This is a data-enriched, interactive PDF that provides the gateway to a world of information when opened in Utopia Documents Download FREE software now
Check for updates

online data

Genomic tagging reveals a random association of endogenous PtdIns5*P* 4-kinases IIα and IIβ and a partial nuclear localization of the IIα isoform

Minchuan WANG*[1], Nicholas J. BOND†[1], Andrew J. LETCHER*, Jonathan P. RICHARDSON*[2], Kathryn S. LILLEY†, Robin F. IRVINE*[3] and Jonathan H. CLARKE*[3]

*Department of Pharmacology, Tennis Court Road, Cambridge CB2 1PD, U.K., and †Cambridge Centre for Proteomics, Department of Biochemistry, Tennis Court Road, Cambridge CB2 1GA, U.K.

PtdIns5*P* 4-kinases IIα and IIβ are cytosolic and nuclear respectively when transfected into cells, including DT40 cells [Richardson, Wang, Clarke, Patel and Irvine (2007) Cell. Signalling **19**, 1309–1314]. In the present study we have genomically tagged both type II PtdIns5*P* 4-kinase isoforms in DT40 cells. Immunoprecipitation of either isoform from tagged cells, followed by MS, revealed that they are associated directly with each other, probably by heterodimerization. We quantified the cellular levels of the type II PtdIns5*P* 4-kinase mRNAs by real-time quantitative PCR and the absolute amount of each isoform in immunoprecipitates by MS using selective reaction monitoring with ^{14}N,^{13}C-labelled internal standard peptides. The results suggest that the dimerization is complete and random, governed solely by the relative concentrations of the two isoforms. Whereas PtdIns5*P* 4-kinase IIβ is >95 % nuclear, as expected, the distribution of PtdIns4*P* 4-kinase IIα is 60 % cytoplasmic (all bound to membranes) and 40 % nuclear. *In vitro,* PtdIns5*P* 4-kinase IIα was 2000-fold more active as a PtdIns5*P* 4-kinase than the IIβ isoform. Overall the results suggest a function of PtdIns5*P* 4-kinase IIβ may be to target the more active IIα isoform into the nucleus.

Key words: DT40 cell, genomic tagging, nuclear signalling, phosphatidylinositol, phosphatidylinositol 5-phosphate kinase.

INTRODUCTION

Among the many known intracellular roles for inositol lipids, the best understood are those in the cytoplasm. However, there are also a number of less well studied functions for these lipids within the nucleus (for reviews, see [1–4]). There is clear evidence in the nucleus for the minor route of PtdIns(4,5)P_2 synthesis [5], the 4-phosphorylation of PtdIns5*P* by type II PtdIns5*P* 4-kinases. The probable major route of PtdIns5*P* synthesis in animals is by 4-dephosphorylation of PtdIns(4,5)P_2 [6,7], with the re-phosphorylation of PtdIns5*P* by type II PtdIns5*P* 4-kinases serving to remove it. Thus the PtdIns(4,5)P_2 4-phosphatases and the type II PtdIns5*P* 4-kinases can form a reversible pathway regulating the levels of PtdIns5*P* (see Lecompte et al. [8] for evolutionary arguments for this pathway). PtdIns5*P* is present in the nucleus [9] and evidence has been presented that it regulates the activity of the transcription factor ING-2 (inhibitor of growth family 2) [10]. Nuclear levels of PtdIns5*P* increase during the cell cycle [9] or when cells are stressed [11], and it is generally accepted that a key regulator of PtdIns5*P* levels in the nucleus is PtdIns5*P* 4-kinase IIβ whose activity in stressed cells is decreased as a result of it being phosphorylated, and thus inhibited, by the p38 MAPK (mitogen-activated protein kinase) [11]. This, together with an increase in nuclear PtdIns(4,5)P_2 4-phosphatase [7], causes the increase in nuclear PtdIns5*P*. Nuclear PtdIns5*P* 4-kinase IIβ has also been reported to associate with and regulate the activity of Cul3 (cullin 3)–SPOP (speckle-type POZ protein) ubiquitin ligase [12].

The nuclear localization of PtdIns5*P* 4-kinase IIβ is mediated by a novel nuclear localization sequence [13] consisting of a 17-amino-acid acidic α-helix, numbered α-helix 7 in the PtdIns5*P* 4-kinase IIβ structure described by Rao et al. [14]. We have shown previously that any disruption of this α-helix in PtdIns5*P* 4-kinase IIβ leads to a cytosolic localization [13,15]. On the other hand, PtdIns5*P* 4-kinase IIα, which is cytosolic when transfected, lacks this α-helix 7 altogether, and merely introducing that helix into PtdIns5*P* 4-kinase IIα is sufficient to target the enzyme to the nucleus [13]. However, there is evidence, though not yet definitive, that some endogenous PtdIns5*P* 4-kinase IIα may be nuclear [13,16,17].

We established previously that endogenous PtdIns5*P* 4-kinase IIβ is indeed mostly nuclear by genomic tagging of the enzyme in DT40 cells [18]. We also showed that when PtdIns5*P* 4-kinase IIα is acutely transfected into DT40 cells it is, as in other cells, cytosolic [18]. In the present study we have used the specificity of genomic tagging together with quantitative MS with internal peptide standards to show that endogenous PtdIns5*P* 4-kinases IIα and IIβ associate *in vivo*, probably by random heterodimerization, and that approximately 40 % of the endogenous IIα isoform is nuclear.

MATERIALS AND METHODS

The culturing of DT40 cells, extraction of genomic DNA, Western blotting, protein purification and immunoprecipitation of tagged proteins were all performed as described in Richardson et al. [18].

Tagging PtdIns5*P* 4-kinase IIα

This was accomplished using the same strategy as for the tagging of PtdIns5*P* 4-kinase IIβ, described previously [18,19].

Abbreviations used: AQUA, absolute quantification; Cul3, cullin 3; DAPI, 4′,6-diamidino-2-phenylindole; qRT-PCR, quantitative real-time PCR; SPOP, speckle-type POZ protein; SRM, selective reaction monitoring; UPLC, ultra-performance liquid chromatography; UTR, untranslated region; WT, wild-type.

[1] These authors contributed equally to this work.

[2] Present address: Ion Channels and Cell Signalling Centre, Division of Basic Sciences, St George's University of London, London SW17 0RE, U.K.

[3] Correspondence may be addressed to either of these authors (email rfi20@cam.ac.uk or jhc30@cam.ac.uk).

© The Authors Journal compilation © 2010 Biochemical Society

Downloaded from http://port.silverchair.com/biochemj/article-pdf/430/2/215/663898/bj4300215.pdf by guest on 07 December 2023

Primers were used for the PCR amplification of genomic DNA upstream and downstream of the stop codon of the gene encoding PtdIns5*P* 4-kinase IIα, *PIP5K2A* (5′ construct, forward primer 5′-GCATGGTACCTCAGAGTCATGTTAGCTGTG-3′ and reverse primer 5′-GCATTCTAGACGTCAAGATGTTGGCAATAAAG-3′; 3′ construct; forward primer 5′-CATGGATCCTCCCCTCATGTCACACCGGACAG-3′ and reverse primer 5′-GCATGCGGCCGCGTCTGTCAGTGCTACAGAAGTG-3′). The subsequent insertion of the puromycin selection cassette, plasmid linearization, transfection and selection of DT40 lines that had incorporated the insert, and PCR confirmation of the correct incorporation, were all as described in Richardson et al. [18].

Fractionation of DT40 cells

DT40 cells were swollen and disrupted by syringing as described previously [18], but the subsequent fractionation was modified as follows. Samples were layered on to 3 ml of 1.8 M sucrose solution and spun at 35000 rev./min for 1 h on a SW55Ti rotor using a Beckman Optima L-100 XP ultracentrifuge (Supplementary Figure S1 at http://www.BiochemJ.org/bj/430/bj4300215add.htm). After centrifugation, two separate volumes were taken from above the buoyant membranes (fraction C, the top 0.4 ml, and fraction P, the remaining volume). The cytoplasmic membranes (fraction M) were added to 0.5 ml of lysis buffer P1 (0.1 % Triton X-100 and 0.5 % protease inhibitor cocktail in PBS buffer). The nuclei were collected, and their purity determined by brightfield microscopic inspection after staining in Trypan Blue solution or by fluorescent microscopic inspection using a wheat germ agglutinin (conjugated to Alexa Fluor® 555) plasma membrane stain in combination with DAPI (4′,6-diamidino-2-phenylindole) nuclear staining. The nuclei were lysed in 0.8 ml of lysis buffer (50 mM sodium phosphate buffer, pH 7.4, containing 300 mM NaCl, 0.1 % Triton X-100 and 0.5 % protease inhibitor cocktail) on ice for 20 min, and the supernatant (fraction N) was collected after centrifugation. Identical proportions of each fraction were taken for SDS/PAGE and immunoblotting with anti-histone or anti-α-actin antibodies, and the remainder was used for affinity purification of the FLAG–$(His_6)_2$-tagged proteins using TALON beads, followed by Western blotting with anti-FLAG antibodies [18].

