Molecular determinants of activation and membrane targeting of phosphoinositol 4-kinase IIβ

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INTRODUCTION

Mammalian cells contain two isoforms of the type II PI4K (phosphoinositol 4-kinase), PI4KIIα and β. These 55 kDa proteins have highly diverse N-terminal regions (approximately residues 1–90) but conserved catalytic domains (approximately from residue 91 to the C-termini). Nearly the entire pool of PI4KIIα behaves as an integral membrane protein, in spite of a lack of a transmembrane domain. This integral association with membranes is due to palmitoylation of a cysteine-rich motif, CCPCC, located within the catalytic domain. Although the CCPCC motif is conserved in PI4KIIβ, only 50% of PI4KIIβ is membrane-associated, and approximately half of this pool is only peripherally attached to the membranes. Growth factor stimulation or overexpression of a constitutively active Rac mutant induces the translocation of a portion of cytosolic PI4KIIβ to plasma membrane ruffles and stimulates its activity. Here, we demonstrate that membrane-associated PI4KIIβ undergoes two modifications, palmitoylation and phosphorylation. The cytosolic pool of PI4KIIβ is not palmitoylated and has much lower lipid kinase activity than the membrane-associated kinase. Although only membrane-associated PI4KIIβ is phosphorylated in the unique N-terminal region, this modification apparently does not influence its membrane binding or activity. A series of truncation mutants and α/β chimaeras were generated to identify regions responsible for the isoform-specific behaviour of the kinases. Surprisingly, the C-terminal approx. 160 residues, and not the diverse N-terminal regions, contain the sites that are most important in determining the different solubilities, palmitoylation states and stimulus-dependent redistributions of PI4KIIα and β.

Key words: acyl protein thioesterase 1 (APT1), catalytic domain, cysteine-rich motif, integral membrane protein, palmitoylation, phosphoinositol 4-kinase (PI4K).

The two PI4KII isoforms have conserved catalytic domains, extending approximately from residue 91 to the C-termini (residues 478 and 481 of rat PI4KIIα and human PI4KIIβ respectively). However, their N-terminal 90 amino acids have almost no sequence similarity. Despite the absence of a transmembrane domain, more than 90% of PI4KIIα is membrane-bound and requires detergent for extraction [6]. This integral association with membranes is due to palmitoylation in a cysteine-rich motif, CCPCC, located within the catalytic domain. Deletion of this motif abrogates catalytic activity and converts the kinase into a tightly bound peripheral membrane protein, extractable by 0.1 M Na2CO3 (pH 11), although not by 1 M NaCl [6]. Although the CCPCC motif is also present in PI4KIIβ, this isoform is only 50% membrane-associated, and at least half of the membrane-bound pool can be solubilized by 0.1 M Na2CO3 (pH 11). When assayed under identical conditions using PtdIns/Triton X-100-mixed micelles as the substrate, membrane-bound PI4KIIβ was found to be approx. 20-fold more active than its soluble counterpart [12].

The different distributions of PI4KIIα and β suggest that PI4KIIα is constitutively active in cells, whereas PI4KIIβ is subject to regulation. Our long-term goals are to understand the basis for this regulation and to explain at a molecular level the different membrane-binding properties of the two isoforms. Here, we show that only the active, membrane-associated pool of PI4KIIβ is palmitoylated and phosphorylated. Palmitoylation, but
not phosphorylation, is required for expression of kinase activity, at least in vitro. We further show that the C-terminal approach of PI4KIIβ compared with PI4KIIα, and for its Rac-dependent redistribution to the plasma membrane.

**MATERIALS AND METHODS**

**Reagents**

L-α-PtdIns was obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Primers were obtained from Sigma/Genosys and IDT (Corvald, IA, U.S.A.). Cloning reagents and reagents for mutagenesis were from Stratagene. Triton X-100 and reagents for electrophoresis were purchased from Bio-Rad. Other reagents, including ATP, buffers and protease inhibitors, were from Sigma. Monoclonal anti-Myc antibody 9E10 was obtained from the National Cell Culture Center (Minneapolis, MN, U.S.A.). Polyclonal anti-Myc antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal anti-HA (haemagglutinin) antibody was from Covance (Berkeley, CA, U.S.A.). Synthetic peptides corresponding to residues 2–17 of rat PI4KIIα and 2–15 of human PI4KIIβ, conjugated to keyhole-limpet (*Diodora aspera*) haemocyanin via an extra N-terminal cysteine residue, were used to immunize rabbits. The antibodies were affinity-purified on SulfoLink prepacked columns (Pierce) with cross-linked peptide according to the manufacturer’s instructions.

