Phosphoinositides and lipid kinases in oxidative stress signalling and cancer
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CHAPTER 6

PIP4Kβ interacts with and modulates nuclear localization of the high-activity PtdIns5P-4-kinase isoform PIP4Kα

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Abstract

The β-isoform of PIP4K (PtdIns5P-4-kinase) regulates the levels of nuclear PtdIns5P, which in turn modulates the acetylation of the tumour suppressor p53. The crystal structure of PIP4Kβ demonstrated that it can form a homodimer with the two subunits arranged in opposite orientations. Using MS, isoform-specific antibodies against PIP4Ks, RNAi (RNA interference) suppression and overexpression studies, we show that PIP4Kβ interacts in vitro and in vivo with the PIP4Kα isoform. As the two isoforms phosphorylate the same substrate to generate the same product, the interaction could be considered to be functionally redundant. However, contrary to expectation, we find that PIP4Kβ has 2000-fold less activity towards PtdIns5P compared with PIP4Kα, and that the majority of PIP4K activity associated with PIP4Kβ comes from its interaction with PIP4Kα. Furthermore, PIP4Kβ can modulate the nuclear localization of PIP4Kα, and PIP4Kα has a role in regulating PIP4Kβ functions. The results of the present study suggest a rationale for the functional interaction between PIP4Kα and PIP4Kβ and provide insight into how the relative levels of the two enzymes may be important in their physiological and pathological roles.

Key words: phosphoinositide, PtdIns(4,5)P₂, PtdIns5P, PtdIns5P kinase.

Abbreviations used: CUL3, Cullin 3; DSS, disuccinimidyl suberate; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EE, Glu-Glu tag (EYMPME); FRB, final resuspension buffer; GST, glutathione transferase; HA, haemagglutinin; HEK, human embryonic kidney; i.d., internal diameter; ING2, inhibitor of growth protein 2; MEL, murine erythroleukaemia; Myr, myristoyl; PBS-T, PBS containing 0.1% Tween 20; PIP4K, PtdIns5P-4-kinase; PIP5K, PtdIns4P-5-kinase; PKB, protein kinase B; PtdSer, phosphatidylserine; RBX1, ring-box 1; RNAi, RNA interference; shRNAi, short hairpin RNAi; SPOP, speckle-type POZ protein.
Introduction

Combinations of phosphorylation at the 3, 4 or 5 position of the inositol head group of phosphatidylinositol generates seven different phosphoinositides that form the basis of a ubiquitous membrane signalling system. An array of tightly regulated phosphoinositide kinases and phosphatases ultimately control the subcellular profile of phosphoinositides [1]. Phosphoinositides can regulate protein localization, ion channel function and protein enzymatic activity, which can have an impact on cellular processes including vesicle transport, cytoskeletal dynamics, cell proliferation and survival, gene transcription, cell polarity and migration [2].

PtdIns(4,5)P$_2$ is central to phosphoinositide signalling, being a substrate for both PLC (phospholipase C) and PtdIns-3-kinase and itself a second messenger [3]. PtdIns(4,5)P$_2$ can be synthesized by two different enzyme families that are highly related. PIP5Ks (PtdIns4P-5-kinases) phosphorylate PtdIns4P [4] on the 5-position, whereas PIP4Ks (PtdIns5P-4-kinases) phosphorylate PtdIns5P on the 4-position [5]. The cellular level of PtdIns4P is much greater than PtdIns5P and it is likely that the majority of PtdIns(4,5)P$_2$ in the cell is synthesized through PIP5K activity. In accordance with this hypothesis, overexpression or suppression of different isoforms of PIP5K impinge on many of the processes that are known to be regulated by PtdIns(4,5)P$_2$ [3]. In contrast with PIP5Ks, the role of PIP4Ks is less well understood. Of the three isoforms of PIP4K [6–9], the α and β isoforms can phosphorylate PtdIns5P and PtdIns3P [10], whereas the γ isoform is likely to be inactive. PIP4Kγ, however, can interact with PIP4Kα and β, probably to target their activities to a particular subcellular localization [11].

Overexpression of PIP4Ks does not lead to phenotypes that are induced by PIP5Ks, suggesting that PIP4Ks do not increase the cellular level of PtdIns(4,5)P$_2$ to the same extent as PIP5Ks. However, this may only reflect the relative levels of PtdIns5P to PtdIns4P in the cell. In megakaryocytes, for example, PIP4Kα-mediated synthesis of PtdIns(4,5)P$_2$ is important in platelet formation [12] and in platelets it is required for secretion [13,14]. Deletion of the only PIP4K in Caenorhabditis elegans and Drosophila melanogaster led to an increase in whole-animal levels of PtdIns5P (Y. Bultsma, W.-J. Keune and N. Divecha, unpublished work) and, in MEL (murine erythroleukaemia) cells, RNAi (RNA interference)-mediated suppression of PIP4Kβ increased, whereas the overexpression of PIP4Kβ decreased, the levels of nuclear PtdIns5P [15]. These data suggest that PIP4Ks, through phosphorylation, probably control the level of PtdIns5P.

The relative expression level of PIP4K isoforms varies in mouse tissues. For example, in brain, PIP4Kα and β are approximately equal, whereas there is nearly 10-fold more PIP4Kβ mRNA in muscle and in the heart. In the spleen, PIP4Kα expression is 3–4-fold higher than PIP4Kβ. PIP4Kγ, on the other hand, is highly expressed in the kidney [11]. The differential expression suggests that each PIP4K may have specific functions within the target organ. At a cellular level, PIP4Kα localizes in the cytosol, whereas PIP4Kγ associates with an endomembrane compartment that partially co-localizes.
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with the Golgi [9,11]. PIP4Kβ appears to localize predominantly in the nucleus [16]. However, PIP4Kβ interacts with both a TNF (tumour necrosis factor) receptor [8] and the EGF (epidermal growth factor) receptor [17], suggesting that it may also be required in the cytosol. Deletion of PIP4Kβ in mice leads to increased insulin-induced PKB (protein kinase B) activation in muscle [18] and, in cells, overexpression of kinase-active but not kinase-inactive PIP4Kβ attenuates insulin-induced PKB activation [19]. Finally, overexpression of IpqD, a bacterial PtdIns(4,5)P₂-4-phosphatase that generates PtdIns5P, also increased PKB activity [19]. Taken together, these data point to a role for cytosolic PIP4Kβ and PtdIns5P in PKB activation, possibly through the regulation of PtdIns(3,4,5)P₃ [19] and/or PKB dephosphorylation [20].

PIP4Kβ is also present in the nucleus and its nuclear import is dictated by a 16-amino-acid α-helical insertion [16] that is not present in other PIP4Ks. In the nucleus, activation of the p38 stress pathway inhibits PIP4Kβ activity leading to an increase in PtdIns5P. PtdIns5P can interact with the PHD finger of ING2 (inhibitor of growth protein 2) [21], which in turn modulates the acetylation and transcriptional activity of the tumour suppressor protein p53 [15]. PHD-finger-containing proteins appear to be nuclear-specific downstream targets of PtdIns5P [22,23]. Through its interaction with SPOP (speckle-type POZ protein), PIP4Kβ can regulate the activity of the CUL3 (Cullin 3) ubiquitination complex. The CUL3 ubiquitination activity also appears to be stimulated by increased levels of PtdIns5P [24].

The differential tissue expression and subcellular localization of PIP4Ks provide a reasonable explanation for the requirement for different isoforms within a cell. However, during a search for proteins that regulate PIP4Kβ, we discovered that PIP4Kβ interacts with PIP4Kα. Previous studies indicate that PIP4Kα and PIP4Kβ phosphorylate the same substrate to generate the same product, suggesting that their interaction may have no functional advantage. In the present paper we have studied the activity of both enzymes in vitro and in vivo and, together with RNAi and overexpression assays, have developed a rationale for a functional interaction between PIP4Kα and PIP4Kβ.