Pull-down using anti-FLAG antibody

The anti-FLAG M2 monoclonal antibody (Stratagene) was coupled to Protein G–Sepharose beads using dimethyl pimelimidate dihyrochloride, and these were incubated for 2 h at 4 °C with 400 μl of DT40 cell lysate [18]. Beads were pelleted by centrifugation and washed twice in PBS buffer, and bound protein eluted with 3× FLAG peptide buffer [100 μg/ml 3× FLAG peptide (Sigma–Aldrich) and 0.5 % protease inhibitor cocktail in TBS (Tris-buffered saline)].

Lipid kinase assays

PtdIns5*P* 4-kinase assays were carried out as described previously [20] with slight adaptations. Substrate (300 pmol of PtdIns5*P*) lipid was dried under vacuum and micelles made by sonication in kinase buffer (50 mM Tris/HCl, pH 7.4, containing 10 mM $MgCl_2$, 80 mM KCl and 2 mM EGTA). Recombinant human type II PtdIns5*P* 4-kinase isoforms, cloned and purified as described in [21], were added to the reaction mixture with 10 μCi of [γ-^{32}P]ATP for 90 min at 30 °C. Lipids were extracted and separated by silica-gel TLC [20] and detected by autoradiography. Radioactivity was quantified by scintillation counting of labelled PtdIns(4,5)P_2.

Table 1 Proteotypic peptides selected for MS internal standards

Two peptides were selected that were unique to each chicken PtdIns5*P* 4-kinase II isoform [IIα; UniProt accession number Q5F356, IIβ; predicted NCBI (National Center for Biotechnology Information) accession number XP_418120.2]. Values in square brackets are the molecular mass (Da) of the unlabelled and labelled peptides respectively; values in parentheses are the mass/charge ratio of the doubly charged precursor ions (Th). *, has a stable isotope charge.

Protein	AQUA peptide
PtdIns5*P* 4-kinase IIα	SAPLANDSQAR* [1128.6/1138.6*] (565/570*)
PtdIns5*P* 4-kinase IIα	FGIDDQDFQNSLTR* [1654.8/1664.8*] (828/833*)
PtdIns5*P* 4-kinase IIβ	SAPVNSDSQGR* [1116.5/1126.5*] (559/564*)
PtdIns5*P* 4-kinase IIβ	FGIDDQDYQNSVTR* [1656.7/1666.7*] (829/834*)

qRT-PCR (quantitative real-time PCR)

Total mRNA was extracted from approx. 3×10^7 DT40 cells from three different cultures of each clone tested, with the SV Total RNA Isolation System (Promega) and cDNA libraries were constructed from each preparation with the Sprint RT (reverse transcriptase) complete kit (Clontech). Primers for singleplex qRT-PCR were designed using Primer3 [22]. Owing to the high sequence similarity between *PIP5K2A* and *PIP5K2B*, primer pairs were designed spanning exon 10 and the 3′-UTR (untranslated region) (*PIP5K2A*, forward 5′-TCAAAGCGCTTCTTGGACTT-3′ and reverse 5′-CTACCCTCGTGGTCACTGCT-3′; *PIP5K2B* forward 5′-GCAGTACTCCAAACGCTTCA-3′ and reverse 5′-GGCAAGTAGCCCCTCTTCTC-3′). Primer pairs specific to the tagged genes were generated from exon 10 and the inserted tag sequence (*PIP5K2A-tag* forward 5′-CGCAGAAATTTCAACCGTTA-3′; *PIP5K2B-tag* forward 5′-AGAGATCTCCACCGTGAACC-3′; common reverse 5′-CTTGTCATCGTCGTCCTTGT-3′). A primer set was also designed against the chicken β-actin housekeeping gene, spanning exons 1 and 2 (forward 5′-TGGCAATGAGAGGTTCAGGT-3′ and reverse 5′-CGGATATCCACATCACACTTCA-3′). All primers were obtained from Sigma–Aldrich and produced single PCR products in conventional reactions. PCR amplification was performed over 40 cycles (95 °C for 15 s, 60 °C for 30 s) in a 96-well plate using SYBR Green I master mix (Applied Biosystems, Warrington, U.K.) and C_t (threshold cycle) values were collected using a CFX96 qRT-PCR detection system (Bio-Rad Laboratories). Dissociation curves for each primer set indicated a single product and no-template controls were negative after 40 cycles. Control reactions using RNA from each extraction as a template were negative, showing that the preparations were free from genomic DNA contamination. Primer and template concentrations were optimized to give C_t values of 20–27. Validation experiments showed equivalent relative amplification efficiencies between products from *PIP5K2* genes and the chicken β-actin reference gene (Supplementary Figure S2 at http://www.BiochemJ.org/bj/430/bj4300215add.htm). Normalized expression ratios were calculated by the $2^{-\Delta\Delta Ct}$ method [23].

MS

Absolute protein quantification of PtdIns5*P* 4-kinase II isoforms was achieved by isotope dilution and SRM (selective reaction monitoring). Briefly, proteotypic peptides unique to each isoform were selected as internal standards giving consideration for mass spectra, size and residue composition (see below and Table 1). Internal standards were synthesized (Anaspec) incorporating a single $^{13}C_6$,$^{15}N_4$-labelled arginine residue at the C-terminus and each was independently quantified by amino acid analysis [24].

Downloaded from http://port.silverchair.com/biochemj/article-pdf/430/2/215/663898/bj4300215.pdf by guest on 07 December 2023

© The Authors Journal compilation © 2010 Biochemical Society

For quantifying total protein in unfractionated cells, 7 mg of lysate digest, with 30 pmol of internal standards was separated by strong cation exchange (0–150 mM KCl over 90 min on a 200 mm NEST column with a 2.1 mm internal diameter). Subsequent fractions were each dried, resuspended in 0.1 % formic acid and analysed by UPLC (ultra-performance liquid chromatography)–SRM. To determine the ratio of the PtdIns5P 4-kinase IIα and IIβ isoforms in pull-downs, protein precipitates were digested by incubation overnight with 40 ng of trypsin, with 100 or 200 fmol of internal standards, and analysed by nano-UPLC–SRM as appropriate.

Nano-flow UPLC (300 nl/min) was used for separation of pull-down digests and normal-flow UPLC (400 μl/min) used for the second dimension of separation for lysate digests. In both cases peptides were eluted from preformed 1.7-mm-diameter BEH packed columns [Waters; nanoUPLC (150 mm column with 75 mm internal diameter) and UPLC (100 mm column with 2.1 mm internal diameter)] using gradients of linearly increasing acetonitrile concentration from 0 to 45 % over 40 min (nano-flow) or 10 min (normal-flow).

SRM was conducted using a Quattro Premier XE (Waters). For each peptide monitored by SRM, up to four transitions were configured to monitor the four most intense fragment ions (Supplementary Table S1 at http://www.BiochemJ.org/bj/430/bj4300215add.htm). Transition dwell times were 5 ms for two-dimensional UPLC–SRM and 50 ms for nanoUPLC–SRM. Signals derived from internal standards and analytes of interest were mean smoothed (window of 2 with 1 iteration) and integrated using QuantLynx (Waters). Ratios of standards and analyte peak integrals were calculated and analyte concentration determined.

RESULTS AND DISCUSSION

Immunoprecipitation of genomically tagged PtdIns5*P* 4-kinase IIβ

We planned to use the DT40 cell line JPR3, in which PtdIns5P 4-kinase IIβ is genomically tagged with a FLAG–$(His_6)_2$ tag [18], to identify protein partners that associate with this enzyme. As a proof of principle that we could specifically pull-down and detect endogenous PtdIns5P 4-kinase IIβ by MS, we conducted a series of pull-downs from 3 l of cells using anti-FLAG antibody beads, followed by three washes of the beads and elution with 3× FLAG peptide. We performed this on triplicate samples from WT (wild-type) and JPR3 cells in parallel, and MS analysis revealed that PtdIns5P 4-kinase IIβ is the major protein pulled down, and that it was detectable only in pull-downs from JPR3 and not from WT cells (results not shown). With one exception (see below), the next 20 most abundant proteins identified (mostly contaminants) were present in much lower amounts and were common to WT and JPR3 pull-downs, establishing that this stringent protocol is highly specific for the tagged PtdIns5P 4-kinase IIβ.

