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PPIP5K1 modulates ligand competition between diphosphoinositol polyphosphates and PtdIns(3,4,5) P_3 for polyphosphoinositide-binding domains

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Abstract

We describe new signalling consequences for PPIP5K1 (diphosphoinositol pentakisphosphate kinase type 1)-mediated phosphorylation of Ins P_6 and 5-Ins P_7 to 1-Ins P_7 and Ins P_8 . In NIH 3T3 cells, either hyperosmotic stress or receptor activation by PDGF (platelet-derived growth factor) promoted translocation of PPIP5K1 from the cytoplasm to the plasma membrane. The PBD1 (polyphosphoinositide-binding domain) in PPIP5K1 recapitulated that translocation. Mutagenesis of PBD1 to reduce affinity for PtdIns(3,4,5) P_3 prevented translocation. Using surface plasmon resonance, we found that PBD1 association with vesicular PtdIns(3,4,5) P_3 was inhibited by Ins P_6 and diphosphoinositol polyphosphates. However, the inhibition by PPIP5K1 substrates (IC₅₀: 5-Ins P_7 = 5 μ M and Ins P_6 = 7 μ M) was substantially more potent than that of the PPIP5K1 products (IC₅₀: Ins P_8 = 32 μ M and 1-Ins P_7 = 43 μ M). This rank order of ligand competition with PtdIns(3,4,5) P_3 was also exhibited by the PH (pleckstrin homology) domains of Akt (also known as protein kinase B), GRP1 (general receptor for phosphoinositides 1) and SIN1 (stress-activated protein kinase-interaction protein 1). We propose that, *in vivo*, PH domain binding of Ins P_6 and 5-Ins P_7 suppresses inappropriate signalling ('noise') from stochastic increases in PtdIns(3,4,5) P_3 . That restraint may be relieved by localized depletion of Ins P_6 and 5-Ins P_7 at the plasma membrane following PPIP5K1 recruitment. We tested this hypothesis in insulin-stimulated L6 myoblasts, using mTOR (mechanistic/mammalian target of rapamycin)-mediated phosphorylation of Akt on Ser⁴⁷³ as a readout for SIN1-mediated translocation of mTORC (mTOR complex) 2 to the plasma membrane [Zoncu, Efeyan and Sabatini (2011) *Nat. Rev. Mol. Cell Biol.* **12**, 21–35]. Knockdown of PPIP5K1 expression was associated with a 40 % reduction in Ser⁴⁷³ phosphorylation. A common feature of PtdIns(3,4,5) P_3 -based signalling cascades may be their regulation by PPIP5K1.

Keywords

diphosphoinositol polyphosphate; inositol pyrophosphate; phosphoinositide 3-kinase (PI3K); phosphoinositide

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AUTHOR CONTRIBUTION

All authors designed and performed the experiments, analysed the data, and contributed to writing the paper.

INTRODUCTION

The phosphate group is a ubiquitous signalling device that establishes specificity in ligand–protein and protein–protein interactions. The phosphate's bulk imposes geometric constraints on these interactions. The phosphate's negative charge also bestows specificity through ionic and hydrogen bonds with certain amino acid residues at physiological pH. An extreme example of these applications for the phosphate group is provided by the *PP*-InsPs (diphosphoinositol polyphosphates), also known as 'inositol pyrophosphates'. These molecules exhibit Nature's most crowded 3D array of phosphate groups. However, there is much that we do not understand concerning the significance of specific proteins interacting with these multiple phosphate groups in *PP*-InsPs.

Three *PP*-InsPs are formed by two groups of enzymes; first, the 5-kinase activities of IP6K (inositol hexakisphosphate kinase) 1/2/3 [1,2] convert InsP₆ and 1-InsP₇ to 5-InsP₇ and InsP₈ respectively. Secondly, the 1-kinase activities of PPIP5K (diphosphoinositol pentakisphosphate kinase) 1/2 [3–5] phosphorylate InsP₆ and 5-InsP₇ to 1-InsP₇ and InsP₈ respectively. These *PP*-InsPs regulate many cellular processes in eukaryotes, including stress responses, apoptosis, vesicle trafficking, cytoskeletal dynamics, exocytosis, insulin signalling and neutrophil activation (for reviews, see [6–9]). It has been challenging to provide a mechanism of action of *PP*-InsPs that adequately explains how these molecules can regulate such a variety of biological processes.

Experiments with yeasts [10,11] have indicated that the Pho80/81/85 cyclin-dependent kinase complex is allosterically inhibited by 1-InsP₇ (inadvertently thought to be 4/6-InsP₇ in those reports, see [12]). However, those observations have not helped us understand the promiscuity of *PP*-InsP signalling in mammalian cells, in which 1-InsP₇ is a minor constituent and 5-InsP₇ is present at much higher levels [12,13]. An alternative mechanism of action of the *PP*-InsPs has been advanced by Snyder, Saiardi and co-workers that does offer multiple signalling opportunities: protein diphosphorylation [14–16]. *In vitro* at least, *PP*-InsPs non-enzymically donate a phosphate to a pre-existing serine phosphate [14–16]. However, that hypothesis does not rationalize why there are several *PP*-InsPs that are all equally capable of phosphorylating proteins (see [17]). In any case, techniques are not yet available to directly determine whether this protein diphosphorylation can occur *in vivo*.

The focus of the present study began with a separate mechanism of action that has been proposed just for 5-InsP₇. Again, it is Snyder and co-workers who have been at the forefront of this work [18–20]. This group has shown that, *in vitro*, 5-InsP₇ can out-compete PtdIns(3,4,5)P₃ for binding to certain PH (pleckstrin homology) domains, most notably that of the protein kinase Akt (or protein kinase B) [18–20]. Moreover, 5-InsP₇ was reported to have at least a 10-fold higher affinity for PH domains than did InsP₆ [20]. Thus PtdIns(3,4,5)P₃-mediated translocation of Akt and other PH domain proteins from the cytoplasm to the plasma membrane, a process that promotes the co-localization of multiprotein complexes and priming of kinase cascades [21], is viewed as being inhibited by the formation of 5-InsP₇ from InsP₆ [18–20]. That hypothesis has been supported by experimental manipulation of IP6K expression in intact cells [18–20]. Nevertheless, the latter phenomenon does not account for the significance of 1-InsP₇ and InsP₈. We considered that such information might be obtained by comparing the relative affinities of 1-InsP₇, 5-InsP₇ and InsP₈ for PtdIns(3,4,5)P₃-binding domains. We selected three PH domain proteins for these studies: (i) Akt, owing to the attention that particular kinase has received from Snyder and co-workers for its binding of 5-InsP₇ [18–20]; (ii) GRP1 (general receptor for phosphoinositides 1), one of several phosphoinositide-dependent guanine-nucleotide-exchange factors [22,23], which show relatively high selectivity for PtdIns(3,4,5)P₃ over other inositol lipids [23,24]; and (iii) SIN1 (stress-activated protein

kinase-interaction protein 1); the structure of its PH domain was solved recently (PDB codes 3ULB, 3ULC and 3VOQ) [25]. SIN1 mediates PtdIns(3,4,5) P_3 -dependent translocation of mTORC2 {mTOR [mammalian (or mechanistic) target of rapamycin] complex 2} to the plasma membrane [26]. The mTOR within mTORC2 phosphorylates Ser⁴⁷³ on Akt [26,27]. Thus genetic ablation of SIN1 eliminates stimulus-dependent Ser⁴⁷³ phosphorylation [27].