**Cell culture and transfection**

COS, HeLa and HEK-293 cells (human embryonic kidney cells) were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) FBS (fetal bovine serum) and antibiotics. The cells were replated 1 day prior to transfection. For transient expression of proteins, cells were transfected for 20 h and antibiotics. The cells were replated 1 day prior to transfection. For transient expression of proteins, cells were transfected for 20 h and antibiotics. The cells were replated 1 day prior to transfection.

**cDNA constructs and generation of mutants**

Rat PI4KIIα (gi:16758553) and human PI4KIIβ (gi:20159767) cDNAs were cloned in pCMV5-Myc vectors as described previously [6,12]. Cat α, the Myc-tagged rat PI4KIIα catalytic domain, was generated as described in [6] where it was designated ‘92–478’. Cat β, the Myc-tagged human PI4KIIβ catalytic domain, was generated by PCR using primers 5′-TGCACCTGCAAGCTTGGTACAGATGATCCTTG-3′ and 5′-GCTCTAAGACTACCCAGAGAAGGAAATGGCTTCTGC-3′, which introduced the PsiI and XbaI restriction sites respectively. The palmitoylation motif of PI4KIIβ, C′′′′′′′PCCC′′′′′′′, was deleted by PCR using primers: 5′-GGACCA-AAATATGCTCATAAAGCTTTGCGGCTGCTGATTC-TAATC-3′ and 5′-GATTAGGAATTCACGGAGCCTCGGC-3′.

To make chimaeric cDNA constructs of full-length PI4KIIα and PI4KIIβ as shown in Table 1, residues 1–91 of rat PI4KIIα were replaced by residues 1–90 of human PI4KIIβ and vice versa. To generate the α/β chimaera, two separate PCR products were generated using primers A (5′-CCATCGATATGACGACCGAGCCCGCTAGTGTACA-3′) and B (5′-CCAGAAGATGCTTGTCGCTCATCCGACTCTGG-3′) as well as primers C (5′-CCAGGGCGCGCGGGGCTTA-CTCGACTACCCAGAGAAGGAAATGGCTTCTGC-3′) and D (5′-GCTCTAAGACTACCCAGAGAAGGAAATGGCTTCTGC-3′). The fragments were combined and the overlap was extended using primers A and D. The final PCR product was subcloned into pcMV5-Myc vector by using Clal and Xbal. To generate the β/α chimaera, two PCR products were generated using primers 1 (5′-CGCACCAGTTTCAATGGAGGATCCTCCGAGCCGCCAC-3′) and 2 (5′-CGCACCAGTTTCAATGGAGGATCCTCCGAGCCGCCAC-3′) as well as primers C (5′-CCAGGGCGCGCGGGGCTTA-CTCGACTACCCAGAGAAGGAAATGGCTTCTGC-3′) and D (5′-GCTCTAAGACTACCCAGAGAAGGAAATGGCTTCTGC-3′). The fragments were combined and the overlap was extended using primers 1 and 4. The PCR product was digested with EcoRI and Xbal and inserted into pcMV5-Myc vector.

The chimaeric catalytic domains, Cat α/β and Cat β/α, were generated by replacing residues 91–311 of human PI4KIIβ with residues 92–314 of rat PI4KIIα and vice versa. First and second round PCRs were performed as described above. The Cat α/β chimaera was generated by using primers 5′-CCATCGATATGACGACCGAGCCCGCTAGTGTACA-3′ and 2 (5′-CGCACCAGTTTCAATGGAGGATCCTCCGAGCCGCCAC-3′) and D (5′-GCTCTAAGACTACCCAGAGAAGGAAATGGCTTCTGC-3′) and the extended product was ligated in pBluescript II SK vector and subcloned in pCMV5-Myc vector after digestion with KpnI and Xbal. The Cat β/α chimaera was generated by

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using primers 5′-CCATCGATACTTTGACTTTGACTGAGATGATAATGACTTATGCTTTGATATCCATTACATCGATGG-3′ and 5′-GGGATGTTATGCTTTGACTTTGACTGAGATGATAATGACTTATGCTTTGATATCCATTACATCGATGG-3′ as well as 5′-GGCAGAATGATTTGCTTTGACTTTGACTGAGATGATAATGACTTATGCTTTGATATCCATTACATCGATGG-3′ and 5′-GCTTATGCTTTGACTTTGACTTTGACTGAGATGATAATGACTTATGCTTTGATATCCATTACATCGATGG-3′. The final PCR product was subcloned into pCMV5-Myc using Clal and PstI.