However, unexpectedly we also detected PtdIns5P 4-kinase IIα, which was identified as the other major protein present, and was again found only in pull-downs from JPR3 cells and not WT cells; quantitative results (Table 2) confirm that this co-precipitation of PtdIns5P 4-kinase IIα with the IIβ form is highly reproducible. Given that only the IIβ form is FLAG-tagged in JPR3 cells, these results unambiguously establish that the two PtdIns5P 4-kinase II isoforms associate with each other and the absence of any detectable third protein suggests that their association is direct. As both PtdIns5P 4-kinase IIβ [14] and IIα [25] are homodimers in solution and the proposed dimerization interface is conserved between the two isoforms [14], it is likely that this association reflects a heterodimerization (Supplementary Figure S3 at http://www.BiochemJ.org/bj/430/bj4300215add.htm), but as yet we have no results directly addressing the structure of the interaction.

Table 2 PtdIns5*P* 4-kinase II isoform ratios determined using two separate sets of internal peptides

Ratios of PtdIns5P 4-kinase IIβ/IIα in anti-FLAG antibody pull-downs from JPR3 cells measured by the relevant peptide sets (559/565 and 829/828) and quantified by internal labelled standards (see Table 1). ND, not determined.

	Ratio IIβ/IIα	
Experiment	Peptide set 559/565	Peptide set 829/828
JPR3 exp. 1	1.39	0.92
JPR3 exp. 2	ND	1.41
JPR3 exp. 3	1.37	ND
JPR3 exp. 4	1.75	1.47

Cellular ratio of PtdIns5*P* 4-kinase IIα and IIβ

There are many examples of different isoforms of the same enzymic activity heterodimerizing to regulate localization and function, such as the myotubularins [26]. However, these phenomena are usually based on associations of transfected proteins and the actual extent of association of endogenous species, and how this may be regulated, have not been quantified. Our genomic tagging approach gave us a unique opportunity to address these issues, and prompts three questions: (i) Is this PtdIns5P 4-kinase II association random and complete, i.e. dictated only by the relative mass levels of the two isoforms? (ii) Given that PtdIns5P 4-kinase IIβ is mostly nuclear [18], are there therefore significant levels of endogenous PtdIns5P 4-kinase IIα in the nucleus? (iii) What is the physiological relevance of these phenomena?

To answer the first question, we needed to know both the mass ratio of the enzymes in the JPR3 DT40 line, and their relative levels in the pull-downs from JPR3 cells. Both PtdIns5P 4-kinase II isoforms are expressed at very low levels in DT40s, and were at the limit of accurate quantification by MS, with PtdIns5P 4-kinase IIα being the predominant isoform (see below). Additionally we quantified the mRNA levels by qRT-PCR of WT and JPR3 DT40 cells, and the results shown in Figure 1 and Supplementary Figure S2 show that the ratio of PtdIns5P 4-kinase IIβ to IIα mRNA is approx. 0.23 and is similar in WT and JPR3 cells. Moreover, the results also suggest that the tagged and untagged PtdIns5P 4-kinase IIβ alleles are expressed in approximately equal amounts. If these proportions are reflected in the protein level, and an association between the isoforms were random, then we can define the cellular PtdIns5P 4-kinase IIβ/IIα ratio as x, and the proportion of PtdIns5P 4-kinase IIβ that is tagged as t, so the ratio of PtdIns5P 4-kinase IIβ to IIα in a pull-down from PtdIns5P 4-kinase IIβ-tagged cells will be $(2-t)x-1$ (see the Supplementary Experimental section at http://www.BiochemJ.org/bj/430/bj4300215add.htm). Thus if we take x as 0.23 and t as 0.5, random association would give a predicted PtdIns5P 4-kinase IIβ/IIα ratio in the pull-downs from JPR3 cells of 1.36:1.

Quantification of PtdIns5*P* 4-kinase IIα and IIβ by quadrupole MS

MS was applied to quantify PtdIns5P 4-kinase II isoforms at the protein level using an AQUA (absolute quantification) [27] isotope dilution strategy. Given the high sequence identity, suitable proteotypic candidate peptides were limited. Unique candidates were ranked according to their perceived fitness for quantification by considering the quality of empirical data and

Downloaded from http://port.silverchair.com/biochemj/article-pdf/430/2/215/663898/bj4300215.pdf by guest on 07 December 2023

© The Authors Journal compilation © 2010 Biochemical Society

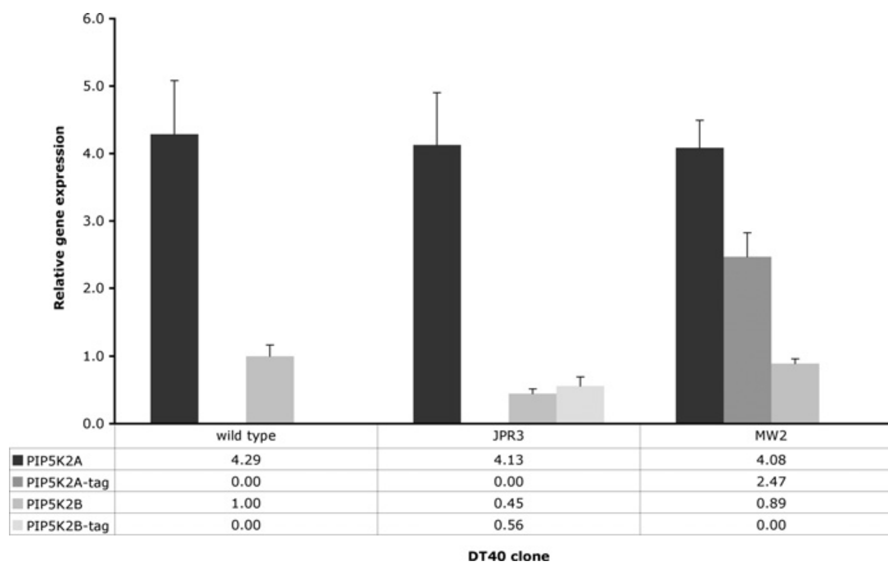

Figure 1 Differential expression of PtdIns5*P* 4-kinase II isoforms from endogenously tagged alleles

Expression from tagged and untagged *PIP5K2A* and *PIP5K2B* alleles determined by qRT-PCR using the comparative threshold cycle (C_t) method. Normalized expression is presented as the relative fold increase above untagged *PIP5K2B* in WT cells ($n = 9$). PCR was performed on cDNA made from RNA extracts of WT, JPR3 and MW2 cells as described in the Materials and methods section.

residue composition. The two best proteotypic peptides for each isoform were selected and AQUA peptides for use as internal standards synthesized (Table 1).

Initially the cellular PtdIns5*P* 4-kinases IIα and IIβ in whole cell lysates were analysed; 7 mg of lysate digest from 2×10^{10} cells, with 30 pmol of AQUA peptides, were separated by two-dimensional LC and peptides were quantified by selective reaction monitoring using a triple quadrupole mass spectrometer. Preliminary experiments indicated which of the first-dimension fractions the AQUA (and analyte) peptides would elute in. Two transitions were monitored for each peptide across the selected first-dimension fractions by UPLC–SRM and quantifiable signals with acceptable signal-to-noise response were integrated (Figure 2). Signals were within the linear response range of the instrument (results not shown) enabling direct calculation of the analyte from the known amount of internal standards. Both diagnostic peptides for PtdIns5*P* 4-kinase IIα were detected and the protein was quantified at 1.5 pmol/mg of total protein, but no quantifiable signals were detected for PtdIns5*P* 4-kinase IIβ.

We then turned to analysis of the anti-FLAG immunoprecipitations from JPR3 cells, and good quantification of both isoforms was achieved. Pre-elution wash steps did not affect the PtdIns5*P* 4-kinase IIβ/IIα ratio observed, but provided a better signal-to-noise response allowing more reliable quantification; the data from four independent experiments using two different sets of internal AQUA peptides (Table 2) yielded a mean $\pm$ S.E.M PtdIns5*P* 4-kinase IIβ/IIα ratio in the immunoprecipitates of 1.39 ± 0.10. This is close enough to the ratio of 1.36 predicted above to suggest strongly that the association between PtdIns5*P* 4-kinases IIα and IIβ is random and complete.