We reasoned further that insight into the relative affinities of the different *PP*-InsPs for PtdIns(3,4,5) P_3 -binding PH domains could also help explain the biological significance of receptor-mediated translocation of PIP5K1 to the plasma membrane [28]. For example, that information could inform us whether the kinase activity of PIP5K (phosphorylation of Ins P_6 and 5-Ins P_7 to 1-Ins P_7 and Ins P_8 respectively) might locally modulate the degree of ligand competition with PtdIns(3,4,5) P_3 for PH domains. Moreover, recruitment of PIP5K1 to the plasma membrane itself involves binding to PtdIns(3,4,5) P_3 [28]. So we also determined the relative abilities of Ins P_6 and the *PP*-InsPs to compete with PtdIns(3,4,5) P_3 for the PBD1 (polyphosphoinositide-binding domain) within PIP5K1.

There has not previously been an analysis of *PP*-InsP specificity for any PtdIns(3,4,5) P_3 -binding domains. Indeed, none of the *PP*-InsPs are commercially available. Additionally, during most of the time that we were working on the present study, we were unaware of any laboratories that were chemically synthesizing these materials (only recently, one group announced that they now produce 5-Ins P_7 [29]). Instead, we prepared *PP*-InsPs enzymatically [12], and using SDS/PAGE methodology [30], our preparations of these molecules were 96–98 % pure [31]. We used SPR (surface plasmon resonance) [32] to assay the relative affinities with which Ins P_6 and the *PP*-InsPs compete with PtdIns(3,4,5) P_3 . Intriguingly, Snyder's group have reported that the potency of 5-Ins P_7 binding to PH domains is complicated by an 'order-of-addition' effect. In their hands, 5-Ins P_7 bound more strongly to Akt when it was added before, rather than after, the PtdIns(3,4,5) P_3 [19]. Thus, in our studies into ligand competition, we followed Snyder's protocol, by premixing either Ins P_6 or *PP*-InsP with the test protein construct, before its injection over immobilized PtdIns(3,4,5) P_3 .

We also considered that these studies might have a broader significance that goes beyond the consequences of receptor activation: cellular levels of PtdIns(3,4,5) P_3 have been shown to increase after cells are subjected to a hyperosmotic challenge [33,34]. The newly formed PtdIns(3,4,5) P_3 acts with other intracellular signals to protect against the potentially lethal consequences of osmotic imbalance, including perturbations to cellular hydration and cytoskeletal integrity, metabolic disruptions, and genomic instability [35,36]. Such stress is not simply restricted to those cell types that reside in anisomotic environments, such as kidney cells, airway epithelial cells, lymphocytes and cells that make up bone and cartilage [35,37–39]. Anisomosis is also a potential hazard for any cell that transports metabolites and ions, or undertakes polymerization or depolymerization of cellular materials [40]. Yet, surprisingly, among the few studies that have considered whether hyperosmotic stress might promote translocation of PH domains to the plasma membrane, several have reported that it does not occur [34,41–43]. Moreover, hyperosmotic stress dramatically activates the synthesis of Ins P_8 [44]. Thus a final goal of the present study was to investigate whether ligand competition between PtdIns(3,4,5) P_3 and *PP*-InsPs might be relevant to cellular responses to hyperosmotic stress.

MATERIALS AND METHODS

Cell lines, cell culture and [³H]inositol radiolabelling

NIH 3T3 cells were cultured in six-well dishes in DMEM (Dulbecco's modified Eagle's medium) (11965–092, Invitrogen) containing 10 % FBS (Invitrogen) and 1 mM sodium

pyruvate (Sigma) at 37 °C with 5 % CO₂. Cells were radiolabelled with 20 μ Ci/ml [³H]inositol as described previously [45]. Rat myoblast L6 cells were purchased from A.T.C.C. (Manassas, VA, U.S.A.) (CRL-1458TM) and cultured in MEM (minimal essential medium) α (Invitrogen; A1049001) supplemented with 10 % FBS at 37 °C with 5 % CO₂. To create a stable L6^{K1-KD} [L6 cell line with reduced ('knocked-down') expression of PPIP5K1], the shRNA construct GI716 021 in the pGFP-V-RS vector (Origene Technologies) was used. The TR30013 non-effective shRNA cassette in the pGFP-V-RS plasmid was used to prepare L6^{CONTROL} (control L6 cells). Puromycin resistance carried by the vector was used for selection. DNA constructs encoding retroviral vectors (12 μ g), CMV gagpol (10 μ g) and pMD2.G (1.2 μ g) were cotransfected into HEK (human embryonic kidney)-293T cells on 100-mm dishes using LipofectamineTM 2000 (Invitrogen). Culture medium was replaced the following day and 24 h later the virus in the supernatants was collected by centrifugation (2000 *g* for 10 min at 4 °C) and used to infect L6 cells [MOI (multiplicity of infection) = 40]. Polyclonal cell lines were selected using 4 μ g/ml puromycin. All strains of L6 cells were limited to 12 passages. The L6 cells were radiolabelled for 5 days in 6-cm dishes with 23 μ Ci/ml [³H]inositol.

Experiments with cultured cells were performed after a 3 h period of serum starvation, in 2 ml medium containing 115 mM NaCl, 5 mM KCl, 1 mM NaH₂PO₄, 0.5 mM MgSO₄, 10 mM glucose, 1.35 mM CaCl₂ and 25 mM Hepes (pH 7.4 with NaOH).

The extraction and HPLC analysis of inositol polyphosphates and inositol lipids in [³H]inositol cells was performed as described [46] using a Partisphere SAX HPLC column [28,47].

DNA subcloning and protein purification

The full-length cDNA for human PPIP5K1 was used as a template for subcloning its PBD1 (amino acid residues 382–917 of PPIP5K1) into the vector pFLAG-CMV 5a (Sigma–Aldrich), with 5'-CGGAATTCATGCCACCACATCTGGCACT-3' as the EcoRI forward primer and 5'-GGGGTACCCTCAACACCTTTC-ACTCC-3' as the KpnI reverse primer respectively. The nucleotides underlined represent the EcoRI and KpnI restriction sites respectively. The mutational forward primers 5'-ATTGCAATT-ATTGCTCATGGGGATCGT-3' and 5'-GTGAAACACCCAGC-TTTTTTTGCTCTG-3' (the nucleotides in bold font represent the mutagenic codons), along with their corresponding reverse complements, were used to generate the double-mutant PBD1^{R399A/R417A} for subcloning into the vector pFLAG-CMV5a, by overlap extension PCR for alanine residue. All constructs were subsequently transformed into DH5 α cells for plasmid isolation and DNA sequencing.

The cDNA encoding GRP1^{PH} (PH domain of GRP1), subcloned into a modified pET-15 vector [48], was a gift from Dr D. Lambright (University of Massachusetts Medical School, MA, U.S.A.). The full-length SIN1 was a gift from Dr E. Jacinto (Robert Wood Johnson Medical School, NJ, U.S.A.). SIN1^{PH} (PH domain of SIN1) (residues 358–522 [25]) was subcloned into pET-21a using 5'-CGGGATCCATGAGGGCAGACGGGGTTTTC-3' as the BamHI forward primer and 5'-CCGCTCGAGCTGCT-GCCCGATTCTT-3' as the XhoI reverse primer. The bases underlined correspond to the restriction sites for BamHI and XhoI. These constructs for either GRP1^{PH} or SIN1^{PH} were each transformed into BL21(DE3) competent cells (Clontech), which were grown at 37 °C in LB broth containing 50 μ g/ml ampicillin. When the *D*₆₀₀ was 0.6, the temperature was reduced to 25 °C and expression was induced overnight with 1 mM IPTG. The next day, cells were collected by centrifugation (10 min, 2400 *g*, 4 °C) and resuspended in ice-cold lysis buffer containing 25 mM Hepes (pH 8), 300 mM NaCl, 10 mM imidazole, CompleteTM protease inhibitor cocktail tablets (1 tablet per 10 ml of the buffer; Roche, 11836170001) and 0.1 % Triton X-100. The

suspension was passed through a cooled continuous-flow cell disrupter (TS series; Constant Systems). The appropriate His₆-tagged peptide was then purified from the cell lysate by FPLC (ÄKTA system, GE Healthcare) first using a 0.5 ml Ni-NTA (Ni²⁺-nitrilotriacetate) column eluted with an imidazole-based gradient (pH 8) and then a 5 ml HiTrap SP HP column (GE Healthcare) using a NaCl gradient (pH 7).

