospholipase C; PtdCho, phosphatidylcholine.

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cilitate PtdIns4*P* production *in vivo* [22,23]; this lipid might also be a critical requirement in protein secretion. In another yeast, *Yarrowia lipolytica*, *SEC14* is not an essential gene and is required only for differentiation from yeast to a mycelial growth form [24].

As a eukaryotic microbe, *Dictyostelium discoideum* offers the opportunity to perform genetic analysis in a micro-organism with a well-studied biochemistry and straightforward molecular genetics. Unlike *S. cerevisiae*, *Dictyostelium* has the complex range of inositol lipids seen in higher eukaryotes; our prediction was therefore that they would contain mammalian homologues of PITP. To establish whether *Dictyostelium* cells use PITPs, we used a biochemical assay of PtdIns transfer activity *in vitro* (which is shared by both mammalian and yeast PITPs) to purify PITP. We purified a protein of 35 kDa to near homogeneity; from a peptide analysis of this protein we cloned the gene. This protein was immunoreactive to a PITPα antibody. We have more recently identified a second gene for PITP, encoding DdPITP2, which is also similar to mammalian PITPs. Finally, we have also identified a random subclone in GenBank® that shows clear homology with yeast SEC14ps. Thus *Dictyostelium* is well endowed with PITP homologues, and all three proteins were found to have transfer activity and to be able to support the reconstitution of G-protein-stimulated PLCβ activity in mammalian cells.

EXPERIMENTAL

Purification of PITP from *Dictyostelium* cytoplasm

Soluble cytoplasm was prepared from *Dictyostelium* cells by filter lysis [25]. Axenically grown AX3 cells (200 ml; 2×10^7 cells/ml) were washed, resuspended in lysis buffer [200 mM sucrose/10 mM Tris/HCl (pH 7.5)/50 mM KCl/1 mM dithiothreitol/0.5 μM PMSF/0.2 μM tosyl-lysylchloromethane ('TLCK')], then lysed with a single pass through a 5 μm Nucleopore filter and centrifuged for 10 min at 10000 ***g***. The supernatant was snap-frozen in solid CO_2/methanol, then stored at -20 °C until needed.

For purification, 150 ml of thawed cytosol was applied in three batches of 50 ml to a Q-Sepharose column by FPLC. A salt gradient (0–1 M NaCl) was used to elute the proteins (NaCl-free buffer for 50 ml, then 0–1 M NaCl for 300 ml); 35 fractions, each of 10 ml, were collected and assayed for PtdIns transfer activity as described previously [15] (see Figure 1A). The active fractions (fractions 11, 12 and 13) from the three separate runs were pooled and concentrated to approx. 10 ml. The pooled material was applied to a gel-filtration column (S 75 gel-filtration column; Pharmacia) in two batches of 5 ml each. The column was equilibriated with 20 mM Pipes/3.7 mM KCl/137 mM NaCl (pH 6.8) and proteins were eluted with the same buffer at a flow rate of 1.5 ml/min. Fractions of 4 ml were collected and assayed for transfer activity (see Figure 1B); peak transfer activities from the two runs were concentrated to 5 ml and rerun with S75 gel filtration. The peak activity was concentrated to 4 ml. After buffer exchange with NAP10 columns (Pharmacia) to 20 mM Tris/HCl, pH 7.6, the active fractions were chromatographed in two batches of 3 ml over a Mono-Q column (elution conditions: 4 ml of NaCl-free buffer, then 0–500 mM NaCl over 25 ml, then to 1 M NaCl over 5 ml). Fraction size collected was 0.5 ml and assayed for activity (see Figure 1C). Fractions containing transfer activity were pooled, concentrated to 2 ml and desalted with NAP10 columns to Tris/HCl, pH 7.6, then chromatographed over a heparin–Sepharose column (20 ml of salt-free buffer, then 0–1 M NaCl over 10 ml at 1 ml/min) and assayed for activity (see Figure 1D).

Fractions obtained after heparin–Sepharose chromatography were analysed by SDS/PAGE and detected by staining with Coomassie Blue (see Figure 1E). The samples were also transferred to nitrocellulose and blotted with the 5F12 monoclonal antibody against mammalian PITPα (see Figure 1F). Fractions 8–10, which contained the PITP activity and protein, were pooled and used for peptide sequencing as described previously [15].

Assay for PITP activity *in vitro*

The activity of PITP was measured by monitoring the transfer of ^{3}H-labelled PtdIns from donor microsomes to acceptor liposomes (PtdCho/PtdIns, 98:2) at 25 °C for 30 min, exactly as described previously [15].

cDNA cloning

The *Dictyostelium* PITP1 cDNA was identified by redundant PCR with primers corresponding to peptides obtained from the purified protein and primers corresponding to conserved regions in mammalian PITPs. The successful primers were 5′-CAGGATCCAAAAAATGAAACXGGTGGTGGTGAAGGT-3′ (corresponding to the conserved sequence Asn-Glu-Thr-Gly-Gly-Gly-Glu-Gly) and 5′-AAATCGAATTCXATACCCATCATAAAATTTTC-3′ (corresponding to the peptide sequence Glu-Asn-Phe-Met-Met-Gly-Ile-Glu-Phe). A 649 bp fragment was generated from these primers and cloned to pBS; a λgt11 library was then screened to obtain a full-length clone [26].