Tagging PtdIns5*P* 4-kinase IIα

To gain independent data of this association, and to answer directly the second question posed above (the likelihood of some endogenous PtdIns5*P* 4-kinase IIα being nuclear), we knocked the same FLAG-$(His_6)_2$ tag into the 3′-end of one allele of the *PIP5K2A* gene by a strategy identical with that which we used previously for the *PIP5K2B* gene [18]. This proved more difficult to do than for the *PIP5K2B* gene, and once we had achieved it, expression of tagged protein in several DT40 lines, including the one we studied most (MW2), declined during the first eight passages (assessed by Western blotting), stabilizing at a level per cell approx. 5-fold lower than the tagged PtdIns5*P* 4-kinase IIβ expression in JPR3 cells (results not shown). Moreover, PCR analysis of the MW2 cells revealed unequal transcription of the tagged and untagged alleles (Figure 1), and an increase in overall PtdIns5*P* 4-kinase IIα transcription, presumably to compensate for the low expression of tagged protein. Clearly there are problems with tagging this isoform that do not apply for PtdIns5*P* 4-kinase IIβ in JPR3 cells, for which expression of tagged protein was always stable, and where no distortion of transcription is evident compared with WT cells (Figure 1). This distortion of both transcription and translation is probably due to a gene-specific effect of the tag, particularly of the puromycin selection cassette in the 3′-UTR. Overall these factors limit the quantification that can be achieved, but the MW2 line nevertheless yielded quantitative results to complement data from JPR3 cells.

In two independent immunoprecipitation experiments where quantification was clear the PtdIns5*P* 4-kinase IIα/IIβ ratios were 11:1 and 8.8:1. As discussed above, our analysis by Western blotting suggested that the level of total tagged PtdIns5*P* 4-kinase IIα protein in MW2 cells was approx. 20 % that of IIβ in JPR3 cells. So if we assume that the untagged PtdIns5*P* 4-kinase IIα allele was translated into protein with the same efficiency in MW2 cells as in WT and JPR3 cells, then from Figure 1 we can to a first degree of approximation estimate that $x = 0.2$ and $t = 0.05$ in MW2 cells. These parameters lead to a predicted PtdIns5*P* 4-kinase IIα/IIβ ratio in pull-downs from MW2 cells of 10.3:1, which is consistent with the mean of 9.9:1 from the above experiments.

Nuclear PtdIns5*P* 4-kinase IIα

Immunocytochemistry in these tagged DT40 cell lines is not possible with such low expression levels [18], and so we

© The Authors Journal compilation © 2010 Biochemical Society

Downloaded from http://port.silverchair.com/biochemj/article-pdf/430/2/215/663898/bj4300215.pdf by guest on 07 December 2023

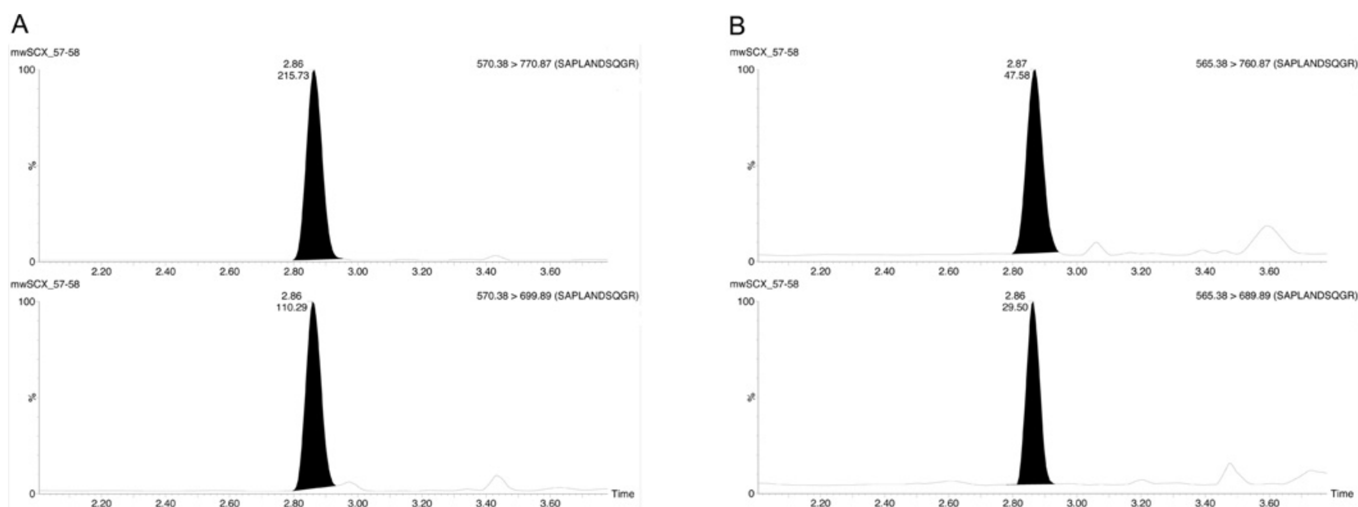

Figure 2 Detection of AQUA and analyte peptides by MS

Integrated signals derived from two selected transitions (see Supplementary Table S1 at http://www.BiochemJ.org/bj/430/bj4300215add.htm) for (**A**) the internal AQUA standard peptide (570.38 > 770.87, top panel; 570.38 > 699.89, lower panel) and (**B**) the corresponding analyte peptide (565.38 > 760.87, top panel; 565.38 > 689.99, lower panel) for quantification of PtdIns5*P* 4-kinase IIα in a whole cell lysate by SRM.

previously used cell fractionation to show that most of the genomically tagged PtdIns5*P* 4-kinase IIβ is nuclear [18]. In the present study we quantified the cytoplasm-to-nuclear ratio of the type II PtdIns5*P* 4-kinase isoforms by another fractionation protocol (Supplementary Figure S1 and see the Materials and methods section) that is more quantitative than that used previously [18] in that we quantified all fractions to give a complete balance sheet of the distribution of the enzymes and markers. Figure 3(A) gives representative images of nuclei obtained from this protocol. The results (Figure 3B) confirm our previous observation [18] that >95 % of PtdIns5*P* 4-kinase IIβ is nuclear. Note that the fractionation protocol has caused significant leakage of histones (as exemplified by histone H1) from the nuclei (Figures 3B–3D), indicating some damage to the nuclear membrane. The retention of most of the PtdIns5*P* 4-kinase IIβ in the nucleus (Figure 3B), in contrast with this histone leakage, suggests that the enzyme is not freely diffusible within the nucleus but is interacting with nuclear structures.

Examination of the nuclear fraction with DAPI staining revealed <1 % nuclei with any detectable cytoplasmic debris attached (Figure 3A). We also probed for remaining plasma membrane using wheat germ agglutinin, and again found <1 % of nuclei had any detectable signal (Figure 3A). Additionally we quantified cytoplasmic contamination of the nuclei by examining for actin by Western blot, and found it to be low (Figure 3D); indeed, the levels of actin (10 % of total) that are detected may be mostly accounted for by *bona fide* intranuclear actin [28] and/or F-actin closely associated with the nucleus [29]. This protocol is therefore apparently effective enough to remove >90 % of the cytoplasm from the nuclei, yet fractionation of MW2 cells showed that 40 % of the tagged PtdIns5*P* 4-kinase IIα is nuclear and 60 % cytoplasmic (Figures 3C and 3D). This suggests that in contrast with transfected PtdIns5*P* 4-kinase IIα [13,18], the endogenous enzyme is significantly nuclear. It also is important to emphasize another difference; we found that endogenous cytoplasmic PtdIns5*P* 4-kinase IIα is in the membranous fraction, not in the cytosol (Figures 3C and 3D). We are not sure to which membrane PtdIns5*P* 4-kinase IIα is attached, but from our current knowledge we suggest that it is most likely to be the plasma membrane, to which it could be targeted by its interaction with type I PtdIns4*P* 5-kinases [30].

Lipid kinase activity of PtdIns5*P* 4-kinase IIα and IIβ

The simplest interpretation of our results overall is that the PtdIns5*P* 4-kinases IIα and IIβ associate randomly, and one consequence may be that PtdIns5*P* 4-kinase IIβ therefore targets the IIα to the nucleus. The third question posed above then remains: what is the physiological significance of these events? One possibility is that the two isoforms could be differentially regulated within the nucleus, e.g. we have shown that PtdIns5*P* 4-kinase IIα is phosphorylated by casein kinase II on a residue that is unique to that isoform [31,32]. However, another (not mutually exclusive) possibility is that the two isoforms have very different enzymic activities. This idea is made more likely by our previous demonstration of an association between PtdIns5*P* 4-kinases IIα and IIγ; the latter had negligible activity when we assayed it in comparison with the former, and IIγ may target IIα to the vesicular compartment in which it (IIγ) is localized [21,33]. To address the issue directly we assayed the PtdIns5*P* 4-kinase activity of recombinant human PtdIns5*P* 4-kinases IIα and IIβ in parallel (Supplementary Figure S4 at http://www.BiochemJ.org/bj/430/bj4300215add.htm and Supplementary Table S2). The specific activity in μmol/min/mg of protein of PtdIns5*P* 4-kinase IIα is 3.25×10^{-2} (S.E.M. $\pm 1.9 \times 10^{-4}$, $n = 4$), and that of IIβ is 1.57×10^{-5} (S.E.M. $\pm 7.9 \times 10^{-7}$, $n = 4$). Thus strikingly PtdIns5*P* 4-kinase IIα is 2000-fold more active than the IIβ isoform, which means that in a PtdIns5*P* 4-kinase IIα/IIβ heterodimer the activity of the PtdIns5*P* 4-kinase IIα is going to be by far the most significant.