**Materials and methods**

**PIP4K plasmids, antibodies and chemicals**

GST (glutathione transferase)–PIP4Kα and β, EE [Glu-Glu tag (EYMPME)]–PIP4Kα, EE–PIP4KαKD (G131L/Y138F), Myr (myristoyl)–PIP4Kα and HA (haemagglutinin)–PIP4Kβ were derived from human sequences. Myc–PIP4Kα, Myc–PIP4Kβ and Myc–PIP4KβKD (D278A) were derived from rat sequences. Peptides (p5, CGVGGNLLCSYG; p6, CNLLSFPRFFGP; and p19, CMATPGNLGSSVL) were coupled to keyhole-limpet haemocyanin and used to immunize New Zealand white rabbits (for specificity of the antibodies see Supplementary Figure S2 at http://www.BiochemJ.org/bj/430/bj4300223add.htm). Antibody 3 is a rat monoclonal antibody that has been described previously [25]. The anti-PIP4Kα-C-term (antibody against the C-terminal of PIP4Kα)
was from Abgent (AP8041b). Anti-HA and anti-Myc antibodies are monoclonal supernatants from clones 12CA5 and 9E10 respectively. Protein G– and Protein A–Sepharose, glutathione–Sepharose and Ni²⁺–Sepharose were purchased from GE Healthcare. DSS (disuccinimidyl suberate) was from Pierce Chemicals. All other chemicals were of analar grade.

**Generation of HEK (human embryonic kidney)-293 cells stably expressing shRNAi (short hairpin RNAi)**

shRNAi constructs, generated by cloning the sequences below into pRetroSuper, were used to generate viral particles in Phoenix ecotropic cells: RNAi-PIP4Kα, 5′-ATAGTGGAATGTCATGGGA-3′; and RNAi-PIP4Kβ: 5′-AGATCAAGGTGGACAATCA-3′. HEK-293 cells stably expressing the ecotropic receptor were infected with retroviral particles using Polybrene. Cell populations were selected using puromycin (2 μg/ml). In general, cells were maintained in the absence of the antibiotic selection for at least 48 h prior to the experiments.

**siRNA targeting of PIP4Ks**

Control siRNA oligonucleotides (siGENOME non-targeting siRNA pool; Dharmacon, catalogue number D-001206-13-20), or those targeting PIP4Kα (PIP4K2A siGENOME SMARTpool; Dharmacon, catalogue number M-006778-01) or PIP4Kβ (PIP4K2B siGENOME siRNA; Dharmacon, catalogue numbers D-006779-02, D-006779-04 and D-006779-06) were transfected into HEK-293 cells using Dharmafect 1 (Dharmacon). After 48 h, cell lysates were used in immunoprecipitation experiments.

**In vitro PIP4K activity measurements and PtdIns5P mass measurements**

In vitro PIP4K activity was measured using liposomes with 1 nmol of PtdIns5P and 10 nmol of PtdSer (phosphatidylserine) as a substrate, 20 μM ATP, 10 μCi of [³²P]ATP and the appropriate amount of enzyme in 100 μl of PIPkin buffer [50 mM Tris/HCl (pH 7.4), 10 mM MgCl₂, 1 mM EGTA and 70 mM KCl] for 10 min. The reactions were terminated and extracted as described previously [26]. [³²P]PtdIns(4,5)P₂, a measure of PIP4K activity, was separated by TLC and quantified using a phosphoimager (Bio-Rad). To determine how PIP4K activity varied with respect to the levels of PtdIns5P, liposomes were made with 50 nmol of PtdSer and various amounts of PtdIns5P. Phosphoimager counts were converted into μmol of PtdIns(4,5)P₂ by quantifying standard amounts of [³²P]ATP spotted on to the TLC. V_max was determined at a saturating concentation of PtdIns5P and the K_m was determined graphically at 50% of the V_max value. PtdIns5P was measured as described previously [26].

**Subcellular fractionation and cross-linking**

MEL nuclei were purified as described previously [27], except that the FRB (final resuspension buffer, see below) was made with 10 mM Hepes instead of Tris/HCl to prevent quenching of the cross-linker. Nuclei, controls or treated with the indicated
concentrations of cross-linking agent (DSS), were terminated by the addition of 1 M glycine, pH 7.5 (to a final concentration of 20 mM). Nuclear proteins were extracted using FRB (see below) containing 1% Chaps and 0.5 M NaCl, and immunoprecipitated using the antibody indicated. Immune complexes were collected on Protein G–Sepharose and were solubilized using 1×SDS/PAGE loading buffer, separated by SDS/PAGE and analysed by Western blotting.

HEK-293 cells were trypsinized, washed with PBS and resuspended in hypotonic swell buffer [5 mM Tris/HCl (pH 7.4), 1.5 mM KCl and 2.5 mM MgCl₂] for 6 min on ice. The cells were then disrupted by passage through a 22-gauge needle. The nuclei were pelleted by centrifugation (352 g for 4 min at 4 °C) and the supernatant was removed and membranes were collected by high-speed centrifugation (14000 rev./min in a microfuge) at 4 °C for 15 min. The supernatant (cytosol) was collected and the membranes were sonicated into FRB (membrane fraction). The nuclei were further purified by centrifugation (352 g for 4 min) through a sucrose cushion [10 mM Tris/HCl (pH 7.4), 1 mM EGTA, 1.5 mM KCl, 5 mM MgCl₂ and 460 mM sucrose] and washed with FRB [10 mM Tris/HCl (pH 7.4), 1 mM EGTA, 1.5 mM KCl, 5 mM MgCl₂ and 290 mM sucrose]. Nuclei were resuspended in FRB or were extracted by resuspending them in Dignam C buffer [20 mM Tris/HCl (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol and 0.5 mM DTT (dithiothreitol)] followed by incubation on ice for 30 min. The insoluble fraction was pelleted by centrifugation (20000 g for 10 min), the supernatant was dialysed against Dignam D buffer [20 mM Tris/HCl (pH 7.9), 0.1 M KCl, 0.2 mM EDTA, 10% glycerol and 0.5 mM DTT], and the samples were stored at −80 °C.

**Sucrose density centrifugation**

For sucrose density centrifugation, 1 ml each of 40%, 31.25%, 22.5%, 13.72% and 5% sucrose solutions were layered sequentially in a centrifuge tube and allowed to form a gradient overnight by standing at 4 °C. The cytosol (50 μl) and nuclear fraction (50 μl) were adjusted to 5% sucrose and loaded on top of the cushions and then centrifuged for 18 h at 45000 rev./min using a Sorvall 5555Ti rotor. After centrifugation, 15×1 ml fractions were isolated from the bottom of the centrifuge tube using a syringe and needle. The samples were mixed with SDS-loading buffer, separated by SDS/PAGE and analysed by staining the gel or by Western blotting.

**Mass spectrometry**

**Gel-band destaining and washing**

Coomassie-Blue-stained gel bands were destained with 3×20 min changes of 1 ml of 200 mM ammonium bicarbonate and 40% (v/v) acetonitrile. Gel bands were the dehydrated by the addition of 500 μl of acetonitrile for 15 min followed by rehydration in 500 μl of water for a further 15 min. This dehydration–rehydration procedure was performed a total of three times, followed by a final dehydration in acetonitrile.
In-gel tryptic digestion
Gel bands were rehydrated in 25 μl of 50 mM ammonium bicarbonate, 9% (v/v) acetonitrile and 20 ng/μl sequencing grade trypsin (Sigma–Aldrich) for 20 min. The bands were then covered in 100 μl of 50 mM ammonium bicarbonate and 9% (v/v) acetonitrile and incubated at 37 °C for 18 h. Following digestion, samples were acidified by the addition of 10 μl of 10% (v/v) formic acid. The digest supernatant was then transferred to a fresh Eppendorf tube and the digest was dried in a vacuum centrifuge at 40 °C for 30 min. The dried peptides were then resuspended in 20 μl of water containing 0.1% trifluoroacetic acid (Sigma–Aldrich) prior to LC-MS analysis.

Nano-LC-MS/MS analysis
Peptides were separated using a Nano-Acquity UPLC system (Waters) as detailed below. A sample was loaded on to a Waters C18 Symmetry trap column [180 μm i.d. (internal diameter), 5 μm, 5 cm] in water, 0.1% (v/v) acetonitrile and 0.1% (v/v) formic acid at a flow rate of 7 μl/min for 5 min. Peptides were then separated using a Waters NanoAcquity BEH C18 column (75 μm i.d., 1.7 μm, 25 cm) with a gradient of 3–30% (v/v) acetonitrile and 0.1% formic acid over 30 min at a flow rate of 400 nl/min.