Identification of *Dictyostelium* PITP2 and Sec14p homologues

DdPITP2 was found by searching the *Dictyostelium* cDNA project in Tsukuba. The complete library of the project was searched by TBLASTN with mammalian PITPα as a target. Several clones were identified, of which SSK416 was both full-length and had an open reading frame throughout its length. The gene encoding DdSec14 had previously been cloned as a random cDNA from a slug-stage library, named *rsc5* and identified as a *SEC14* homologue by Dr N. Iranfar and Dr W. F. Loomis.

Northern blotting

Wild-type (AX3) *Dictyostelium* cells were harvested from axenic medium, washed and deposited on non-nutrient agar plates. Every 4 h, cells were harvested and stored as frozen pellets. Total RNA was prepared as described [27]. RNA (20 μg) from each sample was separated on 1% (w/v) agarose/formaldehyde gel, blotted on a positively charged nylon membrane (Hybond-N$^+$; Amersham) and probed with DdPITP1 and DdPITP2 clones labelled with [^{32}P]dATP made with a random primer kit (Amersham). Hybridization and washing were performed as described [26].

Expression of His-tagged recombinant proteins

Restriction sites were added to the cDNA species for the three PITP clones by using PCR; each was subcloned into the pET 14b and pRSET C Prokaryotic expression vectors. Oligonucleotides were designed with sequence data and synthesized by Genosys Biotechnologies (Europe) Ltd. For DdSec14 in pET14b, the primers were as follows: sense, 5′-GGCGCCCCATATGGAATCAACAGCATCAAC-3′ (*Nde*I); anti-sense, 5′-TTGGGATCCTTAAATAAAGTCGATTAAATTAAT-3′ (*Bam*HI). For DdPITP1 in pRSET C, the primers were as follows: sense, 5′-

CTGAATTCTCGAGCCATGTTAATTAGAGAATTTCGT-3′ (*Xho*I); anti-sense, 5′-ATTAAAGGTACCTATTTATTTTT-CGGCGGC-3′ (*Kpn*I). For DdPITP2 in pRSETC the primers were as follows: sense, 5′-CGTCTCGAGCCATGTTAATTA-AAGAATATAGGATG-3′ (*Xho*I); anti-sense, 5′-CAATAAG-GTACCGTTTATTTATTTGCAGC-3′ (*Kpn*I). PCR reactions were performed in a 100 μl reaction volume for 30 cycles of 30 s at 96 °C, 2 min at 52 °C and 1 min at 75 °C. The PCR products and expression vectors were digested with the appropriate restriction enzymes, ligated at 16 °C overnight and transformed into competent XL1 Blue *Escherichia coli* and plated on ampicillin plates. One clone containing each of the PITP inserts was grown in 200 ml of Luria broth with ampicillin (100 μg/ml) from the remaining 0.5 ml from the minipreps. Purified plasmids were digested to check the insert and re-transformed into competent DE3 pLys *E. coli*.

Recombinant protein preparation

Luria broth (200 ml) with ampicillin (100 μg/ml) was inoculated with the glycerol stock of each DdPITP clone and grown overnight at 37 °C. The 200 ml was diluted into 1 litre of fresh Luria broth and grown for 2 h at 27 °C. The culture was induced with 500 μM isopropyl β-D-thiogalactoside ('IPTG') and grown for a further 4 h at 27 °C. The cultures were centrifuged (3000 ***g***) for 10 min and frozen overnight in buffer [50 mM $NaHPO_4$/10 % (v/v) glycerol/300 mM NaCl (pH 8.0)] at −20 °C. The frozen pellets were thawed on ice and centrifuged at 100000 ***g*** for 30 min; the supernatant was loaded on Qiagen Ni-nitrilo-triacetate resin columns. The columns were washed with 6-fold the bed volume with a low-salt buffer [50 mM $NaHPO_4$/10 % (v/v) glycerol/300 mM NaCl (pH 6.0)], then with 6-fold the bed volume of high-salt wash buffer [50 mM $NaHPO_4$/10 % (v/v) glycerol/525 mM NaCl (pH 6.0)]. The protein was eluted with 1.5-fold the bed volume of the high-salt buffer supplemented with 250 mM imidazole. The protein was then desalted with Bio-Rad desalting columns and tested *in vitro* for PtdIns transfer activity and PLC reconstitution activity. The expression of DdPITPs in *E. coli* was low in comparison with mammalian PITPs; this was a particular problem with DdPITP1 and DdSec14.