Quantitative real-time PCR

Total RNA was isolated from L6^{K1-KD} and L6^{CONTROL} cells using an RNeasy kit with DNase treatment (Qiagen). To quantify mRNA, cDNA was produced using MuLV reverse transcriptase with poly-dT and random hexamer primers. The cDNA was amplified using the Applied Biosystem Fast SYBR[®] Green Master Mix in an Applied Biosystem 7500 Fast Real-Time machine. The following primers were used: rat PPIP5K1 sense, 5'-ACTAAAGTCTGCCAGCGCCACACTGAT-3' and rat PPIP5K1 antisense, 5'-GGCTTTTCAGAGCGATGTCTGAAGTT-3'; and rat GAPDH sense, 5'-AGAGACAGCCGCATCTTCTTG-3' and rat GAPDH antisense, 5'-GGTAACCAGGCGTCCGATAC-3'.

Cell proliferation

Cells (0.5×10⁵) were seeded on to 3.5-cm dishes in MEM α containing 10 % FBS. After 24 h, the FBS concentration was reduced to 2 % FBS. At daily intervals, cells were released by trypsin-treatment and cell numbers were ascertained with a Countess cell counter (Life Technologies).

Cell lysis and immunoblotting

Cells (4×10⁵) attached on to 6-cm dishes were washed twice with ice-cold PBS containing 1 mM Na₃VO₄, then lysed with RIPA buffer (Santa Cruz; 24948) (supplemented with 1 mM Na₃VO₄ and protease inhibitors according to the manufacturer's instructions) plus 25 μ M okadaic acid (LC Laboratories). Soluble fractions of lysates were prepared by centrifugation (10 min, 10 000 g, 4 °C). Samples of the cellular lysates containing equal amounts of proteins were resolved by SDS/PAGE and transferred on to PVDF membrane. Proteins were then visualized by immunoblotting and detected with enhanced chemiluminescence (GE Healthcare; RPN2132), or the WesternBright kit from Bioexpress. The following primary antibodies were used: rabbit anti-phospho-Akt antibody (pSer⁴⁷³) (Cell Signaling; 9271, 1:200 dilution); rabbit anti-Akt antibody (Cell Signaling; 9272, 1:200 dilution); rabbit anti- β -actin antibody (Abcam; 1:1000 dilution); immunoaffinity-purified rabbit anti-PPIP5K1 antibodies (raised against a peptide comprising residues 917–1120 conjugated to keyhole-limpet haemocyanin) purchased from Covance (1:1000 dilution); and anti-SIN1 monoclonal antibody (Millipore; 05–1044, 1:1000 dilution), kindly provided by Dr D. Sarbassov (University of Texas M.D. Anderson Cancer Center, Houston, TX, U.S.A.). For detection, we used one of the following horseradish peroxidase-conjugated (1:5000 dilution) secondary antibodies, which were obtained from Cell Signaling Technology: anti-(mouse IgG) (7076) and anti-(rabbit IgG) (7074) antibodies.

Surface plasmon resonance

Unilamellar vesicles [77:20:3 (v/v) phosphatidylcholine/phosphatidylethanolamine/polyphosphoinositide] were immobilized on a Biacore Pioneer L1 sensor chip [32,49] [the dimensions of the dextran matrix were 2.4 mm (length)×0.5 mm (width)×100 nm (height)]. We injected 90 μ l of the vesicles over the chip surface at a flow rate of 5 μ l/min until the signal reached 5000 AU (arbitrary response units), which corresponds to approximately 50 pg of phospholipid/cm² [50]. This is equivalent to a surface concentration of approximately 3×10¹⁴ phospholipid molecules/cm², close to the value of 2×10¹⁴ phospholipid molecules/

cm² found in a previous SPR analysis of immobilized phospholipid vesicles [51]. As mentioned above, the polyphosphoinositide [usually PtdIns(3,4,5)P₃] comprised 3 % of the total phospholipid.

Next, the protein under study was injected at a flow rate of 5 μ l/min in buffer containing 100 mM KCl, 10 mM NaH₂PO₄, 1 mM MgCl₂ and 25 mM Hepes (pH 7.2). The resulting sensorgram was automatically corrected for any signal arising from protein interacting with control vesicles from which the test phosphoinositide was omitted. The saturating RU values (R_{eq}) were plotted against the protein concentrations (C) and the equilibrium binding affinity (K_d) was subsequently determined by performing a non-linear least squares analysis of the binding isotherm using the equation $R_{eq} = R_{max}/(1 + K_d/C)$, where R_{max} is the maximal R_{eq} value. The S.E.M. of individual R_{eq} determinations did not exceed 1 % of the mean values.

Confocal immunofluorescence

Cells (1.5×10^5) were seeded on to 35-mm glass-bottom microwell dishes and transfected 24 h later with cDNA encoding either FLAG–PPIP5K1 (pDEST515 vector [4]), FLAG–PBD1 (pFLAG-CMV-5a vector [28]), FLAG–GRP1 (pFLAG vector kindly provided by Dr L.C. Santy, Pennsylvania State University, PA, U.S.A.) or EGFP–Akt^{PH} (pEGFP-N1 vector; SigmaGen Laboratories), all using LipofectamineTM 2000 (Invitrogen) according to the manufacturer's instructions. On the following day, cells were serum starved for 3 h and treated with the appropriate agonist as indicated in the Figure legends. For immunostaining, cells were rinsed twice with PBS, fixed with 1 % paraformaldehyde in PBS containing 4 % sucrose for 15 min at room temperature (20 °C) and permeabilized with 0.2 % Triton X-100 for 5 min, and washed with PBS. After blocking with 10 % BSA in PBS for 30 min at 37 °C, cells were incubated with the primary antibody (mouse monoclonal anti-FLAG antibody; Abcam, ab18230) for 2 h at 37 °C and washed with PBS, followed by 1 h incubation at 37 °C with the secondary antibody (Alexa Fluor[®] 488 goat anti-mouse; Invitrogen, A-11001) and washed with PBS before incubation with DAPI (Invitrogen, D1306) for 10 min at 37 °C. After washing the cells twice with PBS, confocal images were taken on a Zeiss LSM 510 META using a Plan-Apochromat X 63/1.4 oil objective. The cellular distribution of the protein constructs was recorded by line intensity profiles [52] using LSM Image Examiner software. Data were exported into SigmaPlot where the different cell widths were normalized for unit length while the total fluorescence intensity of each cell was taken as 100 %. The ratios of pixel intensity (plasma membrane/cytoplasm) were then calculated using an image segmentation protocol [53].