Conclusion

We should note that our suggestion that PtdIns5*P* 4-kinase IIβ may target IIα to the nucleus could throw a new light on experiments where PtdIns5*P* 4-kinase IIβ has been increased by transfection [34] or decreased in a knockout mouse [35], both of which cause alterations in (cytoplasmic) insulin sensitivity, an effect primarily evident in muscle and liver. One consequence of manipulating

Downloaded from http://port.silverchair.com/biochemj/article-pdf/430/2/215/663898/bj4300215.pdf by guest on 07 December 2023

© The Authors Journal compilation © 2010 Biochemical Society

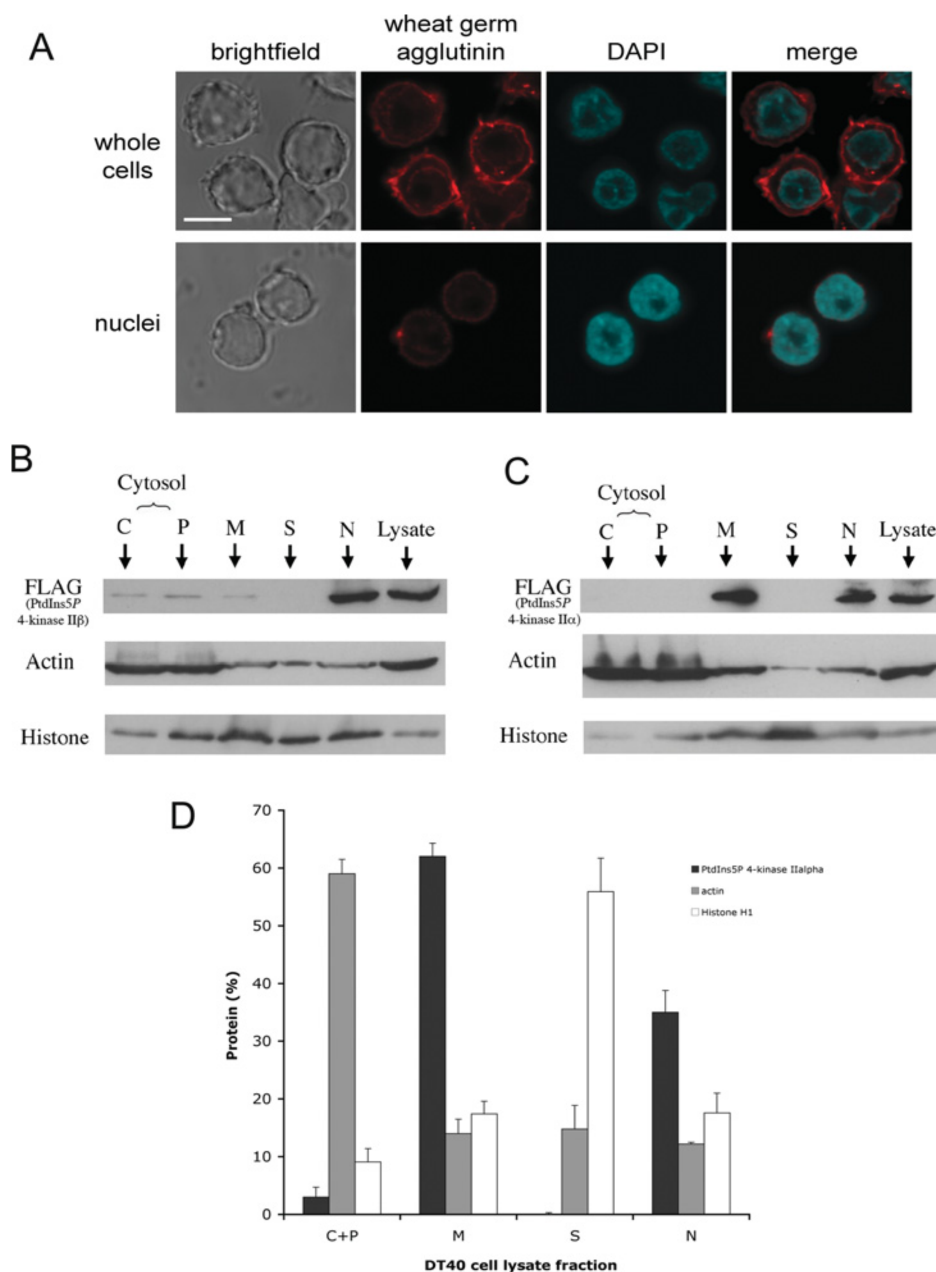

Figure 3 Fractionation and Western blotting of DT40 cell lysates

(**A**) Purity of isolated nuclei were assessed by adhering whole cells or nuclei (prepared as described in the Materials and methods section) to coverslips using cell-tak and decorating with a combination of wheat germ agglutinin plasma membrane stain (conjugated to Alexa Fluor® 555) and DAPI nuclear stain. Scale bar = 5 μm. Representative Western blot for fractionation of (**B**) JPR3 or (**C**) MW2 cell lysates. The same proportion of each cell lysate fraction (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/430/bj4300215add.htm) was either Western blotted for actin or histone H1, or used for immunoprecipitation and detection of FLAG-tagged protein as described in the Materials and methods section. (**D**) Quantification of PtdIns5*P* 4-kinase IIα, actin and histone H1 levels in Western blots of MW2 cell lysate fractionations [the cytosolic fractions (C + P) were combined]. Results are means ± S.E.M. ($n = 4$ for PtdIns5*P* 4-kinase IIα; $n = 3$ for actin and histone H1). C + P, cytosol; M, cytoplasmic membranes; S, sucrose cushion; N, extracted nuclei.

PtdIns5*P* 4-kinase IIβ might be to alter significantly the amount of the IIα isoform present in the cytoplasm, and this would be especially evident in muscle and liver; of the tissues we analysed these have the highest PtdIns5*P* 4-kinase IIβ/IIα ratios [21].

More importantly, the apparently complete and random association (probably heterodimerization) of endogenous proteins that we have revealed and quantitatively examined, plus the much greater enzymic activity of PtdIns5*P* 4-kinase IIα compared with IIβ, make it reasonable to pose the question: is a (or even the) major function of PtdIns5*P* 4-kinase IIβ simply to act as a IIα nuclear-targeting protein? This may not be the whole story, e.g. the requirement for PtdIns5*P* 4-kinase IIβ to be catalytically active to fulfil at least some of its nuclear functions is suggested by a striking dominant-negative effect of a kinase-dead PtdIns5*P* 4-kinase IIβ construct on Cul3–SPOP ubiquitin ligase [12]. As discussed previously, there are precedents for inactive or nearly inactive isoforms of proteins targeting active isoforms to cellular locations, an example taken from phosphoinositide metabolism being the myotubularins [26]. Much of the evidence for this idea is based on association of transfected proteins, and the degree,

© The Authors Journal compilation © 2010 Biochemical Society

Downloaded from http://port.silverchair.com/biochemj/article-pdf/430/2/215/663898/bj4300215.pdf by guest on 07 December 2023

regulation and extent of association and targeting *in vivo* is not always clear. In the present study we have combined the powers of DT40 genomic tagging with MS to place this type of functional relationship, at least for PtdIns5*P* 4-kinases IIα and IIβ, on a new level of quantitative clarity.

While the present work was in progress we learned that another group independently discovered the association between these two PtdIns5*P* 4-kinase II isoforms [36].

AUTHOR CONTRIBUTION

Minchuan Wang performed the pull-downs, made the IIα-tagged cells, did the fractionation experiments and co-wrote the initial draft of the paper. Nicholas Bond co-designed and then performed and co-interpreted all the MS experiments, and co-wrote the inital draft. Andrew Letcher contributed to the pull-downs and fractionation experiments. Jonathan Richardson made the IIβ-tagged cells. Kathryn Lilley co-designed and co-interpreted all the MS experiments, and co-wrote the first draft. Robin Irvine conceived the project, co-designed all the experiments other than the MS and co-wrote all drafts of the paper. Jonathan Clarke performed the qRT-PCR experiments, made the recombinant enzymes and assayed their activity, contributed to the fractionation experiments, co-designed all the experiments other than the MS and co-wrote all drafts of the paper.