Western blotting

Recombinant PITPα (1 μg) was run out on a SDS/14 % (w/v) polyacrylamide gel, as was 10 μg of DdPITP proteins. After transfer to Immobilon-P (Millipore), the blots were probed with the monoclonal antibody 5F12 and polyclonal antibody against PITPα [28], and with polyclonal antibodies against Sec14p, a gift from Dr V. Bankaitis (University of Birmingham, Alabama).

Cell permeabilization and PLC reconstitution

This was performed exactly as described [15]. In brief, HL60 cells were labelled for 48 h in M199 with 1 μCi/ml [^{3}H]inositol. The cells were washed in Pipes buffer [20 mM Pipes/2.7 mM KCl/137 mM NaCl (pH 6.8)] containing 1 mg/ml albumin and 1 mg/ml glucose, then permeabilized with streptolysin O (0.4 unit/ml, purchased from Murex Biotech Ltd., Dartford, U.K.) for 10 min at 37 °C to deplete the cells of endogenous PITP. The permeabilized cells were assayed for inositol phosphate production in an assay volume of 100 μl. The final concentrations present in the assay were 1 mM $MgATP^{2-}$, 2 mM $MgCl_2$, 10 μM Ca^{2+} buffered with 3 mM EGTA, 10 mM LiCl, 10 μM guanosine 5′-[γ-thio]triphosphate (GTP[S]) and recombinant PITPs as indicated. The samples were incubated at 37 °C for 20 min, then quenched with 500 μl of chloroform/methanol (1:1, v/v). Water (250 μl) was added and the samples were vortex-mixed and centrifuged at 1800 ***g*** for 5 min. A 400 μl sample of the aqueous phase was added to 1 ml Dowex columns and washed sequentially with water (6 ml) and 5 mM sodium tetraborate (6 ml); the inositol phosphates were finally eluted with 3 ml of 1 M ammonium formate into 20 ml scintillation vials. The samples were mixed with 10 ml of Ultima Flo and counted in a scintillation counter.

Localization of green fluorescent protein (GFP)-tagged PITPs

The plasmids for DdPITP1, DdPITP2, PITPα and PITPβ were digested with *Xho*1 and *Kpn*1 and the fragments were gene-cleaned and cloned into the corresponding sites of both pEGFP-C1 (Clonetec) vectors. RBL-2H3 mast cells were cultured in Dulbecco's modified Eagle's medium supplemented with 12.5 % (v/v) fetal calf serum, 4 mM glutamine, 50 μg/ml penicillin and 50 i.u./ml streptomycin. The cells were grown as a monolayer in 175 cm^2 vented flasks, at 37 °C with air/CO_2 (19:1) and 100 % humidity. Cells were removed by gentle scraping, washed into Hepes-buffered salt solution and electroporated with 30 μg of DNA. After 24 h, the cells were washed, fixed and examined under an LSR Digital/Confocal Imaging System equipped with an Olympus 1X70 microscope.

RESULTS AND DISCUSSION

Purification of *Dictyostelium* PITP

We set out to purify PITP from *Dictyostelium* lysate and found one major peak of activity that we purified sequentially by anion-exchange chromatograghy (Q-Sepharose), gel filtration, Mono-Q and finally heparin–Sepharose affinity chromatography (Figures 1A–1D). SDS/PAGE analysis of the active fractions eluted from heparin–Sepharose identified a 35 kDa protein by staining with Coomassie Blue (Figure 1E) that was also immunoreactive with a monoclonal antibody 5F12 against mammalian PITPα (Figure 1F). This purification was designed to optimize purity rather than yield; sufficient protein was obtained to allow microsequencing.

Cloning of *Dictyostelium* genes

Peptides that were obviously related to mammalian PITPs, together with conserved regions from mammalian PITP proteins, were used to design redundant oligonucleotides for PCR. The peptides are shown in Figure 2. One oligonucleotide pair produced a 649 bp band from *Dictyostelium* genomic DNA. This fragment was cloned and used as a probe to enable the cloning of the full-length clone of *Dictyostelium* PITP, DdPITP1. The cDNA encoded a protein of 264 amino acid residues with a calculated molecular mass of 31.1 kDa. We subsequently scanned the expressed sequence tag (EST) database from the *Dictyostelium* cDNA project in Japan, and identified several cDNA species encoding a second *Dictyostelium* PITP (DdPITP2). The cDNA for DdPITP2 is predicted to encode a protein of 259 residues with a calculated molecular mass of 30.0 kDa. The genes encoding DdPITP1 and DdPITP2 have been named *pitA* and *pitB* respectively.