Other materials and methods

Human recombinant AKT1 (124006) was purchased from EMD Millipore, insulin was bought from Sigma–Aldrich and PDGF (platelet-derived growth factor) was supplied by R&D Systems. The 1-InsP₇, 5-InsP₇ and InsP₈ were prepared enzymatically as described previously [31]. Cytochalasin B was purchased from Sigma–Aldrich. Sources of other materials are as described previously [28]. Statistical analysis was performed with paired *t* tests ($P < 0.05$ was considered significant). Densitometric analysis of Western blots was performed with ImageJ, and data were normalized to the signal obtained from loading controls.

RESULTS

PBD1 recapitulates receptor-dependent translocation of PPIP5K1 to the plasma membrane *in vivo*

We used confocal microscopy to analyse the impact of receptor activation by PDGF on the cellular distribution of FLAG-tagged PtdIns(3,4,5)P₃-binding domains in NIH 3T3 cells

(Figure 1). The effect of receptor activation on the intracellular distribution of our constructs was quantified using an image segmentation protocol (see the Materials and methods section). In control experiments, FLAG-GRP1 was distributed throughout the cell until PDGF was added, whereupon it translocated to the plasma membrane (Figures 1A and 1B), as in a previous study [54].

In unstimulated cells, FLAG-PPIP5K1 was distributed throughout the cytoplasm, but it was excluded from the nucleus (Figure 1A). We found that 5 min stimulation of NIH 3T3 cells with PDGF caused PPIP5K1 to translocate from the cytoplasm to the plasma membrane (Figures 1A and 1C). Similar data were obtained in a previous study [28].

A modular view of PPIP5K1 [55] led us to previously propose that receptor-dependent translocation of PPIP5K1 is mediated solely by its PBD1 [28]. We have now tested that idea for the first time in intact cells. For these experiments, we transfected NIH 3T3 cells with FLAG-PBD1. Although some of the PBD1 was present in the nucleus, much of this protein was distributed throughout the cytoplasm (Figure 1A). The PBD1 translocated to the plasma membrane in response to PDGF (Figures 1A and 1D). A PBD1^{R399A/R417A} mutant that has reduced binding to PtdIns(3,4,5) P_3 [28] did not translocate to the plasma membrane after the addition of PDGF (Figure 1). These data led us to conclude that PBD1 is sufficient to recapitulate receptor-dependent translocation of PPIP5K1 to the plasma membrane *in vivo*.

Effects of PDGF on cellular levels of inositol lipids and *PP*-InsPs in NIH 3T3 cells

To gain insight into the biological relevance of ligand competition between PtdIns(3,4,5) P_3 and *PP*-InsPs, we next measured the levels of inositol lipids, as well as Ins P_6 and *PP*-InsPs, under the same conditions that we used in the translocation experiments (see above). We found that 5 min stimulation with 50 ng/ml PDGF increased levels of PtdIns(3,4,5) P_3 2-fold (Figure 2A). There was no significant effect on the levels of either PtdIns(3,4) P_2 or PtdIns(4,5) P_2 (Figures 2B and 2C). We also found that 5 min stimulation with PDGF did not alter the levels of either Ins P_6 , Ins P_7 or Ins P_8 (Figures 2D, 2E and 2F). Some of these observations contrast sharply with the results of a previous study [19] with mouse embryo fibroblasts, in which levels of Ins P_6 and Ins P_7 increased 2-fold following 5 min stimulation with IGF-1 (insulin-like growth factor 1) (the levels of Ins P_8 were not described). In that study [19], cells were serum starved overnight before the addition of IGF-1. In contrast, we removed serum from our cells only 3 h before addition of PDGF. However, we must note that Ins P_6 and Ins P_7 may perhaps differentially respond to receptor activation in a cell-type and/or agonist-specific manner.

Effects of hyperosmotic stress on cellular levels of inositol lipids and *PP*-InsPs in NIH 3T3 cells

We next found that 30 min exposure of NIH 3T3 cells to hyperosmotic stress (by treatment with 0.2 M sorbitol) elevated PtdIns(3,4,5) P_3 levels 4.5-fold (Figure 3A). Levels of PtdIns(3,4) P_2 were unaffected by the addition of sorbitol (Figure 3B), but PtdIns(4,5) P_2 levels fell slightly (Figure 3C), perhaps in part reflecting its increased hydrolysis due to PLC (phospholipase C) activation [56]. We also found that hyperosmotic stress slightly reduced Ins P_6 levels (Figure 3D). This effect has been observed previously in a study with HEK cells [4]. Levels of 5-Ins P_7 also decreased following hyperosmotic stress (Figure 3E), whereas Ins P_8 concentration increased 4.5-fold (Figure 3F), apparently reflecting an activation of PPIP5K [4]. Thus the *PP*-InsP profile in osmotically stressed cells (Figure 3) differs significantly from that in PDGF-stimulated cells (Figure 2). Could that impact the spatial dynamics of PtdIns(3,4,5) P_3 -binding domains in the osmotically challenged cells? That was the next question that we addressed.

Hyperosmotic stress promotes translocation of GRP1 and PIP5K1 to the plasma membrane

Several studies have reported that two proteins that contain PH domains, Akt and PDK1 (phosphoinositide-dependent kinase 1), do not translocate to the plasma membrane following a hyperosmotic challenge [34,41–43]. In contrast, we found that GRP1 translocated to the plasma membrane after cells were subjected to hyperosmotic stress (Figures 4A and 4B), to a similar degree to that observed after PDGF treatment (Figures 1A and 1B). To our knowledge, this recruitment of GRP1 reveals a novel cellular response to hyperosmotic stress, and may reflect adaptive responses of membrane trafficking, which GRP1 can regulate [22,23]. The observation that GRP1^{PH} translocated to the plasma membrane (Figures 4A and 4B), despite the accompanying increase in *InsP*₈ levels (Figure 3F), does not support speculation [20] that *InsP*₈ might be an especially potent inhibitor of PH domain association with *PtdIns*(3,4,5)*P*₃.

We found further that PIP5K1 translocated to the plasma membrane after hyperosmotic stress (Figures 4A and 4C). The degree of that translocation (Figures 4A and 4C) was similar to that which was observed after PDGF treatment (Figures 1A and 1C). Control experiments with cytochalasin (Figure 4A) indicated that the translocation of PIP5K was not secondary to cytoskeletal rearrangement, as has been reported for some PH-domain proteins [21]. Stress-induced translocation of PIP5K was recapitulated by the PBD1, but not by the R399A/R417A mutant (Figures 4A and 4D) that had lost its high affinity for *PtdIns*(3,4,5)*P*₃ [28].

*InsP*₆ and *PP-InsP*s compete with *PtdIns*(3,4,5)*P*₃ for binding to the PBD1

Snyder and co-workers [20] have reported that 5-*InsP*₇ competes with *PtdIns*(3,4,5)*P*₃ for certain PH domains. In the latter experiments, ligand competition was determined in co-sedimentation assays with *PtdIns*(3,4,5)*P*₃ in lipid vesicles. We have studied the relative affinities of PBD1 for 5-*InsP*₇ and the other *PP-InsP*s. We used SPR, which, as far as we are aware, has not been applied previously to the study of ligand competition between *PP-InsP*s and *PtdIns*(3,4,5)*P*₃ for any protein.

