Localization of phosphatidylinositol phosphate kinase IIγ in kidney to a membrane trafficking compartment within specialized cells of the nephron

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Clarke JH, Emson PC, Irvine RF. 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, 2008. First published August 27, 2008; doi:10.1152/ajprenal.90310.2008.—PIP4Ks (type II phosphatidylinositol 4-phosphate kinases) are phosphatidylinositol 5-phosphate (PtdIns5P) 4-kinases, believed primarily to regulate cellular PtdIns5P levels. In this study, we investigated the expression, localization, and associated biological activity of the least-studied PIP4K isoform, PIP4Kγ. Quantitative RT-PCR and in situ hybridization revealed that compared with PIP4Kα and PIP4Kβ, PIP4Kγ is expressed at exceptionally high levels in the kidney, the cortex and outer medulla. A specific antibody was raised to PIP4Kγ, and immunohistochemistry with this and with antibodies to specific kidney cell markers showed a restricted expression, primarily distributed in epithelial cells in the thick ascending limb and in the intercalated cells of the collecting duct. In these cells, PIP4Kγ had a vesicular appearance, and transfection of kidney cell lines revealed a partial Golgi localization (primarily the matrix of the cis-Golgi) with an additional presence in an unidentified vesicular compartment. In contrast to PIP4Kα, bacterially expressed recombinant PIP4Kγ was completely inactive but did have the ability to associate with active PIP4Kα in vitro. Overall our data suggest that PIP4Kγ may have a function in the regulation of vesicular transport in specialized kidney epithelial cells.

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Cell culture and transfection. HeLa, HEK 293, COS-7, and NRK cells were maintained in DMEM (GIBCO, Paisley, UK) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin. Cells were transiently transfected with plasmid constructs for 24 h with TransFectin reagent (Bio-Rad, Hercules, CA), following the manufacturer’s protocol. Cell lysates for immunoprecipitation were made by suspending cells in cold lysis buffer (PBS with 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, and 100 µg/ml Sigma P8340 protease inhibitor cocktail) and centrifuging at 10,000 rpm at 4°C.

Tissue sample preparation. Protein and mRNA samples were prepared from adult mouse tissues obtained post mortem and immediately frozen on dry ice after collection. Tissues (50 mg) for mRNA extraction were pulsed in lysing matrix D tubes (Qbiogene) with 1 ml Tri-Reagent (Sigma-Aldrich, Poole, UK) on a Hybaid Ribolyser. mRNA was purified (RNeasy kit; Qiagen, and Turbo DNase; Ambion, Austin, TX), and cDNA libraries were constructed by RT-PCR (Sprint Powerscript kit; Clontech). Protein lysates were prepared in RIPA buffer (150 mM sodium chloride, 50 mM Tris pH 7.4, 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, and 0.1% SDS) with 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 17.5 mM β-glycerophosphate, and 100 µl/mg Sigma P8340 protease inhibitor cocktail, using 10–15 strokes in a 5 ml dounce homogenizer. Cell debris was cleared by centrifugation at 2,900 g for 20 min at 4°C, and lysates were clarified by further centrifugation at 20,000 g for 45 min at 4°C. Kidney tissue was dissected under a binocular microscope to produce cortical and medullary samples. Tissues for immunochromistry from perfused mice were fixed in 4% paraformaldehyde, sectioned on a Leica cryostat at −20°C, mounted on slides, and stored at −80°C.

Quantitative PCR. Singleplex quantitative PCR was carried out using specific primers for each PIP5K2 gene, designed to the 3′-untranslated region (PIP5K2A forward: 5′-AAGAGTCTGTGCAAGAACCTGT-3′ and reverse: 5′-TGCACTGGAACCTTGATGTA-3′; PIP5K2B forward: 5′-CATCTCCACAGAAGACAGTGG-3′ and reverse: 5′-CTTGCATTACCATCGTTCTC-3′; and PIP5K2C forward: 5′-CATCTGCCGGAATGTTGCTCCC-5′ and reverse: 5′-TTGATGTTATG-GCTTGTACCTCCTCCTTCT-3′). Three cDNA libraries were constructed for each tissue tested and analyzed in triplicate. PCR amplification was performed in a 96-well plate using SYBR Green I master mix (Applied Biosystems, Warrington, UK), following the manufacturer’s protocol. Reactions were heated (50°C for 2 min, 95°C for 10 min) and cycled 40 times (95°C for 15 sec, 60°C for 1 min) on an ABI Prism 7700 sequence detection system (Applied Biosystems). Dissociation curves for each primer set indicated a single product, and no-template controls were negative after 40 cycles. Reactions using
Fig. 3. PIP4Kγ is expressed in adult mouse tissues. Lysates were probed with PIP4Kγ-specific antibody (i) and with neutralized antibody after saturation with immunizing peptide (ii). Whole tissue lysates (A) and protein preparations from different kidney regions (B) were probed with the same antibody. Equivalent protein loading was based on total lysate concentration. Purified recombinant PIP4Kγ was used to identify endogenous PIP4Kγ at the correct molecular mass (arrow).