Comparison of the peptide sequences in Figure 2 with DdPITP1 and DdPITP2 shows that the protein that was purified was encoded by DdPITP1, even though the DdPITP1 cDNA has not yet been identified in the cDNA projects. This suggests that DdPITP1 is expressed during growth, whereas DdPITP2 is found later in development, because all the DdPITP2 clones were

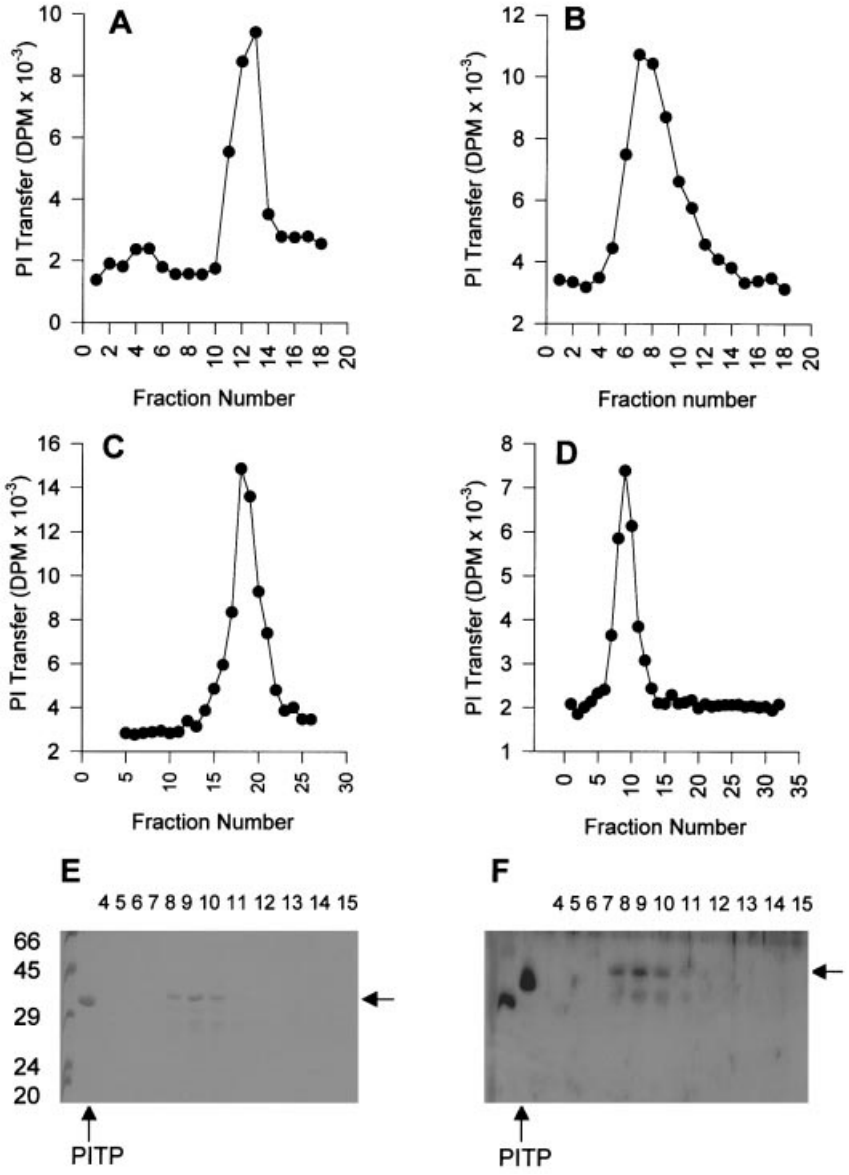

Figure 1 Purification of *Dictyostelium* PITP from cytosol

(**A**–**D**) Cytosol was sequentially chromatographed on Q-Sepharose (**A**), by gel filtration (**B**), on Mono-Q (**C**) and on heparin–Sepharose (**D**). (**E**, **F**) Analysis of fractions 4–15 after chromatography on heparin–Sepharose by SDS/PAGE and staining with Coomassie Blue (**E**) and the same samples probed with a PITPα monoclonal antibody (5F12) (**F**). The first lane contained molecular mass markers (molecular masses are indicated at the left in kDa) and the second lane contained recombinant PITPα, indicated by a vertical arrow. PI, PtdIns.

found in libraries made from slug-stage cells. Northern blotting confirmed this hypothesis (Figure 3). The expression of *pitA* was essentially constant throughout growth and development, whereas *pitB* was barely seen in growing cells but was sharply induced early in the developmental programme.

The sequences of *Dictyostelium* DdPITP1 and DdPITP2 were compared with those of mammalian PITPα and PITPβ and also with the PITP domains of *Drosophila* RdgB (Figure 2). Inspection of the sequence reveals a high degree of conservation between the five sequences. The largest degree of difference was localized to the C-terminus of the protein. This was unexpected, because small changes in the C-terminus cause major changes in the functioning of mammalian PITPs [29]. We also searched the Tsukuba cDNA project and GenBank® for sequences that were similar to that of the yeast PITP, Sec14p, which, as described earlier, is not homologous to mammalian PITPs. Several ESTs were identified, all of which were identical with a full-length random subclone that had been previously deposited with GenBank® (*rsc5*; N. Iranfar and W. Loomis, unpublished work). The predicted DdSec14 protein contains 324 residues, with a calculated molecular mass of 37.5 kDa. We have renamed this gene *pitC* and the protein DdSec14, to correspond with the functional data presented here. The sequence of DdSec14 is homologous with yeast Sec14p throughout its length, although the conservation is less obvious than between mammalian PITPs and DdPITP1 and 2 (Figure 4). On the basis of the structure, Lys-66, Glu-207 and Lys-239 have been suggested as Sec14p residues that participate in inositol headgroup binding [22], and