We first added the PBD1 of PIP5K to immobilized lipid vesicles that contained 3 % (v/v) *PtdIns*(3,4,5)*P*₃. For biological relevance, the mobile phase contained 100 mM KCl, 10 mM NaH₂PO₄ and 1 mM MgCl₂. In these experiments, the size of the sensorgram signal (the broken line labelled 'control' in Supplementary Figure S1 at <http://www.biochemj.org/bj/453/bj4530413add.htm>) corresponds to the degree of association of PBD1 to the lipid vesicles. No signal was observed in the absence of *PtdIns*(3,4,5)*P*₃ (not shown; see also [28]). We found that as little as 1 μM 5-*InsP*₇ (a physiological concentration; [57]) reduced (by approximately 15 %) the extent to which PBD1 associated with vesicle-bound *PtdIns*(3,4,5)*P*₃ (Supplementary Figure S1). We further found that 1 μM *InsP*₆ was approximately half as effective a ligand as 5-*InsP*₇ (Supplementary Figure S1).

Snyder and co-workers [20] used Mg²⁺-free medium in their experiments with *PtdIns*(3,4,5)*P*₃-binding domains. We found that the removal of Mg²⁺ from our buffers did not influence PBD1 binding to *PtdIns*(3,4,5)*P*₃ (Supplementary Figure S1). The absence of Mg²⁺ did slightly increase the potency with which 5-*InsP*₇ and *InsP*₆ competed with *PtdIns*(3,4,5)*P*₃, but without affecting the marginal preference for 5-*InsP*₇ as a ligand (Supplementary Figure S1). The presence of Mg²⁺ is more physiological, so we retained it in our further experiments described below.

More detailed ligand competition experiments (Figure 5) were used to determine the IC₅₀ values for *InsP*₆ and 5-*InsP*₇ competing with PBD1 binding to *PtdIns*(3,4,5)*P*₃ (14 μM and 7

μM respectively). What could be the physiological consequence of ligand competition between $\text{PtdIns}(3,4,5)\text{P}_3$ and InsP_6 or 5-InsP_7 ? In resting cells, in which PPIP5K1 is present in the cytoplasm (Figure 1B), we can predict from the data described in Figure 5 that InsP_6 and/or 5-InsP_7 would likely be associated with the PBD1. We propose that these inositol phosphates would tend to elevate the threshold concentration of $\text{PtdIns}(3,4,5)\text{P}_3$ that would need to be achieved before it would recruit PPIP5K to the plasma membrane. This might be a means by which the cell could ensure that such translocation of PPIP5K1 only occurred in response to a sustained, stimulus-dependent elevation in $\text{PtdIns}(3,4,5)\text{P}_3$, rather than to random fluctuations ('noise') in lipid levels.

Once concentrated at the plasma membrane, the kinase activity of PPIP5K1 could deplete the levels of InsP_6 and 5-InsP_7 and elevate levels of 1-InsP_7 and InsP_8 . These metabolic effects could be localized because of the reduced rates of diffusion in the narrow, subplasmalemmal 'unstirred' space [58]. To explore the possible consequences of such compartmentalization, we next examined the extent to which 1-InsP_7 and InsP_8 might inhibit binding of PBD1 to $\text{PtdIns}(3,4,5)\text{P}_3$. Such experiments represent a new development in inositol pyrophosphate research. To date, 5-InsP_7 is the only member of the $PP\text{-InsP}$ family that has been investigated as a possible ligand for any $\text{PtdIns}(3,4,5)\text{P}_3$ -binding domains, although there has been speculation of the potential biological significance if InsP_8 were to be a more potent ligand [7,9,20].

Our binding data (Figure 5) show that 1-InsP_7 ($\text{IC}_{50} = 43 \mu\text{M}$) and InsP_8 ($\text{IC}_{50} = 32 \mu\text{M}$) exhibit 6–7-fold lower affinity for PBD1 than InsP_6 and 5-InsP_7 . That is to say, the products of PPIP5K kinase activity, 1-InsP_7 and InsP_8 , were considerably weaker ligands than the substrates InsP_6 and 5-InsP_7 respectively. These data raise the possibility that, following its translocation to the plasma membrane, the kinase activity of PPIP5K might generate a microenvironment in which there is a reduction in the degree of competition of inositol phosphates with $\text{PtdIns}(3,4,5)\text{P}_3$ for the PBD1.

InsP_6 and $PP\text{-InsP}$ s compete with $\text{PtdIns}(3,4,5)\text{P}_3$ for the PH domains of GRP1 and Akt

Snyder and co-workers [20] have studied competition of both InsP_6 and 5-InsP_7 for $\text{PtdIns}(3,4,5)\text{P}_3$ binding by a range of PH domains; in each case, 5-InsP_7 was reported to be 10–20-fold more potent at competing with $\text{PtdIns}(3,4,5)\text{P}_3$ than InsP_6 . The PBD1 of PPIP5K1 did not exhibit that high degree of ligand specificity (Figure 5). So we next studied binding of InsP_6 and $PP\text{-InsP}$ s to some PH domains. Both of these inositol phosphates inhibited GRP1^{PH} association with $\text{PtdIns}(3,4,5)\text{P}_3$; the effects of $10 \mu\text{M}$ were particularly dramatic (Supplementary Figure S2 at <http://www.biochemj.org/bj/453/bj4530413add.htm>). Nevertheless, 5-InsP_7 was only marginally more potent than InsP_6 (Supplementary Figure S2). Moreover, as was the case with PBD1, we found that the products of PPIP5K kinase activity, 1-InsP_7 and InsP_8 , were considerably weaker ligands for GRP1^{PH} than InsP_6 and 5-InsP_7 substrates (Supplementary Figure S2). These data led us to hypothesize that the kinase activity of PPIP5K1 at the plasma membrane might generate a localized environment that favours GRP1 association with $\text{PtdIns}(3,4,5)\text{P}_3$. We next considered whether this proposed function of PPIP5K1 might be relevant to other proteins with $\text{PtdIns}(3,4,5)\text{P}_3$ -selective PH domains.

It has been Akt that has received most attention with regard to competition between $\text{PtdIns}(3,4,5)\text{P}_3$ and 5-InsP_7 [18–20]. Thus we next studied the interaction of InsP_6 and the $PP\text{-InsP}$ s with Akt (Figures 6A–6D) from which we obtained IC_{50} data (Figures 6E–6H). In these experiments, InsP_6 ($\text{IC}_{50} = 14 \mu\text{M}$) was half as potent as 5-InsP_7 ($\text{IC}_{50} = 7 \mu\text{M}$) at competing with $\text{PtdIns}(3,4,5)\text{P}_3$. That difference in relative affinities of InsP_6 and 5-InsP_7 for Akt is much lower than the 10–20-fold difference reported previously [20]. We further

found that the affinities of Akt for 1-InsP₇ (IC₅₀ = 45 μ M) and InsP₈ (IC₅₀ > 50 μ M) were severalfold lower than those for InsP₆ and 5-InsP₇ (Figure 6). These results with Akt mirror those that were obtained with GRP1^{PH} (Supplementary Figure S2) and PBD1 (Figure 5): the products of PIP5K kinase activity, 1-InsP₇ and InsP₈, were weaker ligands than the substrates InsP₆ and 5-InsP₇.

Competitive binding of the PH domain of SIN1 to PtdIns(3,4,5)P₃, InsP₆ and PP-InsPs

We next studied the interactions of inositol pyrophosphates with SIN1^{PH}; its crystal structure was published recently [25]. We found that SIN1^{PH} bound to PtdIns(3,4,5)P₃ in lipid vesicles with an affinity (K_d = 141 nM; Figures 7A and 7B) that was similar to the values determined previously by SPR, under comparable experimental conditions, for PBD1 (K_d = 96 nM [28]) and GRP1^{PH} (K_d = 59–170 nM [28,59]); Akt^{PH} has been reported to have a 4-fold higher K_d value for PtdIns(3,4,5)P₃ (35 nM [60]). Some binding of SIN1^{PH} to PtdIns(4,5)P₂ was also detected, but with severalfold lower affinity (K_d = 629 nM; Figures 7B and 7D) than that for PtdIns(3,4,5)P₃.