ACKNOWLEDGEMENTS

We thank Frederic Langevin, K.J. Patel and members of the Irvine laboratory for helpful discussions.

FUNDING

This work was supported by a Wellcome Trust programme grant [grant number 079194/Z/06 (to R.F.I.)]. N.J.B. is supported by a Ph.D. studentship from the Engineering and Physical Sciences Research Council [grant number EP/D013615/1].

REFERENCES

1 Irvine, R. F. (2003) Nuclear lipid signalling. Nat. Rev. Mol. Cell Biol. **4**, 349–360
2 Cocco, L., Faenza, I., Fiume, R., Maria Billi, A., Gilmour, R. S. and Manzoli, F. A. (2006) Phosphoinositide-specific phospholipase C (PI-PLC) β1 and nuclear lipid-dependent signaling. Biochim. Biophys. Acta **1761**, 5095–5021
3 York, J. D. (2006) Regulation of nuclear processes by inositol polyphosphates. Biochim. Biophys. Acta **1761**, 552–559
4 Bunce, M. W., Bergendahl, K. and Anderson, R. A. (2006) Nuclear PI(4,5)P_2: a new place for an old signal. Biochim. Biophys. Acta **1761**, 560–569
5 Rameh, L. E., Tolias, K. F., Duckworth, B. C. and Cantley, L. C. (1997) A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. Nature **390**, 192–196
6 Ungewickell, A., Hugge, C., Kisseleva, M., Chang, S. C., Zou, J., Feng, Y., Galyov, E. E., Wilson, M. and Majerus, P. W. (2005) The identification and characterization of two phosphatidylinositol-4,5-bisphosphate 4-phosphatases. Proc. Natl. Acad. Sci. U.S.A. **102**, 18854–18859
7 Zou, J., Marjanovic, J., Kisseleva, M. V., Wilson, M. and Majerus, P. W. (2007) Type I phosphatidylinositol-4,5-bisphosphate 4-phosphatase regulates stress-induced apoptosis. Proc. Natl. Acad. Sci. U.S.A. **104**, 16834–16839
8 Lecompte, O., Poch, O. and Laporte, J. (2008) PtdIns5*P* regulation through evolution: roles in membrane trafficking? Trends Biochem. Sci. **33**, 453–460
9 Clarke, J. H., Letcher, A. J., D'santos, C. S, Halstead, J. R., Irvine, R. F. and Divecha, N. (2001) Inositol lipids are regulated during cell cycle progression in the nuclei of murine erythroleukaemia cells. Biochem. J. **357**, 905–910
10 Gozani, O., Karuman, P., Jones, D. R., Ivanov, D., Cha, J., Lugovskoy, A. A., Baird, C. L., Zhu, H., Field, S. J., Lessnick, S. L. et al. (2003) The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. Cell **114**, 99–111
11 Jones, D. R., Bultsma, Y., Keune, W. J., Halstead, J. R., Elouarrat, D., Mohammed, S., Heck, A. J., D'Santos, C. S. and Divecha, N. (2006) Nuclear PtdIns5*P* as a transducer of stress signaling: an *in vivo* role for PIP4Kβ. Mol. Cell **23**, 685–695
12 Bunce, M. W., Boronenkov, I. V. and Anderson, R. A. (2008) Coordinated activation of the nuclear ubiquitin ligase Cul3–SPOP by the generation of phosphatidylinositol 5-phosphate. J. Biol. Chem. **283**, 8678–8686
13 Ciruela, A., Hinchliffe, K. A., Divecha, N. and Irvine, R. F. (2000) Nuclear targeting of the β isoform of type II phosphatidylinositol phosphate kinase (phosphatidylinositol 5-phosphate 4-kinase) by its α-helix 7. Biochem. J. **364**, 587–591
14 Rao, V. D., Misra, S., Boronenkov, I. V., Anderson, R. A. and Hurley, J. H. (1998) Structure of type IIβ phosphatidylinositol phosphate kinase: a protein kinase fold flattened for interfacial phosphorylation. Cell **94**, 829–839
15 Clarke, J. H., Richardson, J. P., Hinchliffe, K. A. and Irvine, R. F. (2007) Type II PIP kinases: location, regulation and function. Biochem. Soc. Symp. **74**, 149–159
16 Divecha, N., Rhee, S. G., Letcher, A. J. and Irvine, R. F. (1993) Phosphoinositide signalling enzymes in rat liver nuclei: phosphoinositidase C isoform β1 is specifically, but not predominantly, located in the nucleus. Biochem. J. **289**, 617–620
17 Boronenkov, I. V., Loijens, J. C., Umeda, M. and Anderson, R. A. (1998) Phosphoinositide signaling pathways in nuclei are associated with nuclear speckles containing pre-mRNA processing factors. Mol. Biol. Cell **9**, 3547–3560
18 Richardson, J. P., Wang, M., Clarke, J. H., Patel, K. J. and Irvine, R. F. (2007) Genomic tagging of endogenous type IIβ phosphatidylinositol 5-phosphate 4-kinase in DT40 cells reveals a nuclear localisation. Cell. Signalling **19**, 1309–1314
19 Mosedale, G., Niedzwiedz, W., Alpi, A., Perrina, F., Pereira-Leal, J. B., Johnson, M., Langevin, F., Pace, P. and Patel, K. J. (2005) The vertebrate Hef ortholog is a component of the Fanconi anemia tumor-suppressor pathway. Nat. Struct. Mol. Biol. **12**, 763–771
20 Hinchliffe, K. A., Irvine, R. F. and Divecha, N. (1998) Regulation of PtdIns4P 5-kinase C by thrombin-stimulated changes in its phosphorylation state in human platelets. Biochem. J. **329**, 115–119
21 Clarke, J. H., Emson, P. C. and Irvine, R. F. (2008) Localization of phosphatidylinositol phosphate kinase IIγ in kidney to a membrane trafficking compartment within specialized cells of the nephron. Am. J. Physiol. Renal Physiol. **295**, F1422–F1430
22 Rozen, S. and Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. **132**, 365–386
23 Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods **25**, 402–408
24 Spackman, D. H., Stein, W. H. and Moore, S. (1958) Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. **30**, 1190–1206
25 Hinchliffe, K., Ciruela, A., Morris, J. A., Divecha, N. and Irvine, R. F. (1999) The type II PIPkins (PtdIns5*P* 4-kinases): enzymes in search of a function? Biochem. Soc. Trans. **27**, 657–661
26 Lorenzo, O., Urbe, S. and Clague, M. J. (2006) Systematic analysis of myotubularins: heteromeric interactions, subcellular localisation and endosome related functions. J. Cell Sci. **119**, 2953–2959
27 Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W. and Gygi, S. P. (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc. Natl. Acad. Sci. U.S.A. **100**, 6940–6945
28 Rando, O. J., Zhao, K. and Crabtree, G. R. (2000) Searching for a function for nuclear actin. Trends Cell Biol. **10**, 92–97
29 Khatau, S. B., Hale, C. M., Stewart-Hutchinson, P. J., Patel, M. S., Stewart, C. L., Searson, P. C., Hodzic, D. and Wirtz, D. (2009) A perinuclear actin cap regulates nuclear shape. Proc. Natl. Acad. Sci. U.S.A. **106**, 19017–19022
30 Hinchliffe, K. A., Giudici, M. L., Letcher, A. J. and Irvine, R. F. (2002) Type IIα phosphatidylinositol phosphate kinase associates with the plasma membrane via interaction with type I isoforms. Biochem. J. **363**, 563–570
31 Hinchliffe, K. A., Ciruela, A., Letcher, A. J., Divecha, N. and Irvine, R. F. (1999) Regulation of type IIα phosphatidylinositol phosphate kinase localisation by the protein kinase CK2. Curr. Biol. **9**, 983–986
32 Hinchliffe, K. A. and Irvine, R. F. (2003) CK2 phosphorylation of Type IIa PIPkin. Curr. Biol. **13**, 178
33 Clarke, J. H., Wang, M. and Irvine, R. F. (2010) Phosphatidylinositol 5-phosphate 4-kinases: localization, regulation and function of Type II phosphatidylinositol 5-phosphate 4-kinases. Adv. Enz. Reg. **50**, 12–18
34 Carricaburu, V., Lamia, K. A., Lo, E., Favereaux, L., Payrastre, B., Cantley, L. C. and Rameh, L. E. (2003) The phosphatidylinositol (PI)-5-phosphate 4-kinase type II enzyme controls insulin signaling by regulating PI-3,4,5-trisphosphate degradation. Proc. Natl. Acad. Sci. U.S.A. **100**, 9867–9872
35 Lamia, K. A., Peroni, O. D., Kim, Y. B., Rameh, L. E., Kahn, B. B. and Cantley, L. C. (2004) Increased insulin sensitivity and reduced adiposity in phosphatidylinositol 5-phosphate 4-kinase $\beta^{-/-}$ mice. Mol. Cell. Biol. **24**, 5080–5087
36 Bultsma, Y., Keune, W.-J. and Divecha, N. (2010) PIP4Kβ interacts with and modulates nuclear localization of the high-activity PtdIns5*P*-4-kinase isoform PIP4Kα. Biochem. J. **430**, 223–235