Point mutations in pCMV5-Myc-PI4KIIβ cDNA constructs were performed using the QuikChange® kit (Stratagene) according to the manufacturer’s instructions. pCMV5-HA-Rac2V12 cDNA was kindly provided by Dr Michael White (University of Texas Southwestern Medical Center).

Preparation of the cytosol and membranes

Cells were washed with PBS and scraped in a solution containing 0.25 M sucrose, 20 mM Tris/HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 0.2 mM PMSF, protease inhibitor cocktail (10 μg/ml each of Nα-p-tosyl-l-lysine chloromethyl ester, Nα-p-tosyl-l-arginine methyl ester, Nα-p-tosyl-l-lysine chloromethane, leupeptin and pepstatin A) and phosphatase inhibitors (50 mM NaF, 50 mM glycerophosphate and 1 mM Na3VO4). Cells were lysed by two freeze–thaw cycles and passed through a 27.5 gauge needle. Lysates were then centrifuged at 1000 g for 5 min to obtain PNS (post-nuclear supernatants). The PNS were then centrifuged at 200000 g for 15 min to separate the cytosol from membranes. The resulting membrane pellets were homogenized in one of the following solutions: (i) 0.1 M Na2CO3 (pH 11) to extract peripheral proteins; or (ii) 1% (v/v) Triton X-100, 20 mM Tris/HCl (pH 7.5) and 1 mM EDTA to extract integral proteins. After homogenization, samples were again centrifuged at 200000 g for 15 min at 4°C to remove insoluble material. In some cases, the membrane fractions were prepared sequentially. In these cases, designated in the Figure legends, pellets were first homogenized in 0.1 M Na2CO3 (pH 11) to extract peripheral proteins and then homogenized in solutions containing 1% Triton X-100 to extract integral proteins.

Analysis of [3H]palmitate and [32P]P incorporation

For radiolabelling, transfected COS cells were incubated with phosphate-free or lipid-free medium containing 5% dialysed FBS and either 0.25 mM [3H]palmitate (PerkinElmer Life Sciences) or, as described by Linder et al. [14], [3H]palmitate (0.3 mM/ci/ml) (PerkinElmer Life Sciences) for 4 h. After three brief washes with PBS, labelled cells were scraped with a buffer containing 50 mM Tris/HCl (pH 8.0), 0.15 M NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.05% SDS, 2 mM EDTA, 0.2 mM PMSF and protease and phosphatase inhibitors (RIPA buffer). The cell lysates were immunoprecipitated by a 4 h incubation with anti-Myc antibody that had been chemically cross-linked to Protein G–Sepharose beads (Zymed) using DMP (dimethyl pimelimidate; Pierce). The immunoprecipitates were washed three times with PBS, labelled cells were scraped with a buffer containing 1 M hydroxylamine (pH 7.0) for 16 h at 4°C. Control samples were incubated with 1 M Tris/HCl (pH 7.0) under the same conditions. Enzymatic deacylation was performed by treating the immunoprecipitates with bacterially expressed APT1 (acyl protein thioesterase 1; clone provided by Dr Susanne Mumby, University of Texas Southwestern Medical Center) for 2 h at 30°C.

Depalmitoylation

Prior to chemical or enzymatic deacylation, expressed PI4KIIβ was immunoprecipitated in RIPA buffer. Precipitated kinases were ‘renatured’ by washing in a solution containing 0.1% (v/v) Triton X-100, 50 mM Tris/HCl (pH 7.5), 0.1 M NaCl and 0.2 mM PMSF. Chemical deacylation was carried out by incubation with 1 M hydroxylamine (pH 7.0) for 16 h at 4°C. Control samples were incubated with 1 M Tris/HCl (pH 7.0) under the same conditions. Enzymatic deacylation was performed by treating the immunoprecipitates with bacterially expressed APT1 (acyl protein thioesterase 1; clone provided by Dr Susanne Mumby, University of Texas Southwestern Medical Center) for 2 h at 30°C.