Protein samples (50 μg) were resolved on 10% polyacrylamide gels, and Western blots were carried out as described previously (16), using anti-PIP4Kγ at 0.5 μg/ml. Anti-PIP4Kγ was neutralized by saturating with excess of antigenic peptide for 30 min. Other antibodies used in this study were diluted to the manufacturers recommendations: goat polyclonal antibodies to aquaporin 1 (AQP1), aquaporin 2 (AQP2) and Tamm-Horsfall protein, and polyclonal rabbit antibodies to Igpl10 (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal antibodies to TGN38 were a gift from G. Banting; mouse Mabs to EEA1, BiP, and GM130, and rabbit polyclonal antibodies to p115 (BD Transduction Laboratories, Oxford, UK); rabbit polyclonal antibodies to calreticulin (Calbiochem, La Jolla, CA); mouse Mabs to tubulin (Sigma-Aldrich); and goat polyclonal antibodies to golgin160 and rabbit polyclonal antibodies to catalase, p58K, mannose 6-phosphate receptor, and mannosidase II (Abcam, Cambridge, UK). Alexa Fluor 568 phalloidin and TO-PRO-3 iodide were used to directly stain actin and DNA (Molecular Probes, Paisley, UK). Horseradish peroxidase-conjugated secondary antibodies and SuperSignal West Dura substrate were used for Western blotting (Pierce Protein Research Products, Rockford, IL), and Alexa dye-conjugated secondary antibodies were used for fluorescence microscopy (Molecular Probes, Rockford, IL), and Immunoprecipitation with anti-PIP4Kγ rat Mabs (16) and protein G-Sepharose (GE Healthcare) was carried out for 16 h at 4°C. Beads were washed in cold PBS and used directly for Western blotting or kinase assay.

Immunocytochemistry and immunohistochemistry. Mammalian cells expressing green fluorescent protein-tagged constructs were fixed in 4% paraformaldehyde for 30 min on ice. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with 4% fish skin gelatin (Sigma-Aldrich) in PBS for 30 min and then incubated for 60 min with primary and secondary antibodies (2% gelatin in PBS) with PBS washes between and after incubation. Slides were mounted using ProLong Gold antifade reagent (Molecular Probes), and images were taken on a Leica TCS SP5 laser scanning confocal microscope running LAS AF software (Leica Microsystems, Germany).

RNA as template were negative, showing that the preparations were free from genomic DNA contamination. Primer concentrations were chosen to give threshold cycle (Ct) values of 20–25. Validation experiments showed equivalent relative amplification of specific sequences between each PIP5K2 gene and a primer set for mouse β-actin (forward: 5′-GACGATATCGCTGGCGTGTT-3′ and reverse: 5′-CCACGATTGAGGGAGGATC-3′), and ΔCt values were analyzed using the comparative Ct method (23).

In situ hybridization. Two oligonucleotide probes were designed to the PIP5K2 sequence, one in the 3′-untranslated region (5′-GACTGGGTG-GATTGAGTTATGGCTCTGACTCCTCT-3′) and one in the coding sequence (5′-ATAGGAGATAAGGAAACGGCCATCACTGCCTTCAG-3′), which only identified PIP5K2 when BLAT searched against the European Molecular Biology Laboratory mouse database. Probes were 3′-tail-labeled with [35S]dATP (NEN, Hounslow, UK), hybridized with 20-μm mouse tissue sections and autoradiographed for 5 wk, as described previously (12). Slides of interest were dipped in autoradiographic emulsion and after development (12 wk) were counterstained with hematoxylin and eosin, and images were captured using an Axioskop II light microscope.