The nano-LC effluent was sprayed directly into the LTQ-Orbitrap XL mass spectrometer aided by the Proxeon nano source at a voltage offset of 2.5 kV. The mass spectrometer was operated in parallel-data-dependent mode where the MS survey scan was performed at a nominal resolution of 60000 (at m/z 400) resolution in the Orbitrap analyser between an m/z range of 400–2000. The top six multiply charged precursors were selected for CID (collision-induced dissociation) in the LTQ at a normalized collision energy of 35%. Dynamic exclusion was enabled to prevent the selection of a formally targeted ion for a total of 20 s.

Immunoprecipitations
Washed cells were resuspended in lysis buffer [50 mM Tris/HCl (pH 8.0), 50 mM KCl, 10 mM EDTA and 1% Nonidet P40], and after 15 min the nuclei and cell debris were removed by centrifugation (14000 rev./min in a microcentrifuge at 4 °C for 10 min). Lysates were incubated with the appropriate antibodies overnight and immune complexes were captured using Protein G–Sepharose (1 h at 4 °C). Immunoprecipitates were washed three times with immunoprecipitation wash buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA and 0.1% Tween 20], resuspended in 1× SDS/PAGE loading buffer and subjected to SDS/PAGE and transferred on to nitrocellulose. After incubation with the antibodies indicated, antibody–protein interactions were detected with ECL (enhanced chemiluminescence; GE Healthcare) or Supersignal (Pierce).

SDS/PAGE and Western blotting
Protein extracts were quantified using Bio-Rad Bradford reagent and adjusted to 1× SDS-loading buffer. After boiling, the extracts were either treated or not with
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iodoacetamide (50 mM) to modify cysteine residues and then separated by SDS/PAGE. Proteins were transferred on to nitrocellulose, blocked in PBS-T (PBS containing 0.1% Tween 20) containing 5% (w/v) fat-free milk for 1 h, and then incubated with the primary antibody appropriately diluted in MagicMix [PBS-T, 1% Western blocking reagent (Roche) and 3% BSA]. Antibody dilutions used were: p19 and p6, 1:10000; anti-PIP4Kα-C-term, 1:1000; anti-Myc, 1:100; and anti-HA, 1:100. The blots were washed with PBS-T and then incubated with the appropriate HRP (horseradish peroxidase)-conjugated secondary antibody diluted in MagicMix [anti-rabbit, anti-mouse and anti-rat used at 1:10000 (GE Healthcare), and anti-rabbit-TrueBlot used at 1:10000 (eBioscience)]. Antibody–protein interactions were visualized using ECL (GE Healthcare) or Supersignal (Pierce). Blots were stripped by incubation at 55 °C in 50 ml of strip buffer [50 mM Tris/HCl (pH 7.4), 2% SDS and 50 mM 2-mercaptoethanol] for 20 min followed by extensive washing in PBS-T. The blots were blocked again and then used as above.

Production of recombinant proteins

Bacterial colonies transformed with the appropriate vector were diluted into 5 ml of LB (Luria–Bertani) medium containing ampicillin (50 μg/ml) and grown overnight. Cultures were diluted to 100 ml, grown at 37 °C for 1 h and then IPTG (isopropyl β-D-thiogalactoside; 100 μM final concentration) was added and the cultures were grown overnight at 30 °C. Cells were collected by centrifugation (3345 g for 10 min), washed with PBS and resuspended in 5 ml of PBS. Cells were disrupted by sonication (Diagenode cell disruptor set at the high-intensity setting) and Triton X-100 was added to a final concentration of 1%. Cell debris was removed by centrifugation and the supernatant was incubated with glutathione–Sepharose beads for 2 h. After extensive washing (PBS containing 1% Triton X-100), the bound proteins were eluted using 10 mM reduced glutathione in 50 mM Tris (pH 8) and 0.3 M NaCl. Eluted proteins were quantified using Bio-Rad reagent and by SDS/PAGE and Coomassie Blue staining, and dialysed into an appropriate buffer. GST was cleaved by incubation with thrombin for 2 h at room temperature (20 °C). The uncleaved protein and the cleaved GST portion were removed by an additional incubation with glutathione–Sepharose. The cleaved protein was quantified using standardized amounts of BSA after SDS/PAGE and Coomassie Blue staining.

HEK-293 cells were transfected with the appropriate plasmid encoding an HA-tagged protein using calcium phosphate. The transfected cells were washed once with ice-cold PBS, trypsinized and lysed in lysis buffer. The HA-tagged protein was immunopurified by using the anti-HA antibody followed by Protein G–Sepharose. After extensive washing with IP wash buffer, the HA-tagged protein was eluted with 3×HA peptide (YPYDVPDYA) [1 mg/ml in 50 mM Tris/HCl (pH 8) and 0.3 M NaCl] by incubation at 30 °C for 30 min. The eluted protein was quantified using standardized amounts of BSA after SDS/PAGE and Coomassie Blue staining.
Reconstitution of PIP4Kα–PIP4Kβ complexes using recombinant proteins
Cleaved bacterially expressed PIP4Kα was incubated alone or together with HA–PIP4Kβ in a volume of 150 μl of PIPkin buffer containing 0.1% Nonidet P40, 1 μg/ml BSA and 15 mM DTT for 1 h. The complexes were collected by HA immunoprecipitation overnight at 4 °C, and were separated by SDS/PAGE and analysed by Western blotting.

Ubiquitination assays
HEK-293 cells were transfected using calcium phosphate. MG-132 (10 μM) was added for the final 6 h, then the cells were washed once with PBS and lysed directly into PBS containing 0.2% SDS and 8 M urea, and sonicated. His-tagged proteins were purified on Ni²⁺ columns and separated using SDS/PAGE and analysed by Western blotting.

Confocal microscopy
HeLa cells were transfected on glass coverslips using FuGENE™. HT1080 cells were transduced with the constructs indicated, selected with the appropriate antibiotics and plated onto coverslips. The cells were fixed using PBS containing 4% formaldehyde (20 min), permeabilized with PBS containing 0.1% Triton X-100 (5 min) and blocked with PBS containing 3% BSA (10 min). Appropriate antibodies were diluted in PBS containing 3% BSA and incubated with the coverslips for 30 min at 37 °C in a humidified incubator. The coverslips were washed with PBS and incubated for 30 min with fluorophore-conjugated secondary antibodies diluted appropriately in PBS containing 3% BSA. The coverslips were mounted using Vectashield, sealed using nail varnish and analysed by confocal microscopy (Zeiss).

Results
PIP4Kβ complexes
In order to study the localization of PIP4Kβ and identify proteins that interact with it, a cell line expressing HA-tagged PIP4Kβ (HA–PIP4Kβ) was generated. Cytosolic and nuclear extracts were isolated from these cells and were further fractionated using sucrose gradient centrifugation. Protein molecular masses between 67 and 230 kDa could be separated (Supplementary Figures S1A and S1B at http://www.BiochemJ.org/bj/430/bj4300223add.htm), and the BRG1 chromatin remodelling complex (molecular mass>500000 kDa) eluted in fractions 12–15 (Figure 1A). Cytosolic HA–PIP4Kβ eluted between a molecular mass of 120 and 200, whereas the nuclear HA–PIP4Kβ eluted with a molecular mass between 120 and 150 kDa (Figure 1A); however, the monomeric form of PIP4Kβ (49 kDa), which should elute in fractions 4–6, was not seen. The observed molecular mass suggested that PIP4Kβ exists only in complexes in both the cytosol and in the nucleus.