Inhibition of palmitoylation with 2-BP (2-bromopalmitate)

To obtain unpalmitoylated kinase, COS cells expressing PI4KIIβ were treated with 2-BP according to a procedure described by Webb et al. [17]. Cells were incubated for 16 h in DMEM containing 2.5% dialysed FBS and 0.25% defatted BSA, with or without 100 μM 2-BP dissolved in ethanol. The cells were then labelled for 4 h with [3H]palmitate (in the presence or absence of 2-BP), lysed in RIPA buffer, and the kinases were immunoprecipitated and renatured as above. Each immunoprecipitate was divided into aliquots in order to estimate the amount of kinase by immunoblotting, to measure incorporation of radioactive palmitate, and to assay for kinase activity.

Immunofluorescence microscopy

HeLa cells transfected with different constructs were grown on coverslips. Cells were then washed three times with ice-cold PBS, fixed in 3.7% formaldehyde and permeabilized with 0.1% Triton X-100 on ice and labelled with antibodies in blocking buffer [1% BSA and 3% (v/v) donkey serum in PBS], Cells were examined using a Zeiss 510 laser scanning confocal microscope with a ×63 1.3 NA PlanApo objective. The time of image acquisition, the image gain and enhancement were optimally adjusted at the outset and kept constant for all samples. In most cases, images were collected near the middle of the z-axis. Captured images

and, after three washes with RIPA buffer, the beads were electro-phoresed and visualized by Coomassie Brilliant Blue staining. The slowly migrated upper band was excised and subjected to MS analysis. The lower kinase band was used for the control.

The Coomassie Brilliant-blue stained protein bands were excised from one-dimensional SDS/PAGE gels and subjected to in-gel digestion with trypsin essentially as described in [15,16]. The dried protein digests were dissolved in 5% (v/v) formic acid and loaded on to a pulled capillary filled with Poros R2 resin. After washing three times with 5% formic acid, the peptides were eluted into a nanoelectrospray needle with 1–2 μl of nanoelectrospray sample solution [1% ammonium hydroxide and 60% (v/v) methanol]. All MS analyses are performed on a QSTAR Pulsar-i quadrupole TOF (time-of-flight) tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, ON, Canada) equipped with a nanoelectrospray ion source (MDS Proteomics, Odense, Denmark). For precursor ion scanning experiments, the instrument is set in negative ion mode, to detect the PO3− fragment ion at m/z 79. The optimum collision energies are determined for each experiment by gradually increasing the collision energy in steps corresponding to one-twentieth of the m/z value of the precursor ion. Phosphorylated peptides are detected by nanoelectrospray precursor ion scanning and the exact sites of phosphorylation are determined by manual interpretation of mass spectra in combination with results from site-specific mutagenesis.

Activation and targeting of PI4KIIβ

To identify phosphorylation sites, HEK-293 cells were transfected with pCMV5-Myc-PI4KIIβ for 20 h. Cell lysates were prepared in RIPA buffer containing phosphatase inhibitors. Expressed Myc–PI4KIIβ was immunoprecipitated with anti-Myc antibody
were analysed using MetaMorph® Imaging software (Molecular Devices). Pixel intensity was used to quantify fluorescence in the region of interest [9].

**PI4K assays**

Kinase activity was measured by phosphorylation of PtdIns in PtdIns/Triton X-100-mixed micelles (lipid/detergent molar ratio 1:10), using \( \gamma^{-32} \text{P}\)ATP (10 mCi/ml) as radioactive phosphate donor as described previously [6]. Phospholipids were extracted by the method of Bligh and Dyer [18] and separated by TLC in a solvent system consisting of \( n \)-propyl alcohol/water/NH\(_4\)OH (65:20:15, by vol.). Radioactive PtdIns4P spots were detected by autoradiography using FLA-5100 (Fuji Photo Film) and radioactivity was quantified using the Multi-Gauge V2.3 program (Fuji Photo Film).