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Hs PITPα        MVLLKEYRVILPVSVDEYQVGQLYSVAEASKNETGG-GEGVEVLVPEPYEK-DGEKGQYT 58
Hs PITPβ        MVLIKEFRVVLPCSVQEYQVGQLYSVAEASKNETGG-GEGIEVLKNEPYEK-DGEKGQYT 58
Dm RdgB         -MLIKEYRIPLPLTVEEYRIAQLYMIAKKSREESHGEGSGVEIIINEPYKDGPGGNGQYT 59
DDPITP1         -MLIREFRVVLPLTVEEYRVGQLYSVAKTSSQETSN-GEGVEVLVNEPYKE-PEHEGQYT 57
DDPITP2         -MLIKEYRMVLPLTVEEYQIAQLYMVAKKSKESTKG-GEGIEIIKNEPFDN-EKGKGQYT 57
                 :*::*:*: ** :*:**::.*** :*: * :.: . *.*:*::  **:..    :****
PEPTIDES                 VVLPLTVEEYR

Hs PITPα        HKIYHLQSKVPTFVRMLAPEGALNIHEKAWNAYPYCRTVITNEYMKEDFLIKIETWHKPD 118
Hs PITPβ        HKIYHLKSKVPAFVRMIAPEGSLVFHEKAWNAYPYCRTIVTNEYMKDDFFIKIETWHKPD 118
Dm RdgB         KKIYHVGNHLPGWIKSLLPKSALTVEEEAMECYPYTRTRYTCPFV-EKFSLDIETYYYPD 118
DDPITP1         HKIYHLGSRLPGWIRALIPSSALKLEEKAWNAYPYCKTVLKSPFLGEKFTFIIESRHAQD 117
DDPITP2         EKIIYLANSLPRFAAAILPSSALKIEEKAWNAYPYCKTEYSCPFFGEKLVLSIESMHLPG 117
                .** :: . :* :   : *..:* ..*:* :.*** :*  .  :. :.: : **: :  .
PEPTIDES                                                    FTFIIESR

HsPITPα         LGTQENVHKLEPEAWKHVEAVYIDIADRSQVLSKDYKAEEDPAKFKSIKTGRGPLGPN-- 176
HsPITPβ         LGTLENVHGLDPNTWKTVEIVHIDIADRSQVEPADYKADEDPALFQSVKTKRGPLGPN-- 176
Dm RdgB         NGYQDNVFQLSGSDLRNRIVDVIDIVKDQLWG-GDYVKEEDPKHFVSDKTQRGPLAEDWL 177
DDPITP1         NCKTENIHNLSEKELKERTVEVIDITKPIKDP-KNYKETDPTKIRSEKANRGPLEE--- 173
DDPITP2         RGEVENALKCDAETLKQRHVDPIDIANDQPK---EYIKEEDPKIFKSVKTERGPLDD--- 171
                   :*    . .  :      ***..       :*   ***  : * *: ****

Hs PITPα        -------WKQELVNQKDCPYMCAYKLVTVKFKWWGLQNKVENFIHKQER-RLFTNFHRQL 228
Hs PITPβ        -------WKKELANSPDCPQMCAYKLVTIKFKWWGLQSKVENFIQKQEK-RIFTNFHRQL 228
Dm RdgB         EEYWREVKGKKQPTPRNMSLMTAYKICRVEFRYWGMQTKLEKFIHDVALRKMMLRAHRQA 237
DDPITP1         E---------KWRESTEMPIMTCYKLVTVEFKYFGFQTKVENFMMGIEF-DLFTKFHRQV 223
DDPITP2         ----------PKWRDKVEPVMTCYKLVHAEFKYWGFQTKVENVIQDTGVRDVLLKAHRAL 221
                          . * .**:   :*:::*:*.*:*:.:       :: . **
PEPTIDES                                          VENFMMGIEF-DLFTK

Hs PITPα        FCWLDKWVDLTMDDIRRMEEETKRQLDEMRQKDPVKGMTADD- 270
Hs PITPβ        FCWIDKWIDLTMEDIRRMEDETQKELETMRKRGSVRGTSAADV 271
Dm RdgB         WAWQDEWFGLTIEDIRELERQTQLALAKKMGGGEECS------ 274
DDPITP1         YCWLDEWFGMSMDDVRAFELKTKEDLKKKLEEKDENKAAEK-- 264
DDPITP2         FCWIDEWFGLTIEDIRKIEEETKAELAKKLEENKAANK----- 259
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Figure 2 Comparison of PITP sequences

Alignment of *Dictyostelium* PITPs with human PITPα and PITPβ, the PITP domain from *Drosophila* RdgB (residues 1–274) and peptides obtained from sequencing the purified protein. Protein sequences were aligned by using the CLUSTAL W program (European Bioinformatics Institute, Cambridge, U.K.). Asterisks indicate identity, colons indicate conserved substitutions, and full stops indicate semi-conserved substitutions. Identical residues are also shown in bold. The peptides found in the DDPITP1 sequence are also underlined.