In ligand competition experiments (Figure 8), we found that the IC₅₀ values for InsP₆ and 5-InsP₇ were very similar at 6 and 3.7 μ M respectively. Limitations of ligand supply prevented us from precisely determining the much weaker affinities for 1-InsP₇ and InsP₈, but by extrapolation we estimate the IC₅₀ values to exceed 100 μ M (Figure 8). Compared with the other PH domains that we have tested, SIN1^{PH} exhibited the greatest degree of discrimination between the substrates and the products of PIP5K1 activity. Nevertheless, the identical rank order of affinities for InsP₆ and PP-InsPs exhibited by PBD1, GRP1, Akt and SIN1 suggest that this could be a property of other PtdIns(3,4,5)P₃-selective PH domains.

Effects of PIP5K1 knockdown on a PtdIns(3,4,5)P₃-signalling cascade in intact cells: SIN1-dependent Akt phosphorylation

The data described above led us to hypothesize that, following translocation to the plasma membrane of PIP5K1, its kinase activity may generate a localized environment of inositol phosphate levels that favours the association of PH domain proteins with PtdIns(3,4,5)P₃. We next interrogated this concept in intact cells, by investigating whether a reduction in PIP5K1 expression would inhibit the activity of an endogenously expressed PtdIns(3,4,5)P₃-dependent kinase cascade. Among the proteins that we have studied (see above), it is the PH domain in SIN1 that shows the greatest degree of difference in relative affinities for the substrates versus the products of PIP5K. We therefore considered that an assay for SIN1 function might provide the more sensitive readout of the biological consequences of PIP5K1 knockdown.

SIN1 mediates PtdIns(3,4,5)P₃-dependent translocation of the mTORC2 to the plasma membrane [26]. The mTOR within mTORC2 phosphorylates Ser⁴⁷³ on Akt [26,27]. Thus genetic ablation of SIN1 eliminated stimulus-dependent Ser⁴⁷³ phosphorylation and, significantly, without impacting the degree of phosphorylation of Thr³⁰⁸ by PDK1 [27]. We therefore used Ser⁴⁷³ phosphorylation of Akt as a biological readout in our search for possible changes in SIN1 function following knockdown of PIP5K1 expression. We used the L6 skeletal myoblast cell line for these experiments, since previous work [3] has shown that PIP5K1 is relatively highly expressed in skeletal muscle; we posited, therefore, that the consequences of PIP5K1 knockdown might be particularly significant in a skeletal muscle cell model. L6 cells were also considered suitable for these experiments because, similar to NIH 3T3 cells (see above), there was a persistent translocation of FLAG–PIP5K1 to the plasma membrane when cells were stimulated with an agonist (insulin) that

promotes PtdIns(3,4,5) P_3 accumulation (Supplementary Figure S3 at <http://www.biochemj.org/bj/453/bj4530413add.htm>).

By using retroviral delivery of shRNA against PPIP5K1, we created a new L6 cell line (L6^{K1-KD}) in which the levels of PPIP5K protein were 70 % lower than those in L6^{CONTROL} that was infected with a non-effective shRNA (Figures 9A–9C). Importantly, the degree of expression of SIN1 protein was similar in these two cell lines (Figure 9A). The PPIP5K1 knockdown did not affect the levels of Ins P_6 (Figure 9D), but the levels of Ins P_7 (Figure 9E) and Ins P_8 (Figure 9F) were 17 % ($P < 0.02$) and 37 % ($P < 0.001$) lower in L6^{K1-KD} cells compared with L6^{CONTROL} cells. These data are consistent with PPIP5K1 contributing to both 1-Ins P_7 and Ins P_8 synthesis in intact cells. The fact that the diminution in Ins P_8 was less than the degree of the knockdown may reflect the presence of PPIP5K2 in these cells.

Knockdown of PPIP5K1 expression did not affect insulin-stimulated PDK1-mediated [26] phosphorylation of Thr³⁰⁸ on Akt (Supplementary Figure S4 at <http://www.biochemj.org/bj/453/bj4530413add.htm>), indicating that there was near-normal recruitment of Akt to the plasma membrane. Indeed, we observed similar rates of insulin-dependent translocation of exogenous GFP–Akt^{PH} to the plasma membrane in L6^{K1-KD} and L6^{CONTROL} cells (Supplementary Movie 1 at <http://www.biochemj.org/bj/453/bj4530413add.htm>). On the other hand, we found that at 5 and 15 min after the addition of insulin, the degree of phosphorylation of Ser⁴⁷³ on Akt was 40 % lower in L6^{K1-KD} cells than in L6^{CONTROL} cells (Figures 9G and 9H). These data are consistent with knockdown of PPIP5K1 impairing PtdIns(3,4,5) P_3 -mediated SIN1-dependent recruitment of mTORC2 to the plasma membrane, and hence being responsible for a reduction in the extent to which mTOR phosphorylates Akt. Finally, since the mTORC2 complex to which SIN1 belongs is believed to contribute to the regulation of cell growth, we measured the rates of proliferation of the L6^{K1-KD} and L6^{CONTROL} cells (Figure 9I). The knockdown of PPIP5K1 expression reduced the rate of cell growth by approximately 30 % (Figure 9I).

DISCUSSION

The present study provides new information concerning the participation of PPIP5K in cell signalling. For example, we show that the stimulus-dependent PtdIns(3,4,5) P_3 -mediated translocation of PPIP5K1 to the plasma membrane can be fully recapitulated by its PBD1 (Figure 1). We further show that this translocation of PPIP5K1 is more widespread than was originally appreciated, since it occurs following the increases in PtdIns(3,4,5) P_3 that result from either receptor activation or hyperosmotic stress (Figures 1–4). We have also conducted the first analysis of the specificity with which *PP*-Ins P_s compete for PtdIns(3,4,5) P_3 -binding domains (Figures 5, 6 and 8, and Supplementary Figure S2). Consequently, we have discovered that binding of PtdIns(3,4,5) P_3 is more strongly competed by the substrates of PPIP5K (Ins P_6 and 5-Ins P_7) than by the products (1-Ins P_7 and Ins P_8). We propose that, in resting cells, ligand competition from cytoplasmic Ins P_6 and *PP*-Ins P_s will restrain non-sustained stochastic increases in PtdIns(3,4,5) P_3 from recruiting signalling proteins to the plasma membrane. However, once more prolonged stimulus-dependent increases in PtdIns(3,4,5) P_3 outcompete binding of inositol phosphates, PPIP5K1 will then translocate to the plasma membrane (Figures 1 and 4). The ensuing local increase in PPIP5K1 catalytic activity may sustain a subplasmalemmal microenvironment in which the concentrations of Ins P_6 and 5-Ins P_7 are lower than those in the cytosol. This, in turn, would support further translocation of both PPIP5K1 and PtdIns(3,4,5) P_3 -binding PH domains (Figure 10). That represents a potential positive feedback mechanism; many signalling cascades rely on such amplification in order to prevent mechanisms of noise suppression constraining response sensitivity [61]. Thus optimum signalling by PtdIns(3,4,5) P_3 may depend on coincidence detection: the translocation of both PPIP5K and

a PH-domain protein. We obtained data in support of this model by showing decreased activation of an endogenous PtdIns(3,4,5) P_3 signalling cascade in a cell line in which PPIP5K1 expression was knocked down (Figure 9).