**Other procedures**

SDS/PAGE and immunoblot analysis were carried out by the methods of Laemmli [19] and Towbin et al. [20] respectively. To quantify PI4KII levels in various fractions, samples were electrophoresed and immunoblotted with anti-PI4KII antibodies. The immunoblots were incubated with \( ^{125} \text{I} \)-labelled secondary antibody to estimate relative amounts of proteins. Alternatively, immunoblotted bands were quantified using ImageJ 1.34 n software (National Institutes of Health). Protein concentrations were determined using the modified Lowry method [21] described by Peterson [22] with BSA as a standard.

**RESULTS**

**Post-translational modifications of membrane-associated PI4KIIβ**

In cells, approx. 90% of PI4KIIα and 30% of PI4KIIβ behave as integral membrane proteins, requiring detergent for solubilization. The remaining 70% of PI4KIIβ is almost evenly divided between cytosolic and peripheral membrane pools (Figure 1A). This distribution pattern is similar for the recombinant, overexpressed kinase (Figure 1B). We showed previously that only the membrane-bound pool of PI4KIIβ exhibits enzymatic activity. Here, we test whether post-translational modifications, palmitoylation and phosphorylation, influence the membrane-binding properties and activity of PI4KIIβ.

We first analysed \( ^{1} \text{H} \)-palmitate incorporation into the various PI4KIIβ pools. As expected, only membrane-bound PI4KIIβ incorporates radioactivity in labelled cells (Figure 2A). Deletion of a cysteine-rich motif within the catalytic domain, C\(_{170}^{174}\)CPCC, eliminates both palmitoylation (Figure 2B) and catalytic activity (Figure 2C), as it does in PI4KIIα [6]. However, this CCPCC mutant only slightly redistributes from membranes to the cytosol (Figure 2D), indicating that thioacylation is not essential for association of the kinase with biological membranes. However, deletion of the C\(_{170}^{174}\)CPCC motif eliminates the pool of PI4KIIβ that requires detergent for solubilization (Figure 2E).

To confirm that palmitoylation is required for activity of wild-type PI4KIIβ, we subjected wild-type membrane-bound PI4KIIβ to chemical or enzymatic deacylation using hydroxylamine (NH\(_2\)OH) or bacterially expressed APT1 respectively. These interventions result in losses of activity that correspond closely to reductions in \( ^{1} \text{H} \)-palmitate incorporation (Figure 3). Treatment with 1 M NH\(_2\)OH removes 95% of incorporated label and...
alkaline phosphatase treatment, providing further evidence that a portion of membrane-bound PI4KIIβ precipitates; right panel: autoradiogram to detect phosphorylation. (Figure 4A). The upper electrophoretic band is lost upon deacylation by incubation with APT1 for 2 h at 30°C. Moreover, PI4KIIβ migrates on SDS gels as a single species; hence, phosphorylation of this residue is apparently responsible for the slowed electrophoretic migration (bottom panel in Figure 6A). Neither the S77A nor the S12A/S17A/S77A mutant has impaired kinase activity (Figure 6B), consistent with the lack of inhibition observed upon alkaline phosphatase treatment. Moreover, both mutants are similar to wild-type PI4KIIβ with respect to their distribution between membranes and the cytosol (Figure 6C).

Next, we tested whether there is a relationship between phosphorylation and palmitoylation of PI4KIIβ. To determine if palmitoylation is necessary for kinase phosphorylation, wild-type PI4KIIβ and a deletion mutant lacking the palmitoylation motif (ΔCCPCC) were expressed in 32P-labelled cells. Figure 6(A) shows that there is no difference in 32P incorporation in the two proteins, indicating that palmitoylation is not a prerequisite for phosphorylation. To determine whether kinase phosphorylation influences palmitoylation, we analysed 3H-palmitate incorporation into wild-type PI4KIIβ and the S77A and S12A/S17A/S77A mutants. Again, no difference in labelling between the wild-type and mutant kinases is observed (Figure 6D).

Taken together, these results demonstrate that phosphorylation, at least in the N-terminal region, is not required for the interaction between PI4KIIβ and its yet unidentified protein acyltransferase. Moreover, PI4KIIβ does not have to be integrally bound to the membrane to undergo phosphorylation.