To assess whether the endogenous nuclear PIP4Kβ is also in complexes, isolated nuclei from MEL cells were treated with increasing concentrations of a non-hydrolysable cross-linking agent (DSS), immunoprecipitated and analysed by SDS/PAGE and
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Figure 1. Analysis of PIP4Kβ complexes. (A) Nuclear extracts from HEK-293 cells expressing HA-PIP4Kβ were separated by sucrose density centrifugation and each fraction was separated by SDS/PAGE and the gel was stained with silver. The Western blot below shows the presence of the BRG1 complex (>500 000 kDa) in fractions 12–15. A 2 μl aliquot of every fraction from the sucrose density centrifugation of the cytosolic or nuclear fraction was spotted on to nitrocellulose and probed with the anti-HA antibody, and relevant fractions containing HA immunoreactivity were analysed by Western blotting. (B) MEL nuclei were treated with increasing concentrations of the irreversible cross-linking agent DSS. Nuclear extracts were then immunoprecipitated with either a pre-immune serum or a PIP4Kβ-specific serum (p5 or p6) and Western blotted with an anti-PIP4K rat monoclonal antibody (Antibody 3). Increasing concentrations of DSS induced the formation of the cross-linked dimer (arrow). (C) Nuclei were treated as shown, extracted and immunoprecipitated with the antibodies indicated. The Western blot was probed with a specific rat monoclonal antibody against PIP4K (Antibody 3). The results shown are representative of at least two different experiments. The molecular mass in kDa is indicated. IP, immunoprecipitation; WB, Western blot.

Immunoblotting. Increasing concentrations of the cross-linker induced the loss of the monomeric 49 kDa protein, resulting in the appearance of a 97 kDa complex. Higher concentrations of DSS resulted in disappearance of the dimeric complex, possibly through irreversible cross-linking of PIP4Kβ into the nuclear pellet or cross-linking-
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**Figure 2.** PIP4Kβ interacts with PIP4Kα. (A) HA–PIP4Kβ was purified from HEK-293 cells expressing HA–PIP4Kβ using HA-immunoaffinity chromatography. Eluted proteins were separated by SDS/PAGE and the Coomassie Blue-stained gel was cut into 60 slices and each slice was analysed by MS. The Figure shows the peptides which uniquely defined the presence of PIP4Kα (top) or PIP4Kβ (bottom), in fractions 33 and 34. (B) The lysate from HEK-293 cells expressing Myc–PIP4Kβ (lane 1) was used for affinity purification with either GST (lane 2), GST–PIP4Kα (lane 3), GST–PIP4Kβ (lane 4) or GST–PIP4Kγ (lane 5). Bound proteins were analysed by Western blotting using an anti-Myc antibody. Lane 1 shows 10% of the lysate input used for each affinity purification. (C) EE–PIP4Kα and HA–PIP4Kβ were expressed in HEK-293 cells, as indicated (below) and immunoprecipitated and Western blotted with the antibodies indicated. The experiment is representative of three separate experiments. (D) HA–PIP4Kβ purified from HEK-293 cells was combined with purified bacterially expressed PIP4Kα, after removal of the GST tag by thrombin cleavage, incubated at room temperature for 1 h and then immunoprecipitated using the anti-HA antibody. Immunoprecipitates were analysed by Western blot and were probed first for PIP4Kα (upper panel) after which the blot was stripped and reprobed with an anti-HA antibody (lower panel). Total protein input (10%) was also Western blotted. The arrow denotes PIP4Kα co-immunoprecipitated in the HA immunoprecipitate. IP, immunoprecipitation; WB, Western blot.

dependent loss of the epitope recognized by the antibodies (Figure 1B). The data suggest that endogenous nuclear PIP4Kβ is predominantly a dimer, which is in line with previous data obtained from the crystal structure of the PIP4Kβ [28], although higher molecular mass complexes were also seen (Figure 1C). Extraction of nuclear proteins with Chaps/NaCl or with RIPA buffer before the addition of the cross-linking agent did not prevent the formation of the 97 kDa PIP4Kβ dimer, demonstrating the strength and stability of the complex (Figure 1C).
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**PIP4Kβ associates with PIP4Kα**

To identify potential interacting partners of PIP4Kβ, an immunoprecipitate of HA–PIP4Kβ was separated by SDS/PAGE and stained with Coomassie Blue. The gel was cut into 60 slices, all of which were analysed by MS (Figure 2A). In slices 33 and 34, corresponding to a molecular mass of approx. 49 kDa, 14 peptides were identified, three of which uniquely identified PIP4Kβ and two identified PIP4Kα (Figure 2A). The data suggested that endogenous PIP4Kα interacts with HA–PIP4Kβ. To further investigate the interaction between PIP4Kα and PIP4Kβ, lysates from HEK-293 cells overexpressing Myc–PIP4Kβ were used for affinity purification using bacterially expressed and purified GST, GST–PIP4Kα, GST–PIP4Kβ or GST–PIP4Kγ (Figure 2B). GST–PIP4Kα, β and γ could affinity-purify Myc–PIP4Kβ, but GST alone could not. To demonstrate that the interaction between PIP4Kα and PIP4Kβ occurs in vivo, lysates from HEK-293 cells, expressing EE–PIP4Kα either alone or with HA–PIP4Kβ, were immunoprecipitated with the anti-EE or anti-HA antibody and analysed by Western blotting. Immunoprecipitation with the anti-HA antibody affinity-captured EE–PIP4Kα only when both proteins were co-overexpressed. When the reciprocal experiment was carried out, HA–PIP4Kβ was co-immunoprecipitated by the anti-EE antibody only when both proteins were also co-expressed (Figure 2C).

To demonstrate that the interaction between PIP4Kα and β is direct, both proteins were purified, combined *in vitro* and were analysed by HA immunoprecipitation followed by Western blotting (Figure 2D). The blot also shows 10% of the PIP4Kα and β input. The anti-HA antibody immunoprecipitated approx. 80% of HA–PIP4Kβ and, when HA–PIP4Kβ was combined with PIP4Kα, the anti-HA antibody immuno-affinity purified approx. 80–90% of the PIPKα, showing that PIP4Kα and PIP4Kβ can interact directly (Figure 2D).

To demonstrate that endogenous PIP4Kα and PIP4Kβ can interact *in vivo* we developed two antibodies, p5 and p6, which are specific for PIP4Kβ, and one antibody, p19, which is specific for PIP4Kα (Supplementary Figure S2 at http://www.BiochemJ.org/bj/430/bj4300223add.htm). As the molecular masses of PIP4Kα and PIP4Kβ are identical, HEK-293 cell lines, in which PIP4Kα or PIP4Kβ expression was stably suppressed by RNAi, were used for co-immunoprecipitation studies to further ensure specificity. The shRNAi construct targeting PIP4Kα decreased PIP4Kα protein levels to 54%, whereas targeting PIP4Kβ decreased PIP4Kβ protein levels to 15% (Figure 3A). Pre-immune antibodies did not immunoprecipitate either PIP4Kα or β. Both p5 and p6 immunoprecipitated endogenous PIP4Kβ and co-immunoprecipitated endogenous PIP4Kα. The specificity was demonstrated by the lack of co-immunoprecipitation of PIP4Kα when its expression was suppressed using RNAi (Figure 3B). Immunoprecipitation of endogenous PIP4Kα also co-immunoprecipitated endogenous PIP4Kβ and the interaction was specific as RNAi-mediated suppression of PIP4Kα reduced the co-immunoprecipitation of PIP4Kβ (Figure 3C). The data show that PIP4Kα and PIP4Kβ interact directly and that the interaction occurs *in vivo* between endogenous PIP4Kα and PIP4Kβ.
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**Figure 3.** Endogenous PIP4Kβ associates with PIP4Kα. (A) Lysates from HEK-293 cells, stably expressing the indicated pRetroSuper construct, were probed using the antibodies shown. RNAi to PIP4Kα suppressed its expression to 54% of the control value, whereas the RNAi to PIP4Kβ suppressed its expression to 15% of the control value. (B) Lysates from HEK-293 cells stably expressing the indicated RNAi construct were immunoprecipitated with the antibodies shown and the Western blot was probed with an anti-PIP4Kα antibody. The blot was stripped and reprobed with an anti-PIP4Kβ antibody. (C) Lysates from HEK-293 cells stably expressing the indicated RNAi construct were immunoprecipitated as indicated and the Western blot was probed for the presence of PIP4Kβ. The blot was stripped and reprobed for PIP4Kα. The data are representative of three separate experiments. IP, immunoprecipitation; WB, Western blot.