We determined that there are approximately 2-fold differences in the affinities with which Ins P_6 and 5-Ins P_7 bind to Akt and certain other PH domains; this is a less dramatic discrimination than the 10- to 20-fold differences reported by others [9,20]. Methodological differences may be responsible; we used SPR, a favoured technique for quantitative analysis of ligand–protein interactions [32], and we employed buffers that approximated physiological conditions. Additionally, in our hands, the rank order of relative affinities of Ins P_6 and PP -Ins P_s for PtdIns(3,4,5) P_3 -selective protein domains (5-Ins P_7 > Ins P_6 > 1-Ins P_7 and Ins P_8) did not correlate with phosphate number. This new information concerning the interactions of PP -Ins P_s with PH domains not only helps to determine its biological significance, but could also assist the goal of developing therapeutically relevant drugs that target PH domain–PtdIns(3,4,5) P_3 interactions [62].

Our hypothesis that a localized catalytic activity of PPIP5K1 might regulate PtdIns(3,4,5) P_3 signalling cascades is strengthened by a body of evidence describing reduced rates of diffusion in the narrow, subplasmalemmal ‘unstirred’ space [58]. For example, in the subplasmalemmal space the diffusion coefficient of cAMP has been estimated to be seven orders of magnitude slower than that in the bulk of the cytoplasm [58]. Also of relevance is an emerging opinion [7,16,63] that the free cytoplasmic concentration ratio of Ins P_6 /Ins P_7 is lower than that suggested by assays of total cellular Ins P_6 , due to the latter’s compartmentalization. For example, Ins P_6 is divided between metabolically inert and (smaller) receptor-regulated pools [63]; the latter description of metabolic compartmentalization implies some physical separation of these different Ins P_6 pools. Indeed, the mammalian Ins(1,3,4,5,6) P_5 2-kinase that produces Ins P_6 is predominantly a nuclear enzyme [64]. Furthermore, the only known mammalian Ins P_6 phosphatase (MINPP; multiple inositol polyphosphate phosphatase) is restricted to the lumen of endoplasmic reticulum and Golgi [65,66]. Perhaps Ins P_6 enters a luminal pool through a transmembrane Ins P_6 transport protein such as that which is found in plants [67]. Other evidence of a cellular pool of Ins P_6 that does not freely exchange with the bulk cytoplasm comes from the demonstration that this polyphosphate is a structural cofactor that is deeply buried within adenosine deaminase [68]. There are other known examples of Ins P_6 fulfilling this structural function in plants [69], and so its wider applicability to proteins in mammalian cells can be anticipated. Even electrostatic binding of Ins P_6 to the surface of proteins may significantly reduce its soluble concentration, especially as so many proteins can bind to this inositol phosphate [70].

We have also used a PPIP5K1 knockdown cell model to directly challenge our new hypothesis that PtdIns(3,4,5) P_3 signalling cascades might be regulated by PPIP5K1 (Figure 9). To maximize biological relevance, we looked for an effect of this knockdown on an endogenously expressed PtdIns(3,4,5) P_3 signalling cascade, rather than one in which the component under study was overexpressed. We also anticipated that another important factor might be the relative affinities of a PtdIns(3,4,5) P_3 -binding protein for the substrates and products of PPIP5K1. The latter consideration led us to focus on SIN1; 1-Ins P_7 and Ins P_8 bound to SIN1 with almost two orders of magnitude lower affinity than Ins P_6 and 5-Ins P_7 (Figure 8). This difference in relative affinities was considerably larger than those of the other PtdIns(3,4,5) P_3 -binding domains that we investigated in the present study (Figures 5 and 6, and Supplementary Figure S2).

SIN1 mediates PtdIns(3,4,5) P_3 -dependent translocation of the mTORC2 multiprotein complex to the plasma membrane [26]. The mTOR within mTORC2 phosphorylates Ser⁴⁷³

on Akt [26,27]. Using the degree of Ser⁴⁷³ phosphorylation as a readout for SIN1-mediated PtdIns(3,4,5) P_3 -dependent recruitment of mTORC2, we recorded up to 40 % inhibition of this signalling cascade in the L6^{K1-KD} cells (Figure 9). We interpret these data as evidence in support of our new hypothesis concerning the biological significance of PPIP5K1 translocation. Further work will be necessary to determine whether other PtdIns(3,4,5) P_3 -binding PH domains might be regulated by PPIP5K1 *in vivo*; our SPR data offer Akt (Figure 6) and GRP1 (Supplementary Figure S2) as candidates. If this new action of PPIP5K does regulate many physiological aspects of PtdIns(3,4,5) P_3 signalling, it would represent a somewhat ironic demonstration that the promiscuity of inositol pyrophosphate signalling does not arise from a multiplicity of biological actions of these molecules, but instead depends on both 1-Ins P_7 and Ins P_8 being relatively inactive.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

GRP1	general receptor for phosphoinositides-1
GRP1^{PH}	PH domain of GRP1
HEK	human embryonic kidney
IGF-1	insulin-like growth factor 1
IP6K	inositol hexakisphosphate kinase
L6^{CONTROL}	control L6 cells
L6^{K1-KD}	L6 cell line with reduced ('knocked-down') expression of PPIP5K1
MEM	minimal essential medium
mTOR	mammalian (or mechanistic) target of rapamycin
mTORC	mTOR complex
PBD1	polyphosphoinositide-binding domain
PDGF	platelet-derived growth factor
PKD1	phosphoinositide-dependent kinase 1
PH	pleckstrin homology
PP-InsP	diphosphoinositol polyphosphate
PPIP5K1	diphosphoinositol pentakisphosphate kinase type 1
SIN1	stress-activated protein kinase-interaction protein-1
SIN1^{PH}	PH domain of SIN1

SPR

surface plasmon resonance

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Figure 1. Effect of PDGF on the intracellular distribution of PPIP5K1 and PBD1 in NIH 3T3 cells

After 1 day in which NIH 3T3 cells were transfected with cDNA encoding FLAG-tagged constructs of either GRP1, PPIP5K1, PBD1 or PBD1^{R399A/R417A}, cells were serum starved for 3 h (see the Materials and methods section) and then either vehicle (control) or 50 ng/ml PDGF were added for 5 min. Cells were then fixed and the distribution of each construct between the plasma membrane and the cytoplasm (ratio PM/C) determined by confocal microscopy and an image segmentation protocol [28]. The distribution of the FLAG constructs are shown in green, and blue indicates the DAPI-stained nucleus. The scale bar is 10 μ m (A). The insets show cells from which the nuclear staining was digitally removed. (B–E) The data are represented as means $\pm$ S.E.M. ** $P < 0.01$ compared with control.

Figure 2. Effects of PDGF on the levels of inositol lipids InsP_6 and $PP\text{-InsP}$ s in NIH 3T3 cells Either vehicle (control) or 50 ng/ml PDGF were added for 5 min to NIH 3T3 cells containing [^3H]inositol that had been serum starved for 3 h (see the Materials and methods section). The incubations were then quenched, and either the [^3H]inositol lipids (A–C) or the [^3H]inositol phosphates (D–F) were extracted and quantified by HPLC. The data (either percentage of total inositol lipids or percentage of total inositol phosphates, as appropriate) are represented as means $\pm$ S.E.M. for three experiments. * $P < 0.05$ compared with control.