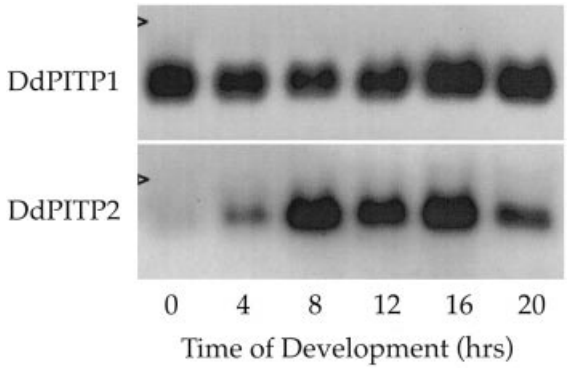

Figure 3 Expression of *pitA* and *pitB* during development

Cells were developed on non-nutrient agar plates. Total RNA was extracted at the indicated times of development; 20 μg of each was blotted to nylon membrane and the filter was probed with *pitA* (top) and *pitB* (bottom). Arrowheads show the position of the 17 S rRNA (1.9 kbp).

indeed mutations of these residues lead to a loss of PtdIns transfer. Whereas Lys-207 and Lys-239 are conserved in DdSec14, we note that Lys-66 is replaced with Asn in DdSec14.

Expression in *E. coli*

The three cDNA species encoding PITPs from *D. discoideum* (DdPITP1, DdPITP2 and DdSec14) were engineered by PCR to add His_6 tags, then expressed in *E. coli*. We found that the expression of DdPITP1 was low in comparison with DdPITP2 and DdSec14. Western blotting with the PITPα monoclonal antibody (5F12) shows cross-reactivity with DdPITP1 but not DdPITP2 (results not shown). Similarly, a polyclonal antibody

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                  #
DdSec14     73..DMCFLRYLRARNYIVSKSEKMLRDTLEWRKKFRPQDIQLGGDIREIGSAGCVY---VNKR 129
Sc Sec14    55..DSTLRFLRARKPDVQLAKEMFENCEKWRKDYGTDTILQDFHYDEKPLIAKFYPQYYHKT 114
Sp Sec14    50..DATLLRFLRARKPNLQQSLEMFIKCEKWRKEFGVDDLIKNFHYDEKEAVSKYYPQFYHKT 109
Ca Sec14    55..DASLLRFLRARKFDIQKAIDMFVACEKWREDPGVNTILKDFHYEEKPIVAKMYPTYYHKT 114
                *  :**:****:: :. : .*:    :**:.:  : :  . .  *    .  *    :*

DdSec14         DKKGRPIIFAVP----RNDTLKNVPSELKFKNLVYWLEQGFSRMDEPKG------IEQFC 179
Sc Sec14        DKDGRPVYPEELGAVNLHEMNKVTSEERMLKNLVWEYESVVQYRLPACSRAAGHLVETSC 174
Sp Sec1         DIDGRPVYVEQLGNIDLKKLYQITTPERMMQNLVYEYEMLALKRFPACSRKAGGLIETSC 169
Ca Sec14        DKDGRPVYPEELGKVDLVKMLKITTQERMLKNLVWEYEAMCQYRLPACSRKAGYLVETSC 174
                * .***: .          .  : .. *  ::***:  *        . .      :*  *
                                   #
DdSec14         FIVDYKDFGSGNMD--MKTNLEAMHFLLDHCPERMGQSLFLDPPALFWFAWKIISPFLNE 237
Sc Sec14        TIMDLKGISISSAYSVMSYVREASYISQNYYPERMGKFYIINAPFGFSTAFRLFKPFLDP 234
Sp Sec14        TIMDLKGVGITSIHSVYSYIRQASSISQDYYPERMGKFYVINAPWGFSSAFNLIKGFLDE 229
Ca Sec14        TVLDLSGISVTSAYNVIGYVREASKIGQDYYPERMGKFYLINAPFGFSTAFKLFKPFLDP 234
                 ::* .... .          :*  :  :: *****:  .::.*  *  *:.::. **:
                  #
DdSec14         VTLSKVRFINSKKVDGKRTFAELLEYVDIENLEQNLGG..275
Sc Sec14        VTVSKIFILGSS------YQKELLKQIPAENLPVKFGG..266
Sp Sec14        ATVKKIHILGSN------YKSALLEQIPADNLPAKLGG..261
Ca Sec          VTVSKIHILGYS------YKKELLKQIPPQNLPVKFGG..266
                .*:.*: ::. .          **: :  :**  ::**
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Figure 4 Alignment of *Dictyostelium* Sec14 with Sec14 proteins from yeasts