**PIP4Kβ has 2000-fold less PIP4K activity compared with PIP4Kα**

PIP4Kα and β phosphorylate the same substrate to generate the same product and so it is questionable why they would interact with each other. The interaction may be fortuitous, as the two proteins are highly related or, alternatively, the interaction may modulate each others activities and/or their localization. In order to rationalize why PIP4Kα and PIP4Kβ interact with each other, their enzymatic activity towards PtdIns5P was reassessed. GST–PIP4Kα and β were expressed in bacteria, purified and quantified by SDS/PAGE and Coomassie Blue staining (Figure 4A). Both enzymes phosphorylated PtdIns5P at the 4-position to generate PtdIns(4,5)P₂, as demonstrated by dephosphorylation of the product with a specific recombinant PtdIns(4,5)P₂-5-phosphatase (results not shown). Therefore both PIP4Kα and β are bona fide PIP4Ks. However, PIP4Kα phosphorylated PtdIns5P approx. 434-fold better than PIP4Kβ (Figure 4A). As the difference in activity between the PIP4Kα and β could be a consequence of their expression in bacteria, Myc-tagged proteins, expressed in and purified from HEK-293 cells, were tested for PIP4K activity. As observed with
PIPK β interacts with and modulates nuclear localization...

### A

![Bar graph showing the specific activity of GST-PIP4KA and GST-PIP4KB](image)

<table>
<thead>
<tr>
<th></th>
<th>Specific activity (counts/μg protein)</th>
<th>fold</th>
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</thead>
<tbody>
<tr>
<td>GST-PIP4KA</td>
<td>4662.2 ± 228.9 *10^3</td>
<td>434</td>
</tr>
<tr>
<td>GST-PIP4KB</td>
<td>10.8 ± 2.4 *10^3</td>
<td>1</td>
</tr>
</tbody>
</table>

### B

![Bar graph showing the specific activity of MYC-PIP4KA and MYC-PIP4KB](image)

<table>
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<tr>
<th></th>
<th>Specific activity (counts/protein)</th>
<th>fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYC-PIP4KA</td>
<td>1139.8 ± 277.8</td>
<td>207</td>
</tr>
<tr>
<td>MYC-PIP4KB</td>
<td>5.5 ± 3.2</td>
<td>1</td>
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</tbody>
</table>
Figure 4. PIP4Kβ has 2000-fold less PIP4K activity than PIP4Kα. Purified GST–PIP4Kα and β were quantified by SDS/PAGE and Coomassie Blue staining (top panel). Matched quantities of protein were then assayed for PIP4K activity (autoradiograph, middle panel and quantification in the histogram and table). (B) Myc–PIP4Kα and β were purified from HEK-293 cells and quantified by Western blots probed with the anti-Myc antibody (top panel). Matched inputs were assayed for PIP4K activity (autoradiograph, middle panel and quantification in the histogram and table). (C) PIP4Kα (left-hand side) or PIP4Kβ (right-hand side) activity was assayed using liposomes containing PtdSer (50 nmol) and increasing amounts of PtdIns5P. The data are represented graphically and the kinetic parameters (Km and Vmax) were determined and are shown in the table. Note that for the graph the phosphoimaging exposure time for the PIP4Kα was 5 min, whereas the exposure time for PIP4Kβ was 90 min. (D) PtdIns5P was measured in HEK-293 cells transfected as indicated. Expression of the various constructs are shown in the top panels, whereas PtdIns5P levels are shown in the histogram. WB, Western blot.
the bacterially expressed proteins, PIP4Kα had approx. 207-fold more activity than PIP4Kβ (Figure 4B). To determine more accurately the difference in PIP4K activity between PIP4Kα and β, we assessed the activity of bacterially expressed PIP4Kα and β at different concentrations of PtdIns5P presented in PtdSer liposomes (Figure 4C). The results show that while both enzymes had approximately the same \( K_m \) with respect to PtdIns5P, the \( V_{max} \) of PIP5Kα was 2000-fold higher than PIP4Kβ (see the table in Figure 4C for quantification). To assess whether PIP4Kα and PIP4Kβ have the same activity in vivo, cellular PtdIns5P levels were measured after the co-expression of each PIP4K isoform with a PtdIns(4,5)P_2 4-phosphatase (IpgD), which increases the level of PtdIns5P in vivo [29]. The co-expression of PIP4Kα partially attenuated an increase in the level of cellular PtdIns5P induced by the expression of GFP (green fluorescent protein)–IpgD. In contrast, expression of an equivalent amount of PIP4Kβ did not attenuate the IpgD-induced increase in PtdIns5P (Figure 4D). This was not a consequence of the nuclear localization of PIP4Kβ as we show here (see Figure 6C) that overexpressed PIP4Kβ is localized in the cytoplasm, nucleus and at the plasma membrane in HeLa cells. These data show that, in vitro and in vivo, PIP4Kβ is much less active compared with PIP4Kα.

**PIP4Kα provides the majority of PIP4K activity in PIP4Kβ immunoprecipitations**

To determine whether PIP4Kα provides the majority of PIP4K activity in a heteromeric complex, PIP4K activity was assessed in complexes containing either wild-type or kinase-inactive EE–PIP4Kα. Overexpression of EE–PIP4Kα led to a dramatic increase in PIP4K activity in the EE immunoprecipitate that, as expected, was not present when an inactive EE–PIP4Kα kinase was expressed (Figure 5A). Co-overexpression of wild-type HA–PIP4Kβ did not lead to any changes in PIP4K activity associated with the EE immunoprecipitation but, as expected, HA–PIP4Kβ was co-immunoprecipitated as assessed by Western blotting (Figure 5A). Overexpression of HA–PIP4Kβ induced a very small increase in PIP4K activity associated with the HA immunoprecipitate; however, when EE–PIP4Kα was co-expressed with HA–PIP4Kβ there was a 20-fold increase in PIP4K activity associated with the HA–PIP4Kβ immunoprecipitate (Figure 5A). Co-overexpression of the kinase-inactive PIP4Kα actually decreased the amount of PIP4K activity associated with the HA immunoprecipitate. The level of HA–PIP4Kβ in the various immunoprecipitates was similar (21163, 16891 and 15280 arbitrary imager units), suggesting that the changes in PIP4K activity were a consequence of co-immunoprecipitation of active EE–PIP4Kα.

To determine to what extent of the PIP4K activity associated with PIP4Kβ is due to its interaction with PIP4Kα, we stably suppressed the expression of PIP4Kα and assessed the PIP4K activity in an immunoprecipitation of endogenous PIP4Kβ. RNAi-targeting of PIP4K decreased the level of PIP4Kα protein by approx. 50% (Figure 3A) and PIP4K activity was also decreased by approx. 50–70% in isolated membrane, cytosol and
nuclear fractions (Figure 5B). When endogenous PIP4Kβ was immunoprecipitated from the cytosol/membrane or nuclear fractions of cells suppressed for the expression of PIP4Kα, the associated PIP4K activity decreased by approx. 50% (Figure 5C), although the amount of PIP4Kβ immunoprecipitated remained similar (6601, 9288, 7838 and 7227 arbitrary image units respectively in the four separate immunoprecipitations). We also carried out a similar analysis using cells transiently transfected with siRNA oligonucleotides targeting either PIP4Kα or β. Suppression of PIP4Kα decreased PIP4Kα protein (6314 to 487 arbitrary imager units) assessed by Western blotting, whereas PIP4K activity in a PIP4Kα immunoprecipitate decreased to 27% of the control (Figure 5D). Similarly, suppression of PIP4Kα decreased the PIP4K activity in a PIP4Kβ immunoprecipitate to 34% of control. Suppression of PIP4Kβ as expected diminished the PIP4K protein levels (33205 in the control to 5554 arbitrary imager units in the siRNA PIP4Kβ) and the PIP4K activity in the PIP4Kβ immunoprecipitate to 28% of the control (Figure 5D). These data are consistent with the PIP4Kα subunit providing the majority of PIP4K activity present in a PIP4Kβ immunoprecipitate.