Received 8 March 2010/4 June 2010; accepted 23 June 2010
Published as BJ Immediate Publication 23 June 2010, doi:10.1042/BJ20100340

© The Authors Journal compilation © 2010 Biochemical Society

Downloaded from http://port.silverchair.com/biochemj/article-pdf/430/2/215/663898/bj4300215.pdf by guest on 07 December 2023

Biochem. J. (2010) **430**, 215–221 (Printed in Great Britain) doi:10.1042/BJ20100340

SUPPLEMENTARY ONLINE DATA

Genomic tagging reveals a random association of endogenous PtdIns5*P* 4-kinases IIα and IIβ and a partial nuclear localization of the IIα isoform

Minchuan WANG*[1], Nicholas J. BOND†[1], Andrew J. LETCHER*, Jonathan P. RICHARDSON*[2], Kathryn S. LILLEY†, Robin F. IRVINE*[3] and Jonathan H. CLARKE*[3]

*Department of Pharmacology, Tennis Court Road, Cambridge CB2 1PD, U.K., and †Cambridge Centre for Proteomics, Department of Biochemistry, Tennis Court Road, Cambridge CB2 1GA, U.K.

Downloaded from http://port.silverchair.com/biochemj/article-pdf/430/2/215/663898/bj4300215.pdf by guest on 07 December 2023

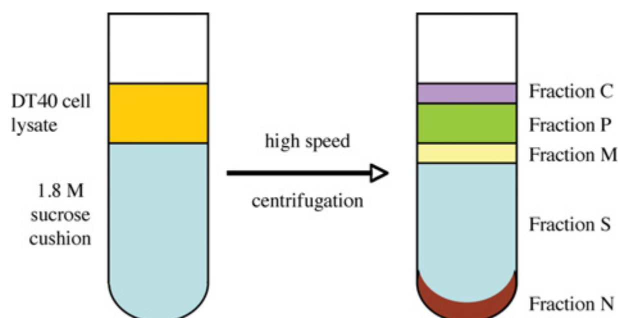

Figure S1 Summary of cell fractionation method

DT40 cell lysate was prepared as described in the Materials and methods section in the main paper, layered on to 1.8 M sucrose and ultracentrifuged. Various fractions were harvested from the preparation. Fraction C and Fraction P were pooled as the cytosolic component. Fraction M consisted of the cytoplasmic membranes. Fraction S was composed of the residual sucrose cushion. The pelleted cell nuclei were further solubilized to make Fraction N. All fractions were used for direct Western blotting or immunoprecipitation.

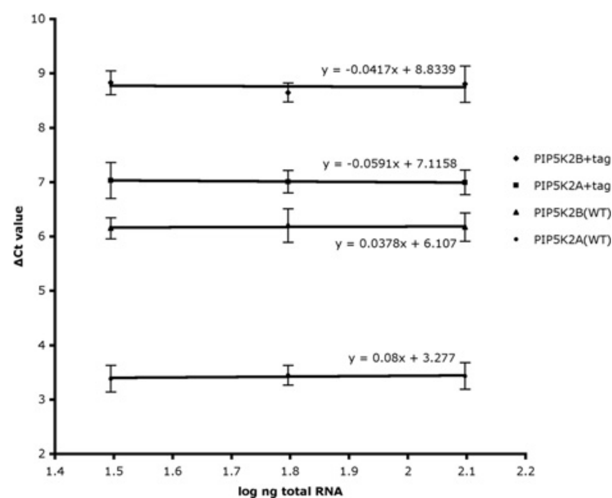

Figure S2 Relative amplification efficiencies of target and reference genes by qRT-PCR

Validation experiments to determine equivalent PCR amplification efficiencies of WT or endogenously FLAG-$(His_6)_2$-tagged mRNA from *PIP5K2A* and *PIP5K2B* genes relative to that of the reference housekeeping gene (chicken β-actin). Line equations of each plot indicate slope, error bars represent S.D. ($n = 9$).

EXPERIMENTAL

Calculations of ratios of isoforms expected in pull-downs from tagged cells and in nuclei

Assuming both alleles are tagged (i.e. all protein is tagged)

If PtdIns5*P* 4-kinase IIβ/IIα is x, let IIβ be 1, so IIα is $1/x$, and so the total PtdIns5*P* 4-kinase II content is $(1 + 1/x)$. If dimerization is random, out of the total (=1) IIβ, $1/[1+(1/x)]$ will homodimerize with IIβ, and $(1/x)/(1 + 1/x)$ will heterodimerize with IIα, and the homodimerized IIα will be $(1/x^2)/[1+(1/x)]$.

In a pull-down from cells with PtdIns5*P* 4-kinase IIβ tagged, the number of IIβ molecules pulled down is 1, and the number of IIα molecules is $(1/x)/[1+(1/x)]$. So the IIβ/IIα ratio in that pull-down is $[1 + (1/x)]/(1/x)$. In a pull-down from cells with PtdIns5*P* 4-kinase IIα tagged, the amount of IIα pulled down is $1/x$ and the amount of IIβ pulled down is $(1/x)/[1+(1/x)]$. So IIα/IIβ = $[1+(1/x)]$

To calculate the proportion of IIα that is cytoplasmic, we assume that only IIα in homodimers is cytoplasmic. This is $(1/x^2)/[1 + (1/x)]$. If we divide this by the total IIα $(1/x)$, then the proportion of total IIα that is cytoplasmic is $(1/x)/[1+(1/x)]$, or $1/(x+1)$ (and the ratio of cytoplasmic to nuclear IIα is $1/x$).

Assuming only one allele is tagged

Any dimers containing only untagged protein will be missed as they will not be pulled down. This will decrease heterodimers pulled down in direct proportion to the degree of tagging, but it will decrease homodimers to a varying degree (dictated by the ratio of isoforms), because only homodimers consisting of two untagged species will avoid the pull-down. If t is the proportion of a protein tagged (and other parameters are as above), for a pull-down from PtdIns5*P* 4-kinase IIβ-tagged cells, the IIβ/IIα ratio will be:

$$\frac{1-(1-t)^2+(t/x)}{t/x}$$

which can be simplified to $2x - tx + 1$. For a pull-down from a PtdIns5*P* 4-kinase IIα-tagged cell, the IIα/IIβ ratio will be

[1] These authors contributed equally to this work.
[2] Present address: Ion Channels and Cell Signalling Centre, Division of Basic Sciences, St George's University of London, London SW17 0RE, U.K.
[3] Correspondence may be addressed to either of these authors (email rfi20@cam.ac.uk or jhc30@cam.ac.uk).