Antibody analysis and Western blotting. A peptide (amino acids 333–352), unique to the variable region of the mouse PIP4Kγ sequence, was used to raise a custom polyclonal antibody (NeoMPS, San Diego, CA), which was subsequently purified from rabbit serum by affinity matrix chromatography. Surface plasmon resonance analysis was carried out on a Biacore 3000 instrument (GE Healthcare San Diego, CA), which was subsequently purified from rabbit serum by affinity matrix chromatography. Surface plasmon resonance analysis was carried out on a Biacore 3000 instrument (GE Healthcare San Diego, CA), which was subsequently purified from rabbit serum by affinity matrix chromatography. Surface plasmon resonance analysis was carried out on a Biacore 3000 instrument (GE Healthcare San Diego, CA), which was subsequently purified from rabbit serum by affinity matrix chromatography. Surface plasmon resonance analysis was carried out on a Biacore 3000 instrument (GE Healthcare San Diego, CA), which was subsequently purified from rabbit serum by affinity matrix chromatography.
Wetzlar, Germany). Golgi dissociation was achieved by treatment with Brefeldin A (10 μg/ml in DMEM) for 30 min at 37°C. Sagittal and horizontal tissue sections for fluorescent labeling were pretreated to reduce autofluorescence (20 min in 1 mg/ml sodium borohydride), blocked for 2 h (1% fish skin gelatin, 0.3% Triton X-100 in PBS), exposed to primary antibody (24 h at 4°C), and fluorophore-labeled secondary antibody (4 h) before being mounted as described previously. Confocal images were spectrally separated by LAS AF software using reference spectra obtained from unstained tissue and cells overexpressing green fluorescent protein. Tissue sections for chemical staining were pretreated for 20 min (20% methanol and 5% hydrogen peroxide), blocked, and incubated with primary antibody as described, and then they were incubated with biotinylated anti-rabbit IgG antibodies before visualization using Vectastain ABC and DAB substrate kits (Vector Laboratories, Burlingame, CA).Slides were treated with Histoclear and mounted with DPX reagent (Sigma-Aldrich) before light microscopy.

**Lipid kinase assay.** PIP4K assays were carried out as described previously (16) with slight adaptation. Substrate lipid (6 μM PtdIns5P) was dried under vacuum, and micelles were made by sonication in kinase buffer (50 mM Tris pH 7.4, 10 mM MgCl2, 80 mM KCl, and 2 mM EGTA). Recombinant PIP4K was added to the reaction mixture with 10μCi [γ-32P]ATP for 90 min at 30°C. Lipids were extracted and separated by silica-gel thin layer chromatography (16), and results were obtained by autoradiography.

**RESULTS**

**PIP5K2 isoforms have different expression profiles.** Expression studies using RT-PCR have previously suggested that levels of PIP5K2 transcription are high in different tissues (1, 19). Using a series of isofrom-specific PCR primer pairs for PIP5K2A, PIP5K2B, and PIP5K2C, we quantitatively assessed the expressed levels of these transcripts in mRNA isolated from a range of mouse tissues (Fig. 1). Automated quantitative PCR of synthesized CDNA libraries was completed using three biological replicates for each tissue. Pooled data were analyzed by the Livak method (23), normalizing PIP5K2 to the housekeeping gene β-actin, and values were calculated as the relative fold increase above the lowest observed tissue expression (Fig. 1A). All three isoforms were expressed at higher levels in the brain, with PIP5K2A expression increased in spleen and PIP5K2B in muscle. PIP5K2C was especially high in kidney, as originally reported by Itoh et al. (19). Tissue analysis by in situ hybridization with a range of PIP5K2C probes confirmed that expression of this isoform was upregulated in brain and kidney, compared with a control tissue, and that the expression was localized to discrete regions of these organs (Fig. 1B).

**Endogenous PIP4Kγ is differentially expressed in mouse tissues.** A polyclonal peptide antibody to the variable region of PIP4Kγ was raised and purified. Due to the similarity of the sequence of the PIP4K isoforms at the protein level, the specificity of the antibody for PIP4Kγ was determined (Fig. 2). The anti-PIP4Kγ antibody has no cross-reactivity with PIP4Kα or PIP4Kβ by Western blot (Fig. 2A) or by immunocytochemistry (Fig. 2B). Purified recombinant PIP4Ks were bound to an NT4 sensor chip by 6×His tag, and surface plasmon resonance analysis of the binding of anti-PIP4Kγ antibody indicated specificity for PIP4Kγ compared with PIP4Kα and PIP4Kβ (Fig. 2C). This was confirmed by binding biotinylated anti-PIP4Kγ antibody to an SA sensor chip and detecting specific binding to PIP4Kγ (data not shown). Direct Western blotting of a bank of tissue lysates confirmed the presence of significant endogenous levels of PIP4Kγ in brain, kidney, ovary, and testis (Fig. 3A, i). Other tissues had little or no detectable levels of PIP4Kγ, assuming representative expression based on equivalent loading of total lysate protein. Endogenous PIP4Kγ was detected as two bands of very similar molecular mass of ~47 kDa, the larger band presumably representing the phosphorylated form of mature PIP4Kγ (19). Crude kidney fractionation and subsequent Western blotting of protein lysates indicated that PIP4Kγ was present throughout this organ, but comparatively higher levels were seen in the medulla (Fig. 3B, i). Nonspecific bands were visualized using neutralized antibody as control, and lower molecular mass immunoreactive bands were presumed to be products of proteolytic cleavage (Fig. 3, ii). Interestingly, PIP4Kγ in heart ran predominantly as a 40-kDa band, suggesting that processing of this isoform may be occurring (Fig. 3A).

**PIP4Kγ expression in kidney is localized to specific cells.** Identification of PIP4Kγ at a cellular level was shown to be specific by the use of peptide-saturated primary antibody controls, which demonstrated an absence