DdSec14 is aligned with proteins from *S. cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp) and *Candida albicans* (Ca). Other regions show no discernible similarity to DdSec14. Protein sequences were aligned with the CLUSTAL W program. Asterisks indicate identity, colons indicate conserved substitutions, and full stops indicate semi-conserved substitutions. Identical residues are also shown in bold. The residues Lys-66, Glu-207 and Lys-239, which have been suggested to be important in binding inositol headgroups, are marked with #; Lys-66 is not conserved in DdSec14.

against PITP, which recognizes both PITPα and PITPβ, immunoreacted only with DdPITP1, not with DdPITP2. However, it should be noted that the polyclonal antibody recognized PITPα more strongly than PITPβ. A monoclonal antibody specific for PITPβ (IB11) did not cross-react with any of the *Dictyostelium* PITPs (results not shown). A polyclonal antibody against *Saccharomyces* Sec14p also cross-reacted with DdSec14 (results not shown).

Assays for PtdIns transfer activity and PLC reconstitution

Analysis of the PtdIns transfer activity of the expressed *Dictyostelium* PITPs *in vitro* indicate that all three proteins are active (Figures 5A–5C). Mammalian PITPα and PITPβ, as well as *Saccharomyces* Sec14p, are capable of reconstituting the G-protein-stimulated PLCβ activity of HL60 cells that have been permeabilized and depleted of cytoplasm [30]. This assay provides a read-out of the ability of these proteins to promote PtdIns(4,5)P_2 synthesis. All three proteins were tested in this assay and were found to be active (Figures 5D–5F).

Localization of PITPs in mammalian cells

Mammalian PITPα is a highly mobile protein, as demonstrated by its ability to leak rapidly out of permeabilized cells [15], and it has been shown to be diffusely localized both in the cytosol and in the nucleus [31]. In contrast, PITPβ is found at the Golgi [31,32]. This differential localization indicates a difference in function of these two mammalian PITPs. To examine whether DdPITPs were functionally similar to PITPα or PITPβ, we tagged both the mammalian and *Dictyostelium* PITPs with enhanced GFP at the N-terminus and transiently expressed these proteins in RBL-2H3 mast cells (Figure 6). PITPα was localized in both the cytosol and the nucleus, whereas PITPβ was found in the Golgi as well as the cell periphery but not in the nucleus. In contrast, both DdPITP1 and DdPITP2 were found in a distribution similar to that of PITPβ and were specifically excluded from the nucleus. Thus although DdPITP1 is immunoreactive with PITPα, functionally it corresponds more closely to PITPβ and might have a specific role in membrane traffic at the Golgi.

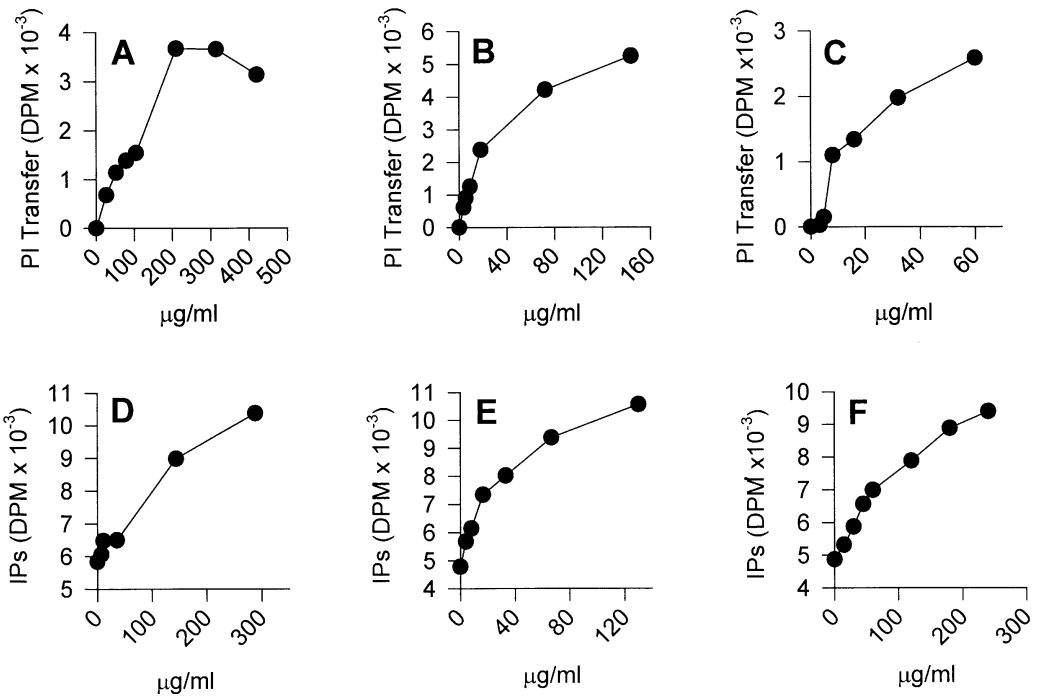

Figure 5 PITP proteins from *Dictyostelium* are all capable of PtdIns transfer *in vitro* and of reconstituting PLCβ activity in cytosol-depleted HL60 cells