To investigate whether active PIP4Kβ is required in the complex, we measured the PIP4K activity of either active or inactive Myc–PIP4Kβ in complex with the endogenous PIP4Kα. Suppression of the expression of PIP4Kα reduced the PIP4K activity associated with the immunoprecipitate of Myc–PIP4Kβ by 75% showing that the majority of activity comes from the endogenous PIP4Kα subunit (Figure 5E). Strikingly, the PIP4K activity associated with kinase-inactive Myc–PIP4Kβ was also reduced to 24% compared with that associated with the active Myc–PIP4Kβ (Figure 5E). Immunoblotting showed that wild-type and kinase-inactive Myc–PIP4Kβ co-immunoprecipitated the same amount of endogenous PIP4Kα (3363 in the wild-type PIP4Kβ immunoprecipitate compared with 3574 arbitrary units in the kinase-inactive PIP4Kβ immunoprecipitate). The specificity of the co-immunoprecipitation of endogenous PIP4Kα was demonstrated by the decrease in the PIP4Kα band in the knockdown (505 and 400 arbitrary units). Both the wild-type and the kinase-inactive PIP4Kβ were immunoprecipitated to similar extents (21878 and 24723 in the pRetroSuper cells compared with 20903 and 23537 arbitrary imager units in the RNAi PIP4Kα cells). These data suggest that PIP4Kα can either activate PIP4Kβ in the complex or that the kinase activity of PIP4Kβ is required for optimal PIP4Kα activity.

**PIP4Kβ can modulate the localization of PIP4Kα**

To determine whether PIP4Kβ can modulate the total amount of nuclear PIP4Kα, the level of endogenous PIP4Kα was determined in cytosol and nuclear fractions from HEK-293 cells stably suppressed for PIP4Kβ expression. The majority of PIP4Kα was cytosolic with a small amount that was nuclear. Although RNAi suppressed the expression of PIP4Kβ to 15% of the control level, the total amount of PIP4Kα present in the nucleus was not reduced significantly. This suggests that translocation of PIP4Kα into the nucleus may not be solely dependent on its interaction with PIP4Kβ.
Figure 5. PIP4Kα provides the majority of activity in the PIP4Kα–PIP4Kβ complex. (A) HEK-293 cells were transfected with the constructs shown and the lysates were divided and immunoprecipitated using an antibody against the EE (left-hand panel) or HA (right-hand panel) tag. Immunoprecipitates were split and PIP4K activity was assessed (histogram) and the rest was analysed by Western blotting using the antibodies indicated (upper blot). It appears that PIP4KαKD interacts less well with PIP4Kβ, however, this is a consequence of its lower expression (see left-hand blot EE-IP WB PIP4Kα). The ratio of PIP4Kα/PIP4KαKD in the input is 3.95 and is similar in the PIP4Kβ immunoprecipitate (4.4). (B) Membrane, cytosol and nuclear →
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D

fractions were isolated from HEK-293 cells expressing either pRetroSuper or RNAi PIP4Kα and lysates from these fractions were immunoprecipitated with p19 (anti-PIP4Kα) and PIP4K activity was measured. (C) Cytosol/membrane and nuclear fractions from the cell lines above were immunoprecipitated with p6 (anti-PIP4Kβ). The immunoprecipitates were divided and assessed for PIP4K activity (histogram), and analysed by Western blotting with antibodies against endogenous PIP4Kα (upper right-hand panel). The blot was stripped and reprobed for PIP4Kβ (lower right-hand panel). (D) HEK-293 cells were transfected with the indicated siRNA oligos and lysates were immunoprecipitated as indicated, split and assayed for PIP4K activity (numbers on the histogram indicate the percentage of the control) and Western blotted with the antibodies indicated. (E) Wild-type or kinase inactive (D278A) Myc–PIP4Kβ was expressed in control cells (pRetroSuper) or cells suppressed for the expression of PIP4Kα (RNAi PIP4Kα). Cell lysates were immunoprecipitated with the anti-Myc antibody and assayed for PIP4K activity (histogram) and Western blotted for the co-immunoprecipitation of endogenous PIP4Kα (arrow in the upper panel). The blot was then stripped and reprobed for Myc–PIP4Kβ (lower panel). The data are representative of at least two separate experiments. IP, immunoprecipitation; WB, Western blot.
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**Figure 6.** PIP4Kβ regulates the localization of PIP4Kα in the nucleus. (A) Lysates from cytosol and nuclear fractions, isolated from HEK-293 cells expressing the RNAi constructs indicated, were immunoprecipitated with an antibody specific against PIP4Kα (p19) and Western blotted using the antibody against PIP4Kα. (B) HeLa cells were transfected with Myc–PIP4Kα, fixed and stained using the anti-Myc antibody. (C) Cells were transfected with HA–PIP4Kβ and stained using the anti-HA antibody. (D) Cells were co-transfected with Myc–PIP4Kα and HA–PIP4Kβ, fixed and stained with rabbit anti-Myc antibody (left-hand micrograph) and mouse anti-HA (middle micrograph) and the merge is shown on the right-hand side. (E) Lysates from HEK-293 cells stably expressing the RNAi constructs indicated, were immunoprecipitated (IP) with p6 (anti-PIP4Kβ antibody) and Western blotted (WB) for PIP4Kα (upper panel). The blot was stripped and reprobed with p6 (anti-PIP4Kβ). The data show that the ratio of PIP4Kα to PIP4Kβ in a PIP4Kβ immunoprecipitate is higher in the nuclear extracts (1.55) compared with the cytosol (0.21). The arrows denote the different molecular masses of PIP4Kα in the membrane, cytosol and nuclear fraction. The data are representative of at least two separate experiments.
(Figure 6A). As the antibodies against PIP4Kα cannot be used to immunolocalize the endogenous protein, Myc–PIP4Kα was expressed alone or together with HA–PIP4Kβ, and their subcellular distribution was determined using confocal microscopy. Overexpressed Myc–PIP4Kα was found predominantly in the cytosol with some staining in the nucleus (Figure 6B). PIP4Kβ on the other hand was present both in the cytoplasm and in nuclear speckles (Figure 6C). When the two enzymes were co-expressed, the localization of PIP4Kβ remained unchanged, but PIP4Kα could be clearly observed in nuclear speckles that co-localized with PIP4Kβ (see merge in Figure 6D). These data indicate that while PIP4Kβ may not control the overall nuclear import of PIPKα, it may modulate the subnuclear localization of PIP4Kα.

Although it is difficult to determine the absolute amounts of PIP4Kα and β using antibodies and Western blotting, we investigated the amount of PIP4Kα associated with PIP4Kβ in each subcellular fraction. Western blotting of PIP4Kβ immunoprecipitates from the membrane fraction revealed that the molecular masses of PIP4Kα and β were smaller when compared with their cytosolic counterparts (Figure 6E). These bands are the correct proteins as they are not present in the RNAi PIP4Kβ-knockdown lines. The decrease in molecular mass may be a consequence of post-translational modification or, alternatively, of proteolytic processing. Strikingly, the ratio of PIP4Kα/PIP4Kβ in the PIP4Kβ immunoprecipitate from nuclear extracts (1.55) was 6-fold higher when compared with the immunoprecipitate from the cytosol (0.21) (Figure 6E). This suggests that that there is a larger percentage of PIP4Kα–PIP4Kβ complex in the nucleus compared with the cytosol. The result is not just a reflection of the total levels of PIP4Kα in the different subcellular fractions as the majority (80%) of PIP4Kα is cytosolic (Figure 6A). How the PIP4Kα–PIP4Kβ complex becomes enriched in the nuclear fraction is not clear. The nucleus may preferentially sequester the PIP4Kα–PIP4Kβ complex compared with the PIP4Kβ homodimers or the nuclear environment might favour exchange of subunits to generate the heterocomplex.