Identification of membrane binding determinants in PI4KIIα and β

Based on the sequence diversity of the N-terminal 90 amino acids of PI4KIIα and β (Figure 5A), we predicted that these regions would determine the differences in distribution and palmitoylation level between the two isoforms. The N-terminal region of PI4KIIα has a pl of 6.9 and contains a long stretch of hydrophobic residues (V8AAQAQLAAVAVHAVQ95), whereas the N-terminal region of PI4KIIβ contains few hydrophobic residues, two stretches of charged residues (E5EEEDEGER82 and E55EGEAGDE89), and has a pl of 4.2. Therefore we speculated that the N-terminal region of PI4KIIα may enhance its interaction with membranes, whereas that of PI4KIIβ could interfere with...
membrane binding. However, this prediction proved to be incorrect, as the catalytic domains of the two kinases, designated Cat α and Cat β, behaved similarly to the full-length proteins despite lacking the N-terminal 90 residues (Table 1). Indeed, Cat β is even more soluble than full-length PI4KII β (71% compared with 44%), indicating that the N-terminal region contributes to membrane binding. However, a Myc-tagged construct consisting of residues 1–90 of PI4KII β is exclusively cytosolic (results not shown), suggesting that it is not sufficient for membrane binding, even though it contributes to binding in the context of the full-length protein. Consistent with these results, an α/β chimaera (residues 1–91 of PI4KIIα fused to residues 91–481 of PI4KIIβ) distributes similarly to PI4KIIβ. Likewise, a β/α chimaera (residues 1–90 of PI4KIIβ fused to residues 91–478 of PI4KIIα) behaves like PI4KIIβ (Table 1). The palmitoylation levels of the truncated chimeras and chimaeras are also determined by their catalytic domains: Cat α and Cat β/α are palmitoylated to the same extent as PI4KIIα; Cat β and α/β are palmitoylated to slightly lower extents than PI4KIIβ. The enzymatic activities of these mutant kinases correspond reasonably closely to their levels of palmitoylation.

Having established that the catalytic domains contain the major membrane binding and palmitoylation determinants of PI4KIIα and β, we next generated a pair of hybrid catalytic domains to more precisely localize these determinants within the two kinases. Because the C-terminal portions of the catalytic domains are slightly less similar than the N-terminal portions (77% compared with 86% similarity; Figure 5), we examined the distribution in cells of a chimaera consisting of residues 91–314 of PI4KIIα fused to residues 312–481 of PI4KIIβ (termed Cat α/β), and another chimaera consisting of residues 91–311 of PI4KIIβ fused to residues 315–478 of PI4KIIα (Cat β/α). As shown in Table 1, Cat α/β is 85% cytosolic and, hence, more similar to PI4KIIβ with respect to solubility, whereas Cat β/α is, like PI4KIIα, almost exclusively bound integrally to membranes. Consistent with these observations, Cat α/β is a poorer substrate than Cat β/α for palmitate incorporation in cells, and expresses negligible kinase activity in vitro. These results demonstrate that the C-terminal approx. 160 amino acids are primarily responsible for the distinct membrane binding properties of PI4KIIα and β and, more specifically, for the presence of a large pool of cytosolic PI4KIIβ that may be available for activation in response to cellular stimuli [12].

Identification of regions in PI4KIIβ that control its Rac-dependent recruitment to the plasma membrane

In quiescent cells, PI4KIIα and β are enriched in the perinuclear region [12] but are also located in other cytoplasmic structures, including the ER [23] and endocytic vesicles [8,24]. However, neither kinase is abundant on the plasma membrane unless cells are treated with PDGF or overexpress the active Rac mutant, RacV12 [12]. In these cases, PI4KIIβ, and to a much lesser extent PI4KIIα, partially translocate to the cell periphery, concentrating in regions of membrane ruffling. To determine if the catalytic domains of the kinase contain information required for stimulus-dependent plasma membrane targeting, we immunolocalized the hybrid kinases described above. Similarly to membrane binding and palmitoylation, the catalytic domains determine the responsiveness of each hybrid to RacV12 overexpression: the α/β hybrid, like wild-type PI4KIIβ, displays much more pronounced plasma membrane distribution than either the β/α hybrid or wild-type PI4KIIα (Figure 7A). Likewise, the isolated catalytic domain of PI4KIIβ (Cat β) shows much more prominent plasma membrane localization than does Cat α (Figure 7B). The C-terminal portion of the PI4KIIβ catalytic domain is responsible for the observed difference in peripheral targeting (Figure 7B), as it is for the greater extent of solubility of PI4KIIβ compared with PI4KIIα.