Dictyostelium proteins were expressed in *E. coli*, purified with the His_6 tag and tested for their ability to transfer ^{3}H-labelled PtdIns from microsomes to liposomes (**A**–**C**) and for PLC reconstitution (**D**–**F**). HL60 cells were prelabelled with [^{3}H]inositol, permeabilized to deplete cytosolic proteins, washed and incubated with GTP[S] and increasing concentrations of PITPs. The release of inositol phosphates was used as a measure of PLC activity. (**A**, **D**) DdPITP1; (**B**, **E**) DdPITP2; (**C**, **F**) DdSec14.

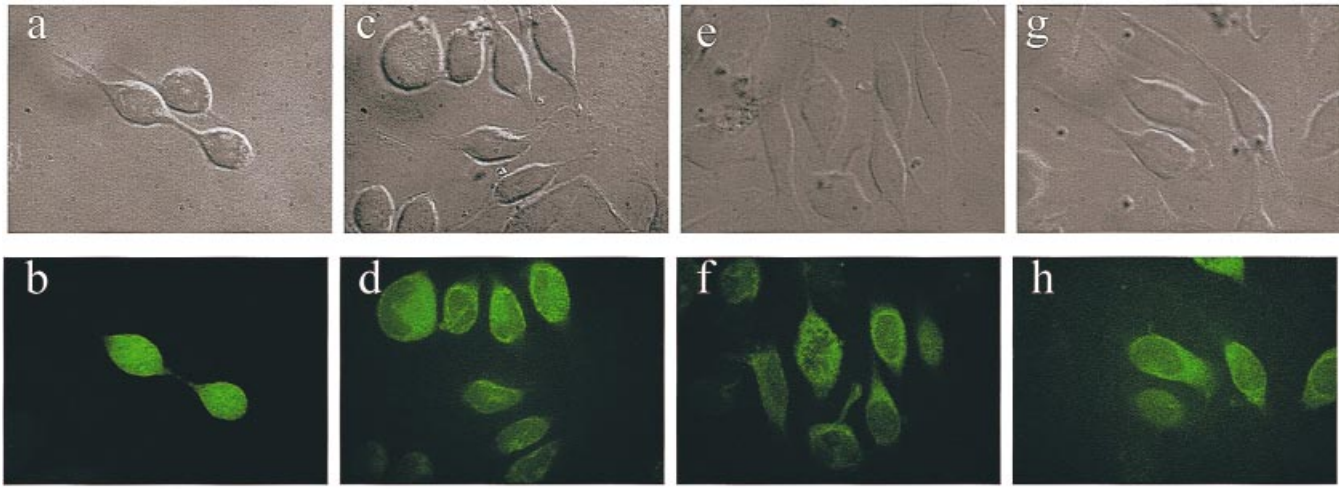

Figure 6 Localization of GFP-tagged PITPs in RBL-2H3 mast cells

PITPα (**a**, **b**), PITPβ (**c**, **d**), DdPITP1 (**e**, **f**) and DdPITP2 (**g**, **h**) were fused with GFP and transiently transfected into RBL-2H3 mast cells. After 24 h the cells were fixed and analysed by confocal microscopy. Nomarski images (**a**, **c**, **e**, **g**) and mid-plane images (**b**, **d**, **f**, **h**) of the cells are shown.

In summary, we have identified the presence of multiple PITPs present in the simple eukaryote *D. discoideum*. Mammalian PITPs and yeast PITPs, although unrelated by sequence, share many biochemical properties, including the ability to bind either PtdIns or PtdCho, and to transfer these lipids between membrane compartments, reconstitute vesicle formation and exocytosis and restore inositol phosphate production via PLC. Mutations in *SEC14* in yeast can be rescued by mammalian PITPs, and indeed PITPβ was identified in screens for the rescue of *SEC14* mutants [33]. *Dictyostelium* seems to contain at least three PITPs, two of which are related to mammalian PITPs and the other to Sec14p. In *S. cerevisiae* the function of *SEC14* is genetically defined as a requirement for vesicle traffic out of the Golgi [34]. Mammalian cells are more complex with respect to their requirements for

phosphoinositides; PITPs are required both for signalling events and for membrane traffic [7]. This includes both vesicle formation and exocytosis [11–13]. *Dictyostelium* systems have more in common with mammalian systems with regard to their use of phosphoinositides in signalling cascades [35]. In addition, an important aspect of this organism is that they specialize in both chemotaxis and phagocytosis, both being processes dependent on membrane movement [36,37]. Thus the identification of PITPs related to both mammalian PITPs and yeast Sec14p in a single organism will provide a unique opportunity to examine the functions of this class of proteins with genetic approaches.

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