Figure 3. Effects of hyperosmotic stress on the levels of inositol lipids InsP_6 and PP - InsP s in NIH 3T3 cells

The serum-free medium bathing NIH 3T3 cells containing [^3H]inositol (see the Materials and methods section) was replaced with 2 ml of fresh medium with or without the addition of 0.2 M sorbitol to induce hyperosmotic stress. After 30 min, the incubations were then quenched, and either the [^3H]inositol lipids (A–C) or the [^3H]inositol phosphates (D–F) were extracted and quantified by HPLC. The data (either percentage of total inositol lipids or percentage of total inositol phosphates, as appropriate) are represented as means $\pm$ S.E.M. for five experiments. * $P < 0.05$, ** $P < 0.01$ compared with control.

Figure 4. Effect of hyperosmotic stress on the intracellular distribution of PPIP5K1 and PBD1 in NIH 3T3 cells

At 1 day after NIH 3T3 cells were transfected with cDNA encoding FLAG-tagged constructs of either GRP1, PPIP5K1, PBD1 or PBD1^{R399A/R417A}, cells were serum starved for 3 h (see the Materials and methods section). Then, the medium was replaced with 2 ml of fresh medium with or without 0.2 M sorbitol added to induce hyperosmotic stress. Where indicated, cells were treated with 5 μ g/ml cytochalasin, 30 min before initiation of osmotic stress. Cells were then fixed and the distribution of each construct between the plasma membrane and the cytoplasm (ratio PM/C) was determined by confocal microscopy and an image segmentation protocol [28]. The distribution of the FLAG epitope is shown in green, and blue indicates the DAPI-stained nucleus. The scale bar is 10 μ m (A). (B–D) The data are represented as means $\pm$ S.E.M. ** $P < 0.01$ compared with control.

Figure 5. SPR-based analysis of the effects of InsP₆ and PP-InsP_s on binding of the PBD1 of PIP5K1 to PtdIns(3,4,5)P₃

(A–D) The PBD1 (100 nM), either alone or after 15 min pre-incubation with the designated inositol phosphate (up to 50 μM as indicated), was injected at a flow rate of 5 μl/min on to immobilized lipid vesicles containing 3 % PtdIns(3,4,5)P₃. The mobile phase contained 25 mM Hepes (pH 7.2), 100 mM KCl, 10 mM NaH₂PO₄ and 1 mM MgCl₂. Representative sensorgrams are plotted in arbitrary units (AU). In (A) and (B), data were also obtained with 0.1 μM inositol phosphate, but the sensorgrams were not significantly different from those obtained in the absence of ligand, and so are omitted for clarity. In additional experiments (results not shown), the effects of chemically synthesized 5-InsP₇ [kindly provided by Dr D. Fiedler (University of Princeton, Princeton, NJ, U.S.A.)] were found to be identical to our enzymatically synthesized 5-InsP₇ (see the Materials and methods section). (E–H) End points are plotted against inositol phosphate concentrations.

Figure 6. SPR-based analysis of the effects of InsP₆ and PP-InsP s on binding of Akt^{PH} to PtdIns(3,4,5)P₃

(A–D) The Akt^{PH} (100 nM), either alone or after 15 min pre-incubation with the designated inositol phosphate (0–50 μM as indicated), was injected at a flow rate of 5 μl/min on to immobilized lipid vesicles containing 3 % PtdIns(3,4,5)P₃. The mobile phase contained 25 mM Hepes (pH 7.2), 100 mM KCl, 10 mM NaH₂PO₄ and 1 mM MgCl₂. Representative sensorgrams are plotted in arbitrary units (AU). In (A) and (B), data were also obtained with 1 μM inositol phosphate, but the sensorgrams were not significantly different from those obtained in the absence of ligand, and so are omitted for clarity. (E–H) End points are plotted against inositol phosphate concentrations.

Figure 7. SPR-based equilibrium-binding analysis of the affinity of SIN1^{PH} for PtdIns(3,4,5) P_3 and PtdIns(4,5) P_2

SIN1^{PH} was injected at 5 μ l/min over immobilized lipid vesicles containing 3 % of either (A and B) PtdIns(3,4,5) P_3 (25, 50, 90, 125, 200, 375, 600 or 700 nM protein) or (C and D) PtdIns(4,5) P_2 (100, 200, 250, 500, 1200 or 1800 nM protein). (A) and (C) show representative sensorgrams, with the same y-axis scale for comparative purposes. (B) and (D) depict the K_d determinations.

Figure 8. SPR-based analysis of the effects of InsP₆ and PP-InsP s on binding of SIN1^{PH} to PtdIns(3,4,5)P₃

(A–D) The SIN1^{PH} (100 nM), either alone or after 15 min pre-incubation with the designated inositol phosphate (0–50 μM as indicated), was injected at a flow rate of 5 μl/min on to immobilized lipid vesicles containing 3 % PtdIns(3,4,5)P₃. The mobile phase contained 25 mM Hepes (pH 7.2), 100 mM KCl, 10 mM NaH₂PO₄ and 1 mM MgCl₂. Representative sensograms are plotted in arbitrary units (AU). (E–H) End points are plotted against inositol phosphate concentrations.

Figure 9. Cellular levels of inositol pyrophosphates and insulin-mediated Akt phosphorylation on Ser⁴⁷³ in L6^{K1-KD} and L6^{CONTROL} cells

L6^{K1-KD} (KD) and L6^{CONTROL} (Con) cells were prepared as described in the Materials and methods section. (A) Representative Western blot analysis of the degree of expression of PIP5K1 and SIN1, with β -actin loading controls. (B) Densitometric analysis of PIP5K1 protein expression, means $\pm$ S.E.M. for three experiments (expression in L6^{CONTROL} cells was normalized to unity). (C) Quantitative real-time PCR analysis of *PIP5K1* mRNA expression (means $\pm$ S.E.M. for four experiments; expression in L6^{CONTROL} cells was normalized to unity). (D) Total [³H]InsP₆. (E) Total [³H]InsP₇. (F) Total [³H]InsP₈. The data in (D), (E) and (F) represent means $\pm$ S.E.M. for six experiments with serum-starved cells. (G) Representative Western blot analysis of Akt phosphorylation on Ser⁴⁷³, and total Akt loading controls, after cells were treated with 10 nM insulin for 0, 5, 15 and 30 min. (H) Densitometric analysis of Akt phosphorylation on Ser⁴⁷³, as a ratio to total Akt (means $\pm$ S.E.M. for five experiments, performed as in G; all values were normalized to the signal in L6^{CONTROL} cells after 30 min insulin treatment, since no Ser⁴⁷³ phosphorylation was detected at zero min). (I) Proliferation assay. * P < 0.05, ** P < 0.01, *** P < 0.001 (compared with control).

Figure 10. Proposed biological significance of stimulus-dependent PtdIns(3,4,5)P₃-mediated translocation of PIP5K1 to the plasma membrane

Recruitment of PIP5K1 to the plasma membrane driven by stimulus-dependent synthesis of PtdIns(3,4,5)P₃ by PI3K (phosphoinositide 3-kinase). The catalytic activity of PIP5K1 is hypothesized to locally decrease subplasmalemmal levels of InsP₆ and 5-InsP₇ through their phosphorylation to 1-InsP₇ and InsP₈ respectively. This, in turn, is predicted to relieve inhibition of the binding to PtdIns(3,4,5)P₃ of signalling proteins with PH domains. Thus PIP5K1 recruitment to the plasma membrane is considered to activate PtdIns(3,4,5)P₃ cascades.