© The Authors Journal compilation © 2010 Biochemical Society

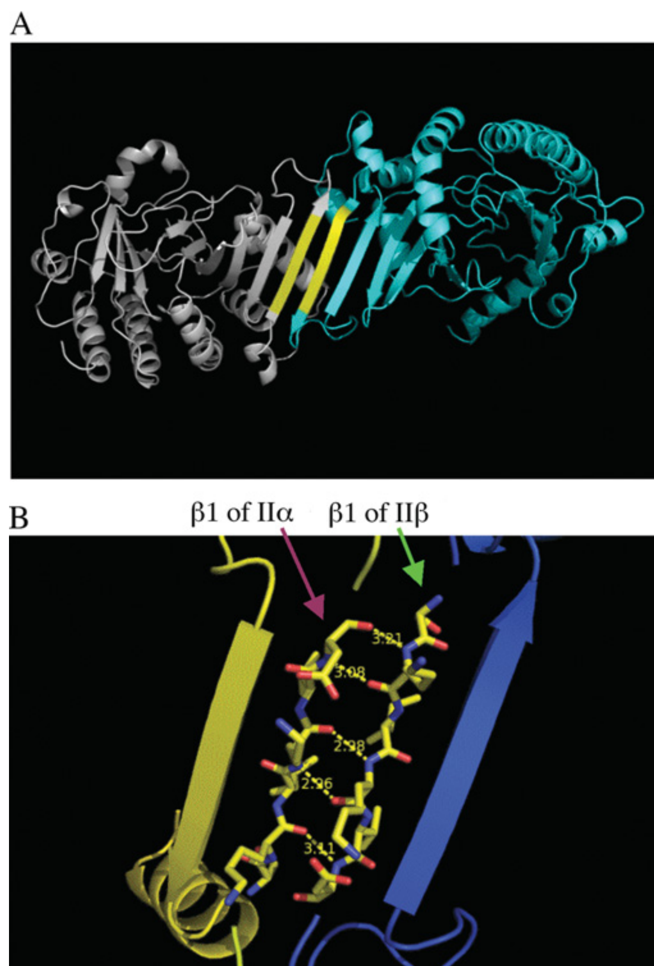

Figure S3 Molecular modelling of putative PtdIns5*P* 4-kinase II heterodimers

(**A**) Comparison of human and chicken orthologues of PtdIns5*P* 4-kinases IIα and IIβ by FUGUE [1] showed protein structure to be identical within 99 % confidence limits. Homology modelling of the three-dimensional structure of the IIα/IIβ heterodimers was performed using MODELLER [2]. The peptide sequences of chicken PtdIns5*P* 4-kinases IIα and IIβ were initially aligned with the chain A and chain B sequences of the homodimer structure (PDB code 1BO1) of human PtdIns5*P* 4-kinase IIβ [3] respectively by ClustalW [4,5]. The resulting alignments were manually adjusted using the BioEdit programme (http://www.mbio.ncsu.edu). The model illustrates the possible three-dimensional structure of the PtdIns5*P* 4-kinase IIα/β heterodimer with the blue subunit representing the structure of the PtdIns5*P* 4-kinase IIα subunit (chain A) and the silver representing the PtdIns5*P* 4-kinase IIβ subunit (chain B). The two subunits are dimerized by forming β-sheet hydrogen bonds between the two β1 strands, which are highlighted in yellow. (**B**) A model illustrating the predicted hydrogen bonds formed between the two β1 strands of the PtdIns5*P* 4-kinase IIα and IIβ subunits. The magenta arrow shows the β1 strand of the PtdIns5*P* 4-kinase IIα subunit and the green the β1 strand of the PtdIns5*P* 4-kinase IIβ subunit. Red, oxygen; yellow, carbon; blue, nitrogen.

$$\frac{1-(1-t)^2+t}{t}$$

which can be simplified to $(2-t+x)/x$.

As we quantify the cytoplasmic and nuclear proportions of tagged PtdIns5*P* 4-kinase IIα in a IIα-tagged cell by Western blotting (Figure 3 in the main paper), which only sees tagged protein, the calculations for expected cytoplasmic IIα are identical with those above under conditions where both alleles are tagged, where $t=1$.

Table S1 Transitions used to detect PtdIns5*P* 4-kinase IIα and IIβ

Four transitions were configured for each PtdIns5*P* 4-kinase II-specific peptide, with either the unlabelled or stable isotope labelled (*).

	Peptide sequence	Transition	Collision energy (eV)
PtdIns5*P* 4-kinase IIα	SAPLANDSQAR	1: 565.38 > 689.89	25
	SAPLANDSQAR	2: 565.38 > 760.87	25
	SAPLANDSQAR	3: 565.38 > 873.89	25
	SAPLANDSQAR	4: 565.38 > 970.88	25
	SAPLANDSQAR*	5: 570.38 > 699.89	25
	SAPLANDSQAR*	6: 570.38 > 770.87	25
	SAPLANDSQAR*	7: 570.38 > 883.89	25
	SAPLANDSQAR*	8: 570.38 > 980.88	25
	FGIDDQDFQNSLTR	1: 828.44 > 864.91	30
	FGIDDQDFQNSLTR	2: 828.44 > 979.86	30
	FGIDDQDFQNSLTR	3: 828.44 > 1222.80	30
	FGIDDQDFQNSLTR	4: 828.44 > 1337.77	30
	FGIDDQDFQNSLTR*	5: 833.44 > 874.91	30
	FGIDDQDFQNSLTR*	6: 833.44 > 989.86	30
	FGIDDQDFQNSLTR*	7: 833.44 > 1232.80	30
	FGIDDQDFQNSLTR*	8: 833.44 > 1347.77	30
PtdIns5*P* 4-kinase IIβ	SAPVNSDSQGR	1: 559.29 > 648.88	25
	SAPVNSDSQGR	2: 559.29 > 762.87	25
	SAPVNSDSQGR	3: 559.29 > 861.86	25
	SAPVNSDSQGR	4: 559.29 > 958.85	25
	SAPVNSDSQGR*	5: 564.29 > 658.88	25
	SAPVNSDSQGR*	6: 564.29 > 772.87	25
	SAPVNSDSQGR*	7: 564.29 > 871.86	25
	SAPVNSDSQGR*	8: 564.29 > 968.85	25
	FGIDDQDYQNSVTR	1: 829.37 > 866.90	30
	FGIDDQDYQNSVTR	2: 829.37 > 981.80	30
	FGIDDQDYQNSVTR	3: 829.37 > 1109.82	30
	FGIDDQDYQNSVTR	4: 829.37 > 1339.74	30
	FGIDDQDYQNSVTR*	5: 834.37 > 876.90	30
	FGIDDQDYQNSVTR*	6: 834.37 > 991.80	30
	FGIDDQDYQNSVTR*	7: 834.37 > 1119.82	30
	FGIDDQDYQNSVTR*	8: 834.37 > 1349.74	30

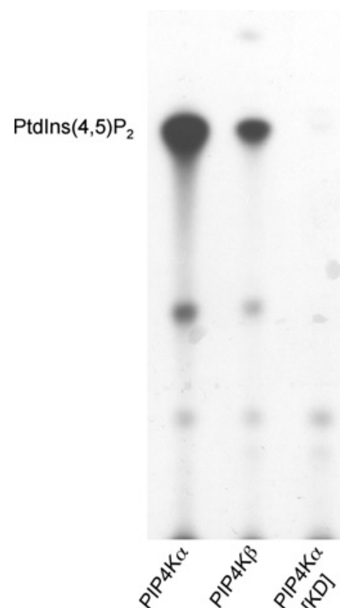

Figure S4 Specific activity of PtdIns5*P* 4-kinase II isoforms

Representative thin-layer chromatogram showing labelled PtdIns(4,5)P_2 product from PtdIns5*P* lipid assays. Recombinant human PtdIns5*P* 4-kinase IIα (0.05 μg), PtdIns5*P* 4-kinase IIβ (12.5 μg) and a control kinase-dead PtdIns5*P* 4-kinase IIα (KD; 12.5 μg) were used in each reaction as described in the Materials and methods section of the main paper.

Downloaded from http://port.silverchair.com/biochemj/article-pdf/430/2/215/663898/bj4300215.pdf by guest on 07 December 2023

© The Authors Journal compilation © 2010 Biochemical Society

Table S2 Results used to calculate the specific enzyme activity

Raw scintillation results used to calculate specific kinase activities of PtdIns5*P* 4-kinase IIs. Assays using 12.5 μg of recombinant PtdIns5*P* 4-kinase IIβ or 50 ng of recombinant PtdIns5*P* 4-kinase IIα were completed as detailed in the Materials and methods section of the main paper. Counts collected for 5 min in the ^{32}P channel were corrected for background and used to calculate mean specific activity.

Protein	Radioactivity (c.p.m)
PtdIns5*P* 4-kinase IIα	32073.47
	26319.75
	28414.37
	24523.23
PtdIns5*P* 4-kinase IIβ	3114.77
	3611.66
	3436.27
	2879.39

REFERENCES

1 Shi, J., Blundell, T. L. and Mizuguchi, K. (2001) FUGUE: sequence–structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties. J. Mol. Biol. **310**, 243–257

2 Sanchez, R. and Sali, A. (2000) Comparative protein structure modeling. Introduction and practical examples with modeller. Methods Mol. Biol. **143**, 97–129

3 Rao, V. D., Misra, S., Boronenkov, I. V., Anderson, R. A. and Hurley, J. H. (1998) Structure of type IIβ phosphatidylinositol phosphate kinase: a protein kinase fold flattened for interfacial phosphorylation. Cell **94**, 829–839

4 Fukami-Kobayashi, K. and Saito, N. (2002) How to make good use of CLUSTALW. Tanpakushitsu Kakusan Koso **47**, 1237–1239

5 Thompson, J. D., Gibson, T. J. and Higgins, D. G. (2002) Multiple sequence alignment using ClustalW and ClustalX. Curr. Protoc. Bioinformatics **2**, Unit 2.3

Received 8 March 2010/4 June 2010; accepted 23 June 2010
Published as BJ Immediate Publication 23 June 2010, doi:10.1042/BJ20100340

Downloaded from http://port.silverchair.com/biochemj/article-pdf/430/2/215/663898/bj4300215.pdf by guest on 07 December 2023

© The Authors Journal compilation © 2010 Biochemical Society