**PIP4Kα affects the ubiquitination of PIP4Kβ by the SPOP–CUL3 complex**

As PIP4Kα can interact with PIP4Kβ, PIP4Kα should play a role in functions that are primarily associated with PIP4Kβ. The nuclear SPOP–CUL3 ubiquitination complex [CUL3, SPOP and RBX1 (ring-box 1)] can interact with and ubiquitinate PIP4Kβ. Furthermore, the ubiquitination activity of the complex appears to be stimulated by an increase in the level of PtdIns5P [24]. How does overexpression of wild-type PIP4Kβ, which should decrease the level of PtdIns5P, increase its own ubiquitination? We considered that overexpression of PIP4Kβ will increase the level of the PIP4Kβ homodimer, which will have much less PIP4K activity than the PIP4Kα–PIP4Kβ complex, and therefore could potentially act as a dominant-negative kinase when recruited to the SPOP–CUL3 complex. This could increase the local level of PtdIns5P and therefore increase SPOP–CUL3-mediated ubiquitination of PIP4Kβ. If so, then overexpression
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of PIP4Kα should rebalance the ratio of the PIP4Kα–PIP4Kβ complex and therefore reduce PIP4Kβ ubiquitination. Furthermore, overexpression of a kinase-inactive PIP4Kα would not be expected to suppress PIP4Kβ ubiquitination.

As shown previously [24], ubiquitination of Myc–PIP4Kβ was only observed when it was co-expressed with all three CUL complex proteins (Figure 7A, compare lane 1 with lane 2, and lane 7 with lane 8) and ubiquitination was enhanced when the kinase-inactive PIP4Kβ was expressed (Figure 7A, compare lane 2 with lane 8). Strikingly, co-expression of the wild-type PIP4Kα enzyme suppressed ubiquitination of PIP4Kβ (Figure 7A, compare lane 2 with lane 4, and lane 8 with lane 10); however, co-expression of the kinase-inactive PIP4Kα (EE–PIP4KαKD) did not (compare lane 2 with lane 6, and lane 8 with lane 12). To test whether nuclear PIP4Kα is required to suppress PIP4Kβ ubiquitination, PIP4Kα was targeted to the plasma membrane by a myristoylation sequence and PIP4Kβ ubiquitination was assessed. When co-expressed with PIP4Kβ, Myr–PIP4Kα was not present in the nucleus, localized predominantly on the membrane and did not influence the nuclear localization of PIP4Kβ (Supplementary Figure S3 at http://www.BiochemJ.org/bj/430/bj4300223add.htm). Myr–PIP4Kα is active and in vivo can attenuate lpgD-induced PtdIns5P generation to the same extent as the

**Figure 7.** PIP4Kα regulates CUL3-mediated ubiquitination of PIP4Kβ. HEK-293 cells were transfected as indicated and His-tagged proteins were affinity-purified using Ni2+-Sepharose and analysed by Western blotting with an anti-Myc antibody to visualize Myc–PIP4Kβ and its ubiquitinated products. The arrow denotes where non-ubiquitinated Myc–PIP4Kβ would migrate. Total cell lysates were also separated by SDS/PAGE and probed as shown. (B) His-tagged proteins were purified from lysates of cells expressing the constructs indicated and analysed by Western blotting using an anti-Myc antibody. Total cell lysates were also Western blotted with the antibodies indicated. Note all cells received the three CUL complex proteins (CUL3, RBX1 and SPOP). The data are representative of three separate experiments. IP, immunoprecipitation; WB, Western blot.
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wild-type PIP4Kα (results not shown). Overexpression of the Myr–PIP4Kα, however, did not suppress the ubiquitination of the PIP4Kβ (Figure 7B), suggesting that the interaction with PIP4Kβ and targeting of PIP4Kα to the nucleus is required to suppress SPOP–CUL3-mediated PIP4Kβ ubiquitination.

Discussion

PIP4Kβ exists as a dimer through the interaction between two anti-parallel β-sheets at the N-terminus of the protein [28,30]. When expressed in cells, we also found that PIP4Kβ forms dimers as assessed by sucrose density centrifugation studies. Under these conditions, no monomers were detected. The dimer appeared to be extremely stable as extraction with RIPA buffer containing 0.2% SDS before cross-linking did not prevent dimer formation. The dimeric surface creates a large flat positively charged surface able to interact electrostatically with the membrane [30]. The interaction of PIP4Kβ with the membrane occurs in order to present the enzyme to its substrate PtdIns5P, which is then phosphorylated to generate PtdIns(4,5)P2. However, our studies show that when compared with the PIP4Kα isoform, the PIP4Kβ dimer is 2000-fold less active in vitro. This led us to question whether PIP4Kβ is less active in vivo than PIP4Kα. Overexpression of IpgD, a bacterial PtdIns(4,5)P2 4-phosphatase, induced PtdIns5P generation which could be partially attenuated by the expression of PIP4Kα, but not by an equivalent level of expression of PIP4Kβ, showing that PIP4Kβ activity is also much lower in vivo than PIP4Kα. The inability of PIP4Kβ to decrease IpgD-induced PtdIns5P levels is not due to its nuclear localization as we show that PIP4Kβ is present in the cytosol, membrane and in the nucleus.

If the role for both PIP4Kβ and PIP4Kα is to regulate the level of PtdIns5P, then the much reduced PIP4K activity of PIP4Kβ is surprising. However, the unexpected discovery that PIP4Kβ interacts with and targets PIP4Kα provides an explanation as to how the low activity PIP4Kβ isoform can regulate PtdIns5P levels. They also suggest that the level of nuclear PtdIns5P may depend on nuclear PIP4Kα. Although we show that PIP4Kα and β can interact with each other, the nature of the interaction is not clear. It is possible that it exists as a heterodimer, or that a PIP4Kα monomer may interact with the PIP4Kβ dimer in a trimeric complex. Indeed, PIP4K purified from erythrocytes showed a native molecular mass of 150000 kDa, suggesting the presence of a trimer [31]. Quantitative proteomic studies of PIP4Kα and PIP4Kβ in chicken DT40 cells suggest that the complex of α and β is a dimer. Furthermore, the amino acid sequence of the β-sheet that forms the dimer interface in PIP4Kβ is identical in PIP4Kα, and in silico modelling studies confirm the suggestion that PIP4Kα and β could form heterodimers [32].

The much lower PIP4K activity of PIP4Kβ compared with PIP4Kα begs the question as to whether there is a role for active PIP4Kβ in the complex. We show that the PIP4K activity is higher when active PIP4Kβ, compared with the inactive PIP4Kβ, is complexed
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with endogenous PIP4Kα. These data suggest either that PIP4Kα can dramatically stimulate the activity of PIP4Kβ, or that PIP4Kβ kinase activity is required to activate PIP4Kα in the complex. The conformation of the active PIP4Kβ may also be different to the inactive kinase, which may be important for the regulation of the activity of the PIP4Kα subunit. In the absence of specific inhibitors against each isoform and crystal structures of the various complexes it is difficult to conclude the exact role of the active PIP4Kβ subunit in the complex.

It is also pertinent to question whether the formation of the PIP4Kα–PIP4Kβ complex is regulated. If the affinities of interaction between PIP4Kβ homodimers and the heterocomplex are similarly equal then the formation of the heterocomplex may just be controlled by the ratio of the levels of expression of PIP4Kα to PIP4Kβ. In this case changes in the ratio of expression of PIP4Kα to PIP4Kβ will modulate the amount of PIP4Kα–PIP4Kβ complex, and therefore the amount of PIP4K activity, targeted by PIP4Kβ. Physiologically, the ratio of the levels of PIP4Kα and PIP4Kβ differ dramatically between tissue types. Perhaps more importantly, the gene encoding PIP4Kβ is located in the ERBB2 amplicon that is often amplified in human breast tumours, which can lead to increased expression of PIP4Kβ [33]. The increased expression of PIP4Kβ could: (i) increase the concentration of the homodimer, which may have a specific function within the cell, independent of PIP4Kα; (ii) increase targeting of PIP4Kα to decrease PtdIns5P levels; or (iii) increase the homodimer concentration, which may act in a dominant-negative manner and thereby lead to an increase in the level of PtdIns5P. Clearly, what the outcome is will very much depend on the absolute ratio of PIP4Kα to PIP4Kβ. Being able to control the expression of PIP4Kβ, together with measurement of the PtdIns5P level in both the cell and in the nucleus, should clarify some of these issues. Quantitative mass spectrometric evidence for random dimerization of PIP4Kα and β in chicken DT40 cells are presented in another study [32].