DISCUSSION

The present study follows our previous report [12] and that of Balla et al. [8] that PI4KIIβ is more soluble and less active than PI4KIIα and our finding that a portion of PI4KIIβ translocates to membrane ruffles in response to PDGF treatment or overexpression of RacV12 [12]. In an effort to explain these observations, we examined the effects of two post-translational
modifications of PI4KIIβ, thioacylation and phosphorylation, and identified the region in PI4KIIβ that is responsible for determining its association with cellular membranes.

PI4KIIα and β differ significantly in their extent of thioacylation, with PI4KIIβ incorporating only 30% of [3H]palmitate. Because palmitoylation is essential for expression of catalytic activity, at least in vitro (Figure 3), this result suggests that cells contain a large pool of inactive PI4KIIβ that may be mobilized and activated in a stimulus-dependent manner. It is interesting that Balla et al. [8] reported that the specific activity of PI4KIIβ is 30% that of PI4KIIα, corresponding exactly to the differences in palmitoylation state determined here.

We also observed that only membrane-associated PI4KIIβ is phosphorylated, and that this modification accounts for the more slowly migrating electrophoretic band seen in SDS gels of membrane-bound but not cytosolic PI4KIIβ. The major phosphorylation site was identified by MS and mutational analysis as Ser77, located in the N-terminal segment, which is most diverse between the two PI4KII isoforms. There was no effect on catalytic activity, palmitoylation state or subcellular distribution when PI4KIIβ phosphorylation was reduced by alkaline phosphatase treatment or by mutation of Ser77 to alanine (or to the potentially phosphomimetic residue, glutamate; results not shown). However, it remains possible that phosphorylation of the kinase regulates specific protein–protein interactions that are yet to be defined.

In view of the sequence diversity of the N-terminal segments of PI4KIIα and β (Figure 5A), we expected that these regions would account for the differences in acylation and distribution between the two isoforms. However, the differences were maintained by the isolated catalytic domains, which are relatively conserved in sequence (64% identity and 77% similarity). The C-terminal approx. 160 residues of these catalytic domains are most responsible for the greater solubility and lower level of palmitate incorporation of PI4KIIβ than PI4KIIα. In addition, they are responsible for the preferential redistribution of PI4KIIβ to plasma membrane ruffles in response to RacV12 overexpression. Within the C-terminal segments of the kinase, these are two short stretches having essentially no sequence similarity. In future
Figure 7  Redistribution of wild-type and truncated/hybrid PI4KIs in response to expression of activated RacV12

The constructs shown in Table 1 ([A] full-length; [B] catalytic domains) were expressed in HeLa cells and visualized using anti-Myc antibodies followed by rhodamine-conjugated anti-rabbit antibody. Actin filaments were stained with FITC-phalloidin and HA–RacV12 was visualized using anti-HA antibodies followed by Cy5-conjugated anti-mouse antibodies. Membrane ruffles are highlighted with arrowheads. Scale bars, 50 \( \mu \)m. The histogram at the bottom shows the proportion of cells that display plasma membrane staining. The numbers indicate the number of cells showing anti-Myc staining at the plasma membrane (PM) compared with the total number of cells expressing HA–RacV12. The values shown were obtained from two independent experiments that gave similar results.

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studies, we will examine the importance of these two stretches in determining the different properties of PI4KIIα and β.

Although the different solubilities of PI4KIIα and β are determined primarily by their C-terminal approx. 160 residues, the unique N-terminal segment of PI4KIIβ nevertheless contributes to its affinity for cellular membranes. Deletion of this highly acidic region, residues 1–90, results in the redistribution to the cytosol of approximately half of the hitherto membrane-associated portion of the intact kinase, shifting from 44 to 71 % cytosolic. This is consistent with the immunofluorescence microscopy data of Balla et al. [8], who show that a truncated version of PI4KIIβ lacking the N-terminal 96 amino acids is predominantly cytosolic.

Our observations highlight the role of the C-terminal portions of the type II kinases in membrane targeting. However, it is possible that the unique N-terminal regions are important in mediating specific protein–protein interaction within membranes. For example, Xu et al. [25] recently showed that the N-terminal region of PI4KIIα is essential to target the kinase to a specific subset of glucose transporter-4-containing vesicles in HEK-293 cells, i.e. those which also contain cellugyrin.

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