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Type IIα phosphatidylinositol phosphate kinase associates with the plasma membrane via interaction with type I isoforms

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The phosphatidylinositol phosphate kinases (PIPkins) are a family of enzymes involved in regulating levels of several functionally important inositol phospholipids within cells. The PIPkin family is subdivided into three on the basis of substrate specificity, each subtype presumably regulating levels of different subsets of the inositol lipids. The physiological function of the type II isoforms, which exhibit a preference for phosphatidylinositol 5-phosphate, a lipid about which very little is known, is particularly poorly understood. In the present study, we demonstrate interaction between, and co-immunoprecipitation of, type IIα PIPkin with the related, but biochemically and immunologically distinct, type I PIPkin isoforms. Type IIα PIPkin interacts with all three known type I PIPkins (α, β and γ), and in each case co-expression of the type I isoform with type IIα results in recruitment of the latter from the cytosol to the plasma membrane of the cell. This change in subcellular localization could result in improved access of the type IIα PIPkin to its lipid substrates.

Key words: membrane interaction, phosphatidylinositides, subcellular localization.

INTRODUCTION

Inositol phospholipids play important roles in many diverse cellular processes, including signal transduction, membrane trafficking and cytoskeletal regulation. However, despite their central importance to cellular function, their physiological regulation is incompletely understood. Unsurprisingly, regulation of these lipids is complex, and multiple enzymes responsible for inositol lipid synthesis are present within cells. The best studied of these are the various members of the phosphoinositide 3-kinase (PI 3-kinase) family [1], which regulate production of lipids phosphorylated at the 3-position of the inositol ring. In particular, intense interest has focused on the members of this family that phosphorylate the multi-functional inositol lipid PtdIns(4,5)P_2 to produce the lipid second messenger, PtdIns(3,4,5)P_3, an important intermediate in signalling pathways activated by a variety of stimuli. However, there exists a second, less well-known family of inositol lipid kinases, unrelated to the PI 3-kinases, which also have an essential role in regulating inositol lipid synthesis: the phosphatidylinositol phosphate kinases (PIPkins) [2].

The PIPkin family is subdivided into three subtypes: types I, II and III. Type III PIPkins control production of PtdIns(3,5)P_2, a lipid that appears to have a role in membrane trafficking [3]. In contrast, the type I PIPkins are responsible for the bulk of PtdIns(4,5)P_2 production by phosphorylation of its major cellular precursor PtdIns4P. However, these isoforms also phosphorylate other inositol lipids *in vitro* [4,5], and were demonstrated recently to regulate an alternative route for PtdIns(3,4,5)P_3 synthesis in cells exposed to oxidative stress, achieved via the 5-phosphorylation of another inositol lipid, PtdIns(3,4)P_2 [6]. This suggests that the physiological functions of the PIPkins may be more diverse than was previously realized.

The ability to phosphorylate more than one lipid is also a feature of the third PIPkin subclass, the type II isoforms. The preferred substrate of these enzymes is the minor inositol lipid PtdIns5P, which they convert into PtdIns(4,5)P_2 [7]. It is possible that this pathway constitutes an alternative route for PtdIns(4,5)P_2 production, providing the cell with more flexibility in the regulation of functionally distinct pools of this lipid. Given that roles have been proposed for PtdIns(4,5)P_2 in cytoskeletal dynamics, endocytosis, exocytosis, vesicle trafficking and ion-channel regulation (reviewed in [2]), there is clearly a requirement for multiple mechanisms controlling its synthesis. However, little is known about the physiological role of PtdIns5P, so it is possible that the type II PIPkins actually function to regulate levels of this lipid. Levels of PtdIns5P increase severalfold in human platelets on stimulation with thrombin [8], and during G_1 of the cell cycle in nuclei of mouse erythroleukaemia cells [9]. However, whether this reflects a signalling function of PtdIns5P, or up-regulation of PtdIns(4,5)P_2 synthesis via type II PIPkins for a particular purpose, is not yet known.

Type II PIPkins also phosphorylate PtdIns3P to PtdIns(3,4)P_2 *in vitro* [7], although it is not known if they do so in intact cells. The physiological role of PtdIns(3,4)P_2 is also incompletely understood, but it is known to activate protein kinase B/Akt [10], and is produced in Swiss 3T3 cells in response to oxidative stress [11], and in aggregating platelets [12]. It is unclear at present whether PtdIns5P or PtdIns3P is the main physiological substrate of the type II PIPkins, although a PtdIns3P 4-kinase activity in human platelets, though still unidentified, has been shown not to be a type II PIPkin [12].

In an attempt to clarify the physiological function of the type II PIPkins, we here describe the construction of a kinase-dead mutant of the type IIα isoform. Unexpectedly, this mutant associates with type I PIPkin activity when expressed in cultured cells. This association with a type I isoform is also a feature of the wild-type IIα PIPkin. Interestingly, co-expression with type I PIPkins alters the subcellular distribution of type IIα PIPkin, and possibly reflects a mechanism for recruiting the type II isoform to the plasma membrane.

Abbreviations used: D273K; site-directed mutant containing the amino acid substitution $Asp^{273} \rightarrow Lys$; FCS, fetal calf serum; GFP, green fluorescent protein; GST, glutathione S-transferase; HRP, horseradish peroxidase; PAE, pig aortic endothelial; PI 3-kinase, phosphoinositide 3-kinase; PIPkin, phosphatidylinositol phosphate kinase.

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EXPERIMENTAL

Materials

The Quik change site-directed mutagenesis kit and the anti-FLAG M2 antibody were from Stratagene. The vector pGEX-4T1, thrombin protease, Protein G–Sepharose, [^{3}H]Ins(1,4,5)-P_3 and horseradish peroxidase (HRP)-coupled anti-mouse antibodies were from Amersham Biosciences. [γ-^{32}P]ATP was from NEN. HRP-coupled anti-rat antibodies were obtained from Sigma. The vectors pEGFP-C1 and pEGFP-N1 were from Clontech. PBS, Dulbecco's modified Eagles Medium, Ham's F-12 medium and fetal calf serum (FCS) were from Invitrogen. Tissue culture plasticware was from Costar Corning. Maxi-prep kits were from Qiagen. FuGENE 6 was from Roche. Synthetic inositol lipids were from Echelon. Prolong antifade, Alexafluor-568 phalloidin and anti-green fluorescent protein (GFP) antibody 3E6 were from Molecular Probes. The anti-GFP antibody used in Western blots was from Clontech. Enhanced chemiluminescence (ECL®) reagents were from Pearce. TLC plates were from Merck. Dye-conjugated secondary antibodies were from Jackson Labs. All other materials used were of reagent grade.

Molecular biology

Site-directed mutagenesis was performed using the Quik-change kit. Mutations were generated in type IIα PIPkin constructs cloned into either the bacterial expression vector pGEX-4T1 or the mammalian expression vectors pEGFP-C1 and pEGFP-N1. Glutathione S-transferase (GST)-tagged wild-type and mutant PIPkins were expressed in *Escherichia coli*, purified on glutathione–Sepharose columns and cleaved *in situ* with 50 units/ml thrombin. The cleaved proteins were stored at -20 °C in a 50% (v/v) solution of glycerol in PBS.

Cell culture and manipulation

Pig aortic endothelial (PAE) cells were maintained in 5% CO_2 in Ham's F-12 medium supplemented with 10% (v/v) FCS. HeLa cells and Cos-7 cells were grown in Dulbecco's modified Eagle's Medium with 10% FCS, also in 5% CO_2. For immunoprecipitation experiments, cells (1.5×10^6) were seeded on 100 mm culture dishes and allowed to attach overnight. The cells were transfected with PIPkin constructs prepared using an endotoxin-free Maxi-prep kit, using FuGENE 6 transfection reagent. Lysates were prepared (see below) 24 h after transfection.

For microscopy, HeLa cells were seeded on to 20 mm^2 glass coverslips in 30 mm tissue-culture dishes the day before transfection. Transfection was performed using a standard calcium phosphate precipitation method. Cells were fixed and prepared for microscopy 24 h after transfection.

Cell lysis and immunoprecipitation

Cultured cells were washed once with PBS, then lysed with 2 ml of ice-cold PBS containing 1% (v/v) Triton X-100 plus 5 μM leupeptin, 1 mM PMSF, 5 mM EDTA and 5 mM EGTA. Cells were scraped on dry ice, thawed and centrifuged at 10000 ***g*** for 10 min at 4 °C. Supernatants were transferred to 2 ml tubes and stored at -80 °C. Unstimulated human platelets were isolated, and lysates were prepared for immunoprecipitation as described previously [13].

When required, thawed lysates were centrifuged at 10000 ***g*** (10 min, 4 °C), and aliquots were immunoprecipitated with anti-GFP antibody 3E6, anti-FLAG M2 antibody, anti-myc antibody 9E10 or the anti-type II PIPkin antibody, MAC 334 [14] and Protein G–Sepharose beads. In some experiments, antibodies were crossed-linked to the beads using dimethylpimelimidate. For platelet immunoprecipitates, control samples (which received immunoprecipitation beads without added antibody) were produced. All immunoprecipitation samples received equal amounts of platelet lysate. After immunoprecipitation, beads were washed twice with ice-cold Tris-buffered saline [50 mM Tris/HCl (pH 7.4)/150 mM NaCl] and once with kinase buffer [40 mM KCl/50 mM Tris (pH 7.4)/10 mM $MgCl_2$/2 mM EGTA] before either assaying for PIPkin activity (see below) or preparing for SDS/PAGE.

PIPkin assays

Inositol lipids (crude PtdIns*P* from pig brain, or synthetic dipalmitoyl PtdIns4*P* or PtdIns5*P*) were dried down under vacuum in 1.5 ml tubes. All assays using PtdIns4*P* also included phosphatidic acid (10 μM), which was dried down with the PtdIns4*P*. Micelles were formed by sonication in kinase buffer. Lipid micelles (final concentration 10 μM) were mixed with recombinant PIPkins or immunoprecipitates in the presence of [γ-^{32}P]ATP (50–100 μCi·ml^{-1}; total ATP concentration of 10 μM in 100 μl) and incubated at 30 °C for the indicated times. Reaction was stopped with 500 μl of chloroform/methanol (1:1, v/v) and 125 μl of 2.4 M HCl. After brief centrifugation at 10000 ***g***, the aqueous upper phase was removed and replaced with an equal volume of theoretical upper phase (chloroform/methanol/1 M HCl, 2:48:47, by vol.). Centrifugation was repeated and the organic lipid containing the lower phase was removed and evaporated to dryness under vacuum. The lipids were re-dissolved in chloroform and spotted on to silica-gel-coated TLC plates impregnated with 1% potassium oxalate containing 4 mM EGTA and heated to 110 °C for 1 h before use. Lipids were resolved by TLC in a pre-equilibrated tank containing methanol/chloroform/water/ammonia solution (40:28:10:6, by vol.). After the chromatography, the plates were dried and exposed to X-ray film at -80 °C.

Analysis of lipid head groups

^{32}P-labelled PtdInsP_2 produced by phosphorylation of PtdIns4*P* by the co-immunoprecipitating lipid kinase was de-acylated, de-glycerated and treated with inositol phosphate 5-phosphatase, as described previously [8]. A [^{3}H]Ins(1,4,5)P_3 standard was used to generate [^{3}H]Ins(1,4)P_2 by treatment with the same enzyme. Samples were run on Partisil-10 SAX column. Sodium phosphate buffer, pH 3.75, was used to elute inositol phosphates, as described previously [8,9].

SDS/PAGE and Western blotting

Samples were resolved on 10% (w/v) acrylamide gels and transferred to nitrocellulose blotting membranes, as described previously [13]. Blots were blocked with 5% (w/v) non-fat dried milk and 0.05% (v/v) Tween 20 in TBS, before probing with antibodies. Bound HRP-linked secondary antibodies were visualized by ECL®.

Preparation of cells for microscopy

Coverslips were washed once with PBS, and the cells fixed with 4% (w/v) paraformaldehyde in 0.1 M NaH_2PO_4, pH 7.4. After washing with PBS, the cells were permeabilized with 0.2% (w/v) Triton X-100 in PBS, washed again and then blocked with 5% (v/v) goat serum in PBS. Antibodies or Alexafluor-568-conjugated phalloidin were applied in blocking mixture. Stained coverslips were mounted in Prolong anti-fade reagent, and kept at 4 °C in the dark.

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RESULTS

While investigating type IIα PIPkin function, we generated a kinase-dead mutant: on the basis of the structure of the closely related type IIβ PIPkin [15], we attempted to block activity by mutating an aspartate residue in the active site (Asp^{273} of the type IIα sequence) to lysine. As expected, when expressed in bacteria as a GST fusion protein, the $Asp^{273} \rightarrow Lys$ (D273K) mutant lacked detectable activity against synthetic PtdIns4*P* or PtdIns5*P* (Figure 1A). In fact, it produced no detectable phosphorylation of either lipid [3 ± 10 and -3 ± 7 (means ± S.E.M.) pmol of PtdInsP_2 $\cdot$ min^{-1} $\cdot$ mg^{-1} produced from PtdIns4*P* and PtdIns5*P* respectively, compared with phosphorylation rates of 119 ± 45 and 6074 ± 2269 pmol $\cdot$ min^{-1} $\cdot$ mg^{-1} for the wild-type enzyme ($n = 3$)]. This demonstrates that the D273K mutation abolishes activity of type IIα PIPkin towards both PtdIns4*P* and PtdIns5*P*, and further, in agreement with other studies [7,8], that PtdIns4*P* is a very poor substrate of the wild-type enzyme compared with PtdIns5*P*.

We then expressed a GFP-tagged version of type IIα PIPkin (D273K) in eukaryotic cells. Transient transfection using this construct caused the appearance in anti-GFP immunoprecipitates from cell lysates of an 80 kDa band that cross-reacted with both type II PIPkin (results not shown) and GFP antibodies on Western blots (see Figure 1C), corresponding to the mutant protein. Surprisingly, we observed that immunoprecipitates from transfected PAE cells, HeLa cells or Cos-7 cells contained considerable PIPkin activity towards pig-brain PtdIns*P*, a mixture of PtdIns4*P* and PtdIns5*P* (results not shown). To investigate this result further, we therefore determined whether the PtdInsP_2 produced was due to phosphorylation of either PtdIns4*P* or PtdIns5*P* (Figure 1B). This demonstrated that anti-GFP immunoprecipitates from cells transfected with GFP-type IIα PIPkin (wild type) or GFP-type IIα PIPkin (D273K) both contained significant PtdIns4*P* kinase activity ([113.5 ± 7.9 % and 330.5 ± 73.9 % of the PtdIns5*P* kinase activity in immunoprecipitates from cells transfected with wild-type IIα PIPkin respectively ($n = 3$)]. However, immunoprecipitates from cells transfected with the D273K mutants contained greatly reduced PtdIns5*P* kinase activity (12.5 ± 4.4 % of the wild type; $n = 3$). Immunoprecipitates from cells transfected with GFP alone contained little activity against either lipid: 11.2 ± 3.0 % and 2.2 ± 0.5 % of the PtdIns5*P* kinase activity in cells transfected with the wild-type enzyme for PtdIns4*P* and PtdIns5*P* respectively ($n = 3$; Figure 1B), despite very similar expression levels of the transfected proteins (Figure 1C).

The profound effect of the D273K mutation on PtdIns5*P* kinase activity in immunoprecipitates (Figure 1B), and the complete abolition of PtdIns4*P* and PtdIns5*P* phosphorylation in the bacterially expressed protein (Figure 1A), suggest that the phosphorylation of PtdIns4*P* by immunoprecipitates is due to the additional presence of a cellular PtdIns*P* kinase. The fact that PtdIns4*P* kinase activity is also present in immunoprecipitates from cells transfected with wild-type type IIα PIPkin (despite the marked preference of the latter for PtdIns4*P* demonstrated in Figure 1A) suggests that this co-precipitating protein also interacts with the wild-type enzyme. Co-precipitation of the PtdIns4*P* kinase is not due to its interaction with GFP, or with the anti-GFP antibody, as the activity is absent from anti-GFP immunoprecipitates from untransfected cells (results not shown) or cells transfected with empty vector (Figure 1B). Moreover, the association persists if FLAG-tagged IIα PIPkin is used, and whether the GFP is attached at the N- or the C-terminus (results not shown). Significantly, the presence of PtdIns4*P* kinase activity in anti-type II PIPkin (MAC 334) immunoprecipitates from human platelets (Figure 2) also demonstrates that the observed interaction is not simply an artefact of transfection. The amount of PtdIns4P kinase activity present in the MAC 334 immunoprecipitates is 250.4 ± 99 % ($n = 3$) of the PtdIns5P kinase activity present (after correction for the small amount of lipid kinase activity that adheres to the antibody-free beads alone). Together, these pieces of evidence suggest that an endogenous PtdIns4*P* kinase present in several cell types associates with the type IIα PIPkin itself, although whether this interaction is direct or mediated via another protein is unclear.

Type II PIPkins are known to homodimerize [15,16], but the substrate specificity of the co-immunoprecipitating kinase activity (utilizing PtdIns4*P*, but not PtdIns5*P*) suggests that it is not a type II isoform. Alternative possibilities include a PI 3-kinase [which would produce PtdIns(3,4)P_2 from PtdIns4*P*] or a type I PIPkin [which would produce PtdIns(4,5)P_2]. To discriminate between these possibilities, we analysed the ^{32}P-labelled

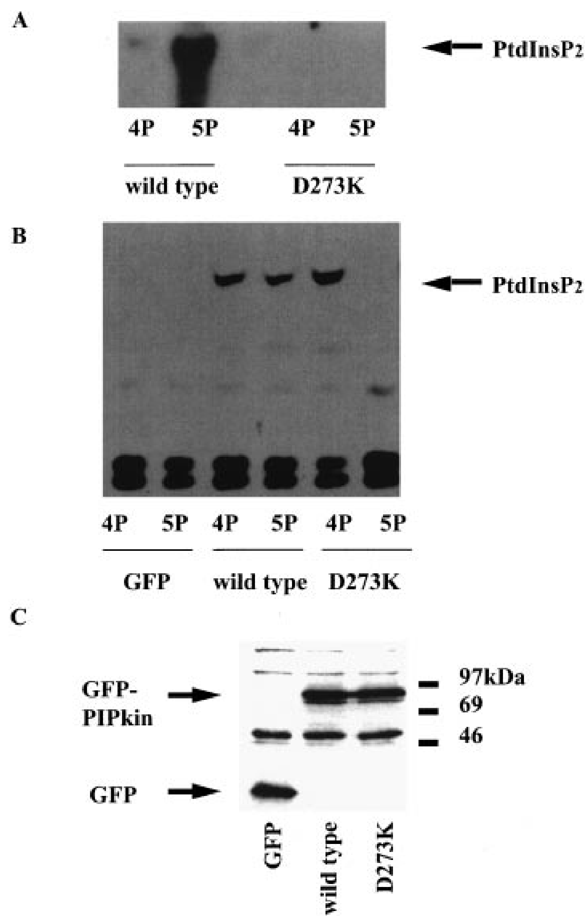

Figure 1 Expression of type IIα PIPkin (D273K) in bacteria and Cos-7 cells

(**A**) Wild-type type IIα PIPkin or the D273K mutant were expressed in bacteria as GST fusion proteins, cleaved with thrombin, and 50 ng of each was assayed for their ability to phosphorylate PtdIns4*P* or PtdIns5*P* in the presence of [γ-^{32}P]ATP. Phosphorylation was allowed to proceed for 5 min. Phosphorylated products were resolved by TLC. The position of migration of PtdInsP_2 is indicated. (**B**) Lysates from Cos-7 cells, either transfected with GFP vector alone or transfected with GFP-type IIα PIPkin or GFP-type IIα PIPkin (D273K) as indicated, were immunoprecipitated with anti-GFP antibodies. The washed immunoprecipitates were assayed for 10 min for kinase activity towards PtdIns4*P* (4P) or PtdIns5*P* (5P). The position of migration of PtdInsP_2 is indicated. (**C**) Rather than being subjected to immunoprecipitation, a small proportion (37.5 μl each) of the lysates used in (**B**) was resolved by SDS/PAGE. A Western blot of the gel was probed with an anti-GFP antibody. The positions of migration of GFP-tagged type IIα PIPkin and of GFP alone are indicated.

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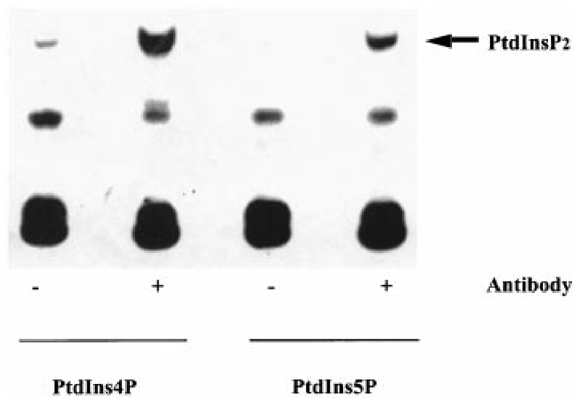

Figure 2 Presence of PtdIns4*P* kinase activity in anti-type II PIPkin immunoprecipitates from platelets

Lysates from human platelets were incubated with MAC 334 (+ antibody) or with beads alone (− antibody). The washed beads were incubated and assayed for kinase activity towards PtdIns4*P* or PtdIns5*P*, as described in the legend to Figure 1(B).

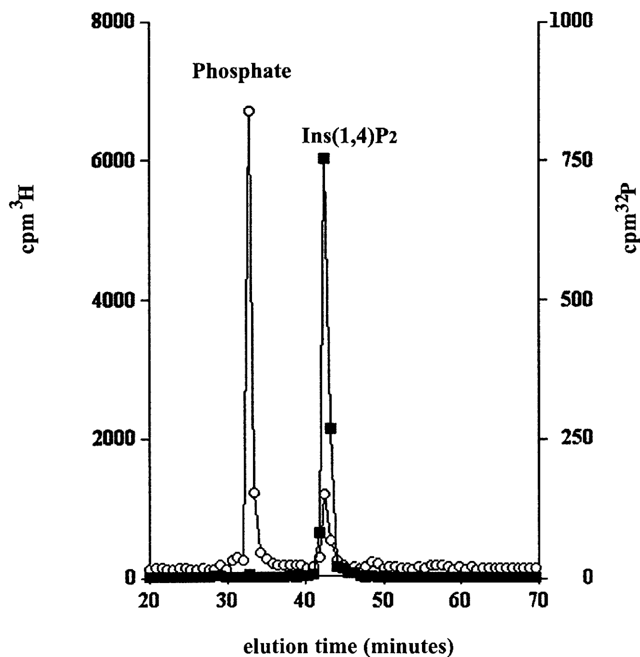

Figure 3 Release of ^{32}P from labelled PtdInsP_2 by inositol phosphate 5-phosphatase

The ^{32}P-labelled PtdInsP_2 produced by phosphorylation of PtdIns4*P* by the lipid kinase that co-immunoprecipitates with type IIα PIPkin (D273K) was converted into Ins(1,4,5)P_3. This was then treated with inositol phosphate 5-phosphatase and the digestion products were resolved by HPLC. A [^3H]Ins(1,4)P_2 standard, generated by 5-phosphatase treatment of [^3H]Ins(1,4,5)P_3, was run as a comparison. The amount of ^3H or ^{32}P in each fraction was quantified by scintillation counting.

inositol phosphate head group of the PtdInsP_2 by HPLC. This co-migrated with Ins(1,4,5)P_3 (results not shown), showing the PtdInsP_2 to be PtdIns(4,5)P_2, and suggesting that the endogenous PtdIns4*P* kinase is not a PI 3-kinase. Moreover, as expected, the ^{32}P label was released from the Ins(1,4,5)P_3 as inorganic phosphate by inositol phosphate 5-phosphatase (Figure 3). This shows that the immunoprecipitates contain a PtdIns4*P* 5-kinase activity, most probably a type I PIPkin. This identification is supported further by the observation that the activity was consistently more robust in the presence of phosphatidic acid, a known positive regulator of type I PIPkins, which we routinely include in assays of PtdIns4*P* phosphorylation.

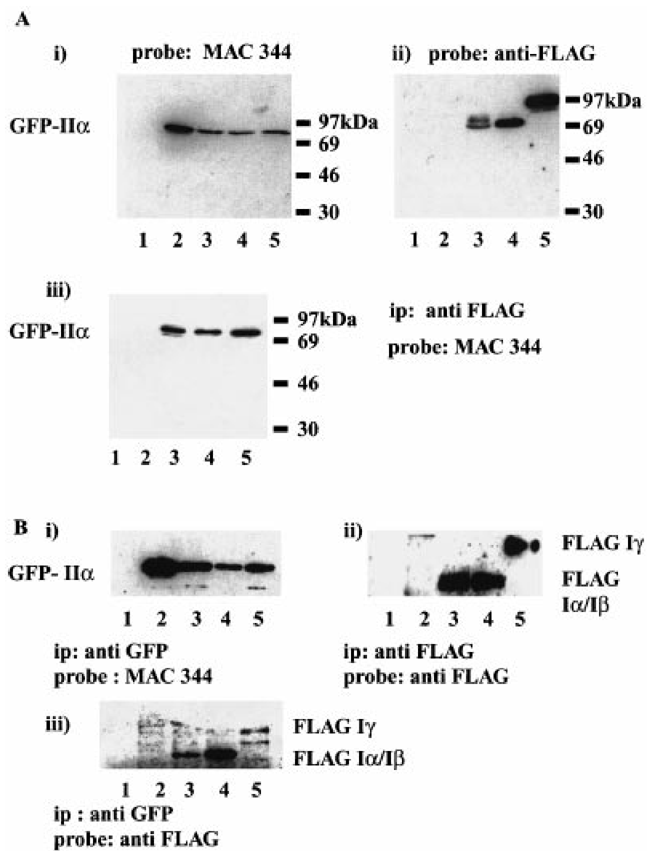

Figure 4 Co-immunoprecipitation of GFP-type IIα PIPkin and FLAG-tagged type I PIPkins

Cos-7 cells (**A**) or PAE cells (**B**) were transfected with the following constructs: 1, no transfection; 2, GFP-type IIα PIPkin; 3, GFP-IIα PIPkin and FLAG-Iα PIPkin; 4, GFP-IIα PIPkin and FLAG Iβ PIPkin; 5, GFP-IIα PIPkin and FLAG Iγ PIPkin. (**A**) Western blots of whole lysates (50 μl each) were probed with anti-type II PIPkin antibody MAC 344 (i) or with anti-FLAG antibody (ii) to reveal the relative levels of expression of transfected proteins. Longer exposure of (i) reveals the presence of endogenous type II PIPkins at 53 kDa (results not shown). In (ii), the band migrating of molecular mass 97 kDa in lane 5 is FLAG-type Iγ PIPkin, whereas the 68 kDa band in lane 3 is FLAG-type Iα PIPkin, and that in lane 4 is FLAG-Iβ PIPkin. The third panel (iii) shows a blot of anti-FLAG-immunoprecipitated lysates (1 ml each) probed with MAC 344, demonstrating co-precipitation of type IIα with each type isoform. (**B**) Western blots of immunoprecipitates. (i) Anti-GFP immunoprecipitates (from 100 μl of lysate each) probed with MAC 344 to illustrate GFP-type II PIPkin expression. (ii) Anti-FLAG immunoprecipitates (from 100 μl of lysate) probed with anti-FLAG antibodies, illustrating type I PIPkin expression. (iii) Anti-GFP immunoprecipitates (from 1 ml of lysate each) probed with anti-FLAG, demonstrating co-precipitation of each type I isoform with type IIα PIPkin.

Three mammalian type I PIPkin isoforms, α, β and γ, are known, each being alternatively spliced [17,18]. In an attempt to identify which associate with type IIα PIPkin, we co-expressed FLAG-tagged versions of each type I isoform with this protein in Cos-7 cells (Figure 4A) and PAE cells (Figure 4B). Surprisingly, this showed that all three type I PIPkins can co-immunoprecipitate with the type II enzyme. To investigate possible associations between these proteins within cells, HeLa cells were co-transfected with GFP-tagged type IIα PIPkins and FLAG-tagged type I PIPkin isoforms, and examined using confocal microscopy.

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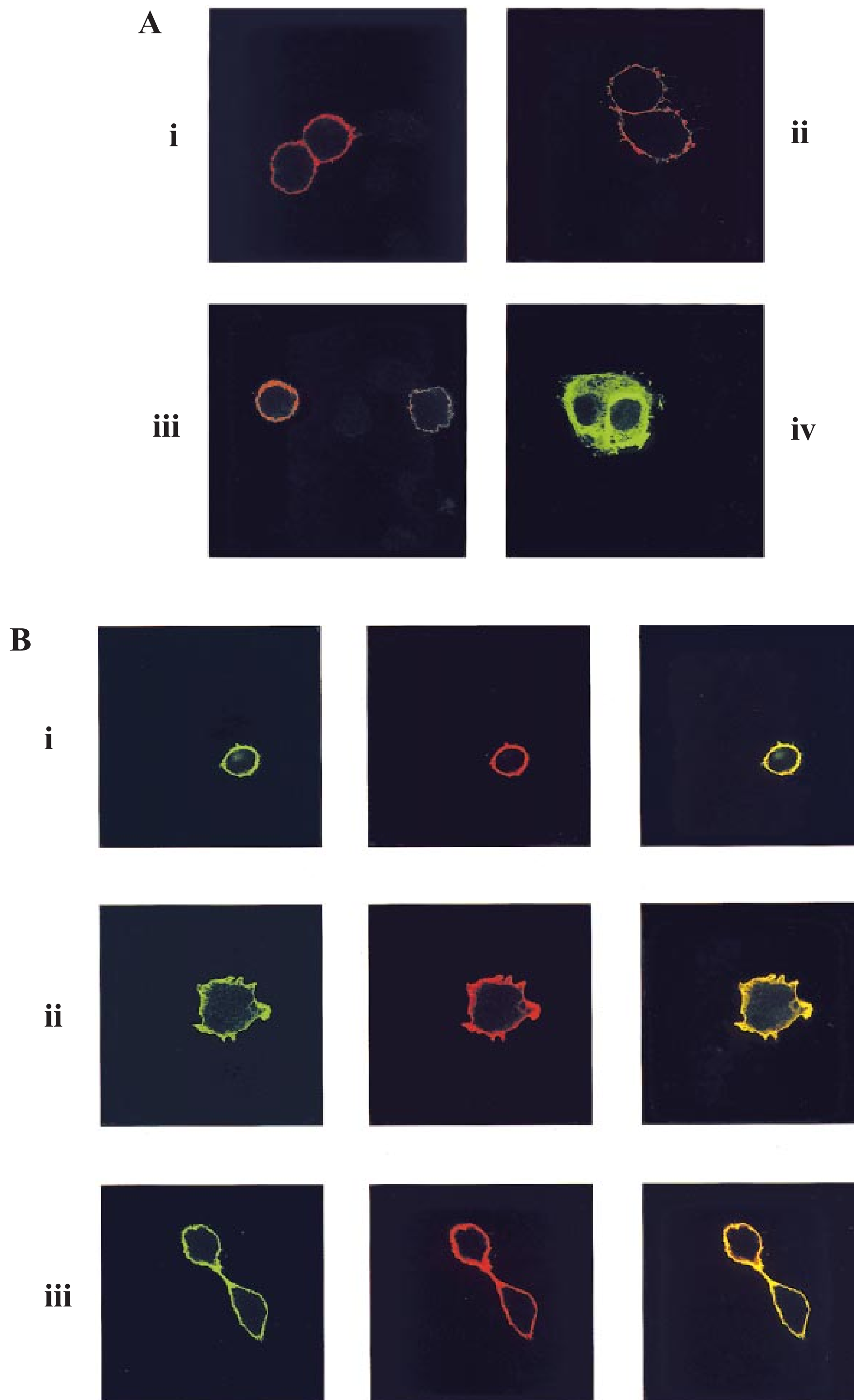

Figure 5 Co-expression with type I PIPkins promotes translocation of type IIα PIPkin to the cell periphery

(**A**) FLAG-tagged type Iα PIPkin (i), type Iβ PIPkin (ii), type Iγ PIPkin (iii) or GFP-tagged type IIα PIPkin (iv) were transfected into HeLa cells. The FLAG epitope was visualized by staining with a specific primary antibody, followed by a Texas-red conjugated second antibody. (**B**) HeLa cells were co-transfected with GFP-type IIα PIPkin and the following FLAG-tagged constructs: (i) type Iα; (ii) type Iβ; (iii) type Iγ. The epitope was recognized as above. The signals from the green and red channels are shown in the first two columns; an overlay of these images is shown in the third. The yellow colour indicates co-localization of the signals.

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Figure 5 shows that co-expression in HeLa cells with any of the type I PIPkins causes a profound shift in the subcellular localization of the type IIα isoform, confirming that all three are able to interact with it. In the absence of a co-transfected protein, GFP-tagged type IIα PIPkin has a diffuse cytosolic localization, whereas the type I PIPkins all localize at the cell periphery (Figure 5A). However, when co-expressed with type Iα, type Iβ or type Iγ PIPkin, type IIα PIPkin adopts a more restricted localization at the periphery of the cell, coinciding with the type I PIPkin immunofluorescence (Figure 5B). This change in subcellular localization also occurs if the type IIα (D273K) mutant is co-transfected with type I isoforms (results not shown). It is noteworthy that GFP-type IIα PIPkin fails to localize at the cell periphery in the absence of a co-transfected type I PIPkin (Figure 5A), despite the fact that the immunoprecipitation data indicate association with endogenous type I PIPkin. However, this probably reflects the fact that levels of ectopically expressed proteins in transfected cells far exceed those of endogenous proteins. Thus, although a small percentage of the GFP-type IIα PIPkin is probably recruited to the cell periphery by endogenous type I PIPkins, most of the GFP-type IIα PIPkin remains cytosolic as the endogenous material has become saturated.

While performing these experiments, we consistently found that cells overexpressing type I PIPkin isoforms, although not the type IIα PIPkin alone, adopted a rounded morphology. Morphological changes have also been reported previously in Cos-7 cells transfected with type I PIPkins [18,19], where type I PIPkins provoked disassembly of stress fibres and the appearance of short actin filaments termed 'pine-needles' [19]. The alteration in HeLa cell morphology seen upon transfection with type I PIPkins raised the possibility that the apparent change in subcellular localization of the co-transfected type IIα isoform is simply a consequence of the alteration in cell shape. To test this, we performed the experiments shown in Figure 6. Here, cells were again co-transfected with type IIα and type Iγ PIPkin, but now the actin cytoskeleton was visualized with Alexa-fluor568-conjugated phalloidin. This clearly shows that, when transfected alone, the majority of the GFP-type IIα PIPkin is cytosolic and does not co-localize with the bulk of the f-actin, which in these cells forms a cortical network underlying the plasma membrane (Figure 6A). In contrast, when co-transfected with type Iγ PIPkin, the IIα and cortical f-actin signals co-localize (Figure 6B). In agreement with the data from Cos-7 cells [19], disassembly of some actin structures seems to have occurred in HeLa cells transfected with type Iγ PIPkin, though the appearance of actin 'pine-needles', as seen in Cos-7 cells, does not occur.

These data demonstrate that the subcellular localization of the type II PIPkin has indeed been altered by the co-transfected type I isoform. Despite its co-localization with the f-actin signal, however, the recruitment of IIα PIPkin to the cell periphery actually reflects association with the plasma membrane, not the cytoskeleton. This is demonstrated by the fact that treatment of cells with 0.2% (w/v) Triton X-100 before fixation removes both the type I and type II signals while leaving the f-actin intact (results not shown).

The association between two proteins that both generate the same product is unexpected. Given that both type IIβ and type IIα PIPkins homodimerize [15,16], one possibility is that overexpression of the type I and type II isoforms drives their artefactual heterodimerization. However, comparison of the sequences of the type I and type II PIPkins suggests that this is unlikely. Homodimerization of type IIβ PIPkin is due to associations between the α-helix $\alpha 1$ on each subunit, and similarly between the two β-strands designated $\beta 1$ [15]. The sequences of $\alpha 1$ and $\beta 1$ are highly conserved between type IIα and type IIβ PIPkin, the β-strands having identical sequence and the α-helices exhibiting only two conservative substitutions over 16 amino acids [15]. However, even these modest differences are sufficient to prevent heterodimerization of type II α and β PIPkins co-expressed in PAE cells (J. B. Morris, K. A. Hinchliffe and R. F. Irvine, unpublished work). Importantly, the protein primary structures corresponding to the $\alpha 1$ helix and the $\beta 1$ strand in type IIβ PIPkin are not conserved between the type I and type II PIPkins, and it is not known whether the type I isoforms even form dimers, as their structures have yet to be solved. It is therefore extremely unlikely that the observed co-precipitation of the type I and type II PIPkins is due to aberrant heterodimerization, especially as it also occurs in lysates of human platelets.

DISCUSSION

The fact that type I PIPkins interact with type IIα is at first sight difficult to understand, given that the type I and type II PIPkins can both generate the same product, PtdIns(4,5)P_2. However, the persistence of the interaction when different epitope tags are used, and its occurrence in immunoprecipitates from human platelets, suggest that it does indeed occur physiologically. Furthermore, this finding explains a previous report that PtdIns4P 5-kinase activity is depleted from platelet lysates by immunoprecipitation with the anti-type II PIPkin antibody, MAC 334 [12]. MAC 334 does not cross-react with type I PIPkins, but the ability of this antibody indirectly to deplete type I PIPkin levels by removing type II PIPkin to which it is bound provides a plausible explanation for the reported finding.

One possible explanation for association between type I and type II PIPkins might be that the type II PIPkin supplies substrate to the type I. Type I PIPkins phosphorylate PtdIns-(3,4)P_2 to PtdIns(3,4,5)P_3 *in vivo* [6], a reaction which does not occur in unstimulated cells, but which is promoted by oxidative stress. Furthermore, type II PIPkins can phosphorylate PtdIns3P to PtdIns(3,4)P_2 *in vitro*, although it is still unclear whether they do so in living cells. Preliminary experiments in which PtdIns3P was added to immunoprecipitates from transfected cells failed to generate PtdIns(3,4,5)P_3 (results not shown), suggesting that type IIα PIPkin does not fulfil this role in this system.

It is also possible that the cellular function of type II PIPkins is to remove PtdIns5P by conversion into PtdIns(4,5)P_2. It may be, therefore, that the function of the type I PIPkin in the complex is to generate PtdIns(4,5)P_2 from PtdIns4P, whereas the role of the type II PIPkin is actually to remove PtdIns5P. Certainly, since PtdIns4P levels in cells far exceed those of PtdIns5P [7], the activity of the latter pathway would be unlikely to make a major contribution to bulk levels of PtdIns(4,5)P_2. Interestingly, since type I PIPkins have been reported to produce PtdIns5P from PtdIns *in vitro* [4], it is possible that the observed interaction between type I and type II PIPkins may constitute a multi-protein complex responsible for both the synthesis and removal of this enigmatic lipid. However, until the physiological function of PtdIns5P is elucidated this idea must remain hypothetical.

While performing these experiments, we consistently found that the amount of PtdIns4P kinase activity in immunoprecipitates from type IIα PIPkin (D273K)-transfected cells was significantly greater than that from cells transfected with the wild-type enzyme. This does not seem to be due simply to differences in transfection efficiency, and may therefore reflect a greater degree of interaction of the type I and type IIα (D273K) proteins under

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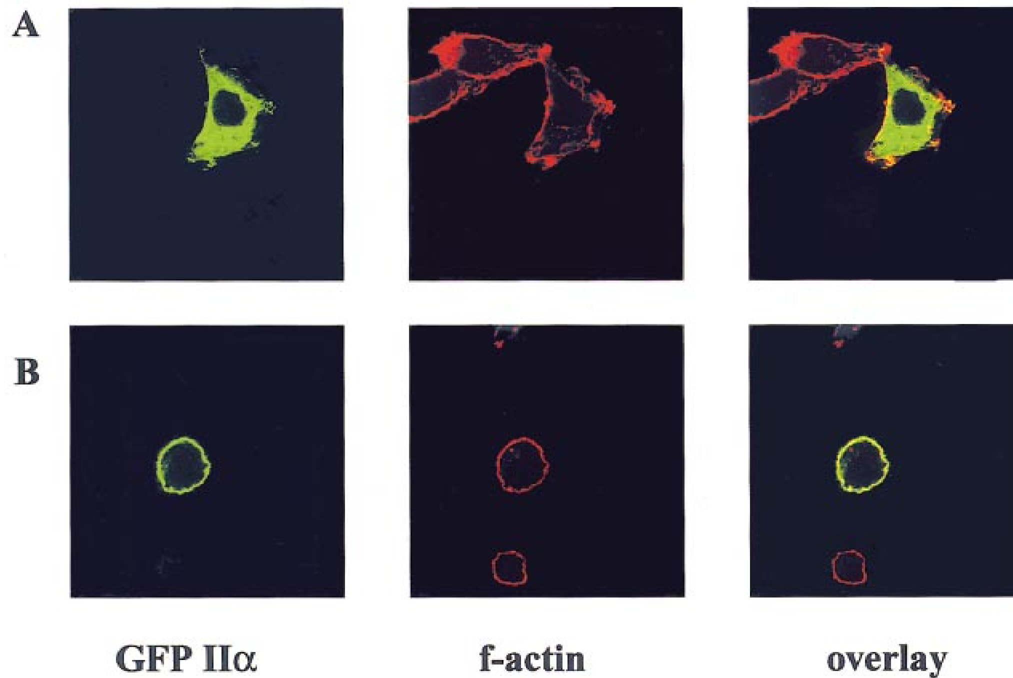

Figure 6 HeLa cells co-transfected with GFP IIα PIPkin alone (A) or with GFP IIα PIPkin and FLAG Iγ PIPkin (B)

Fixed permeabilized cells were stained with Alexa-fluor568-conjugated phalloidin to visualize the actin cytoskeleton (red), and were examined using confocal microscopy.

the immunoprecipitation conditions. However, it is not clear whether this situation also exists under intracellular conditions, as in intact cells most of the wild-type type IIα PIPkin associates with co-transfected type I isoforms (see Figures 5B and 6). Although this does not give a direct insight into interaction between the type II PIPkin and the endogenous type I activity, it does provide evidence that the type I–type II interaction is robust. The significance (if any) of the observed enhancement of type I activity in immunoprecipitates from type IIα (D273K)-transfected cells therefore remains unclear.

Whether the interaction of the type I and type II PIPkins is direct or involves the interaction of both with another binding protein(s) remains to be determined. However, the ability of co-transfected type I PIPkins to alter the subcellular distribution of co-transfected type IIα PIPkin suggests that any such putative binding protein must be present at high levels within cells. Whether or not the binding is direct, the interaction provides a plausible mechanism for allowing access of the type II PIPkins to their substrate. Type IIα PIPkin adopts a cytosolic localization when overexpressed in cells (Figures 5A and 6A), which is surprising at a superficial level, given that its lipid substrate(s) are presumably integral to membranes. Interaction with a type I PIPkin may therefore be necessary for positioning type IIα PIPkin at the membrane surface, where it has access to lipids.

The ability of type IIα PIPkin to interact with more than one type I isoform is also surprising, but the data clearly show that it associates with all three known mammalian type I PIPkins. Interestingly, the small G-protein Rac-1 shares this ability to interact with more than one type I PIPkin isoform [20]. Identification of the site on the type I PIPkins that mediates their interaction with type IIα remains to be performed. However, the α, β and γ isoforms of type I PIPkin only possess homology with each other in limited regions, outside of which they lack similarity [17,18]; it is likely to be within the regions of homology that the interaction domain lies.

Little published evidence exists for the incorporation of type II PIPkins into multi-enzyme complexes, although the type IIβ isoform co-immunoprecipitates with the tumour necrosis factor-α receptor [21], the epidermal growth factor receptor and ErbB2 [22]. Also, evidence for the ability of type IIα PIPkin to interact with other, as yet uncharacterized, proteins comes from a recent study on bovine rod outer segments [23]. In this preparation, conditions that promote tyrosine phosphorylation also stimulate the appearance of type IIα PIPkin in anti-phosphotyrosine immunoprecipitates, despite the fact that the PIPkin itself is not tyrosine-phosphorylated. Type II PIPkin immunoreactivity (isoform unknown) has also been shown to translocate from a Triton X-100-soluble location to the cytoskeleton of aggregating human platelets [13], suggesting further the participation of these isoforms in multi-protein complexes.

In contrast with the paucity of interactions recognized for type II PIPkins, a number of studies report interactions of the type I PIPkins with a variety of binding partners. Types Iα and Iβ both interact with Rac-1 (see above), and type I PIPkin–Rac-1 complexes from rat brain also contain a diacylglycerol kinase that binds directly to the PIPkin [24]. Furthermore, a type I PIPkin activity forms a complex with a PtdIns 4-kinase and active protein kinase Cμ [25], whereas both phospholipase D1 (PLD1) and PLD2 associate with the Iα isoform [26]. The functional consequences of these interactions are not completely understood, but it seems likely that the ability of type I PIPkins to interact with enzymes involved in other aspects of lipid regulation is significant. Whether a type I PIPkin molecule can interact with multiple binding partners at once, perhaps forming

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a multi-enzyme complex able to regulate several aspects of lipid metabolism, remains to be determined.

In conclusion, we have demonstrated a previously undescribed interaction between type IIα and type I PIPkins. Interaction occurs with all three known type I PIPkins, in each case altering the subcellular localization of the type IIα isoform. The result of this is a recruitment of the type II PIPkin from the cytosol to the plasma membrane, potentially giving it greater access to lipid substrates, either for the purposes of modulating PtdIns(4,5)P_2 [or possibly PtdIns(3,4)P_2] levels or, alternatively, to allow removal of PtdIns5P.

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