Alternatively or additionally, the heterocomplex formation may be regulated. PIP4Kβ is extensively post-translationally modified (Y. Bultsma, W.-J. Keune and N. Divecha, unpublished work) and these modifications may be able to regulate complex formation. We previously showed that p38 MAPK (mitogen-activated protein kinase) phosphorylates PIP4Kβ at Ser326 and induces a decrease in its PIP4K activity [15]. As PIP4Kα accounts for the majority of PIP4K activity in a PIP4Kβ immunoprecipitate, we considered that the decrease in activity might be due to phosphorylation-dependent inhibition of the interaction between PIP4Kα and β. We assessed this and found that UV stimulation, okadaic acid, etoposide, doxorubicin or H2O2 treatment did not change the interaction between PIP4Kα and PIP4Kβ (Supplementary Figure S4 at http://www.BiochemJ.org/bj/430/bj4300223add.htm), although they did induce Ser326 phosphorylation of PIP4Kβ (results not shown). Phosphorylation of Ser326 on PIP4Kβ may lead to a conformational change that inhibits the activity of the associated PIP4Kα.
Interestingly, in HEK-293 cells and in MEL cells (results not shown) the PIP4Kα–PIP4Kβ complex appears to be more prevalent in nuclear extracts than in the cytosolic fraction. This does not reflect the total level of PIP4Kα in the two fractions as the majority of PIP4Kα is cytosolic. Notwithstanding that during the nuclear isolation procedure there may be a preferential loss of the PIP4Kβ dimer or of the PIP4Kα enzyme, this may suggest that heterocomplex formation is regulated in the nucleus. We do not believe that there is significant redistribution of the enzymes on subcellular fractionation as immunofluorescence analysis of overexpressed PIP4Kα or β show a similar subcellular distribution to subcellular fractionation. Why then is the PIP4Kα–PIP4Kβ complex more prevalent in the nucleus? If the PIP4Kα isoform can only enter the nucleus as a consequence of its interaction with PIP4Kβ, we would expect that the nuclear PIP4Kα/PIP4Kβ ratio would reflect the cytosolic PIP4Kα/PIP4Kβ ratio. The PIP4Kα–PIP4Kβ complex, however, may be preferentially imported into the nucleus or preferentially sequestered in the nucleus compared with the PIP4Kβ homodimer. Alternatively, if PIP4Kα can be imported into the nucleus independently of PIP4Kβ, then the nuclear environment may favour rapid exchange of subunits to generate the PIP4Kα–PIP4Kβ complex. This may be a direct consequence of subcellular specific post-translational modification of PIP4Kβ. PIP4Kα may be imported into the nucleus independently of PIP4Kβ as, compared with control cells, we were unable to detect significant differences in the total amount of PIP4Kα present in the nucleus after PIP4Kβ expression was suppressed by RNAi.

It is clear, however, that control of the nuclear localization of both PIP4Kβ and α is complex. Previous studies on the overexpression of PIP4Kβ in HeLa cells [16] and on the localization of FLAG-tagged endogenous PIP4Kβ in chicken DT40 cells [34] suggested that the majority of PIP4Kβ was nuclear. However, in most cells that we have studied, overexpressed PIP4Kβ localizes to the nucleus, the cytosol and the plasma membrane as observed by others [24] and in the present study in HeLa cells. However, in MCF7 cells the majority of PIP4Kβ appears nuclear, whereas in T47D, another breast cancer cell line, PIP4Kβ is mainly cytosolic (results not shown). The reason for these differences is not clear, but may be a consequence of differential expression of import factors or post-translational modification of PIP4Kβ. In the case of PIP4Kα in HT1080 cells, overexpressed PIP4Kα is predominantly cytosolic. Co-overexpression of PIP4Kα and PIP4Kβ leads to a clear increase in total PIP4Kα targeted to the nucleus (Supplementary Figure S5 at http://www.BiochemJ.org/bj/430/bj4300223add.htm), suggesting that in some cells, overexpressed PIP4Kβ will target more PIP4Kα into the nucleus. Our results also show that PIP4Kβ can target PIP4Kα to nuclear speckles and that PIP4Kβ-mediated targeting of PIP4Kα is functional and can regulate the ubiquitination activity of the SPOP–CUL3 complex.

The ability of inactive mutant proteins to localize or modulate active counterparts has been reported in numerous signalling cascades and has been well-characterized.
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in the myotubularin family. Myotubularins are a family of lipid phosphatases some of which are inactive, such as MTMR13, due to a mutation in a critical active-site cysteine residue [35]. MTMR13 interacts with and activates MTMR2 [36] and mutation in either MTMR2 or the inactive MTMR13 can induce Charcot–Marie–Tooth syndrome 4B [37]. These data exemplify the importance of how the interaction between the active PIP4Kα and less-active PIP4Kβ may impinge on phosphoinositide regulation in physiological and pathological conditions.

We would like to note that while the present study was in progress we became aware that another group independently discovered the association between PIP4Kα and PIP4Kβ [32].

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References

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Supplementary materials

Supplementary Figure 1. Characterization of the separation of different molecular mass proteins by sucrose density gradient centrifugation. (A) SDS/PAGE showing molecular mass standards used in the sucrose gradients. Alb, albumin; Ado, aldolase; Cat, catalase; Fer, ferritin; Thy, thyroglobin; Mix, mixture of standards. (B) Separation of the standards by sucrose density gradient centrifugation.

Supplementary Figure 2. Characterization of specific antibodies against PIP4K isoforms. (A) The indicated amounts (µg) of purified GST–PIP4Kα and β were separated by SDS/PAGE and the gel was stained with Coomassie Blue. (B) On the basis of the gel in (A), the indicated amounts of each recombinant protein (ng) were separated by SDS/PAGE, transferred on to nitrocellulose and probed with the antibodies indicated. The upper panel of each blot shows a longer exposure. The data show that p19 and the PIP4Kα C-terminus antibodies specifically recognize only PIP4Kα, whereas p5 and p6 recognize only PIP4Kβ. WB, Western blot.
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**Supplementary Figure 3.** Localization of PIP4Kβ and Myr–PIP4Kα. Myr–PIP4Kα was co-expressed with GFP–PIP4Kβ, fixed and stained for PIP4Kβ (green channel) or for PIP4Kα (red channel). It demonstrates that the Myr–PIP4Kα is predominantly on the membrane, but does not translocate to the nucleus with PIP4Kβ nor does it prevent the localization of GFP–PIP4Kβ in the nucleus.

**Supplementary Figure 4.** Cellular stressors do not modulate the interaction between PIP4Kα and PIP4Kβ. HEK-293 cells expressing the indicated RNAi constructs were treated as shown and cell lysates were immunoprecipitated with the p6 antibody (anti-PIP4Kβ), separated by SDS/PAGE and Western blots were probed for PIP4Kα. The blot in the top panel was stripped and then reprobed for PIP4Kβ. None of the treatments changed the interaction between PIP4Kα and PIP4Kβ. The arrow denotes the migration of endogenous PIP4Kα. CTL, control; IP, immunoprecipitation; WB, Western blot.
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Supplementary Figure 5. PIP4Kβ targets PIP4Kα to the nucleus in HT1080 cells. (A) HT1080 cells were transduced with Myc–PIP4Kα (human), selected and plated on to coverslips. The anti-Myc antibody was used to immunolocalize PIP4Kα and the cells were co-stained with DAPI to reveal the nuclei. Confocal analysis shows that Myc–PIP4Kα predominantly localizes to the cytosol and plasmamembrane. (B) HT1080 cells were transduced with Myc–PIP4Kα and HA-PIP4Kβ, selected with the appropriate antibiotics and plated on to coverslips. Anti-Myc and p6 were used to immunolocalize PIP4Kα and PIP4Kβ respectively, and DAPI was used to counterstain the nuclei. Confocal analysis revealed that when co-expressed with PIP4Kβ, PIP4Kα now localizes in the nucleus. There is also co-localization between PIP4Kα and β in the plasma membrane. The data suggest that PIP4Kβ can target PIP4Kα to the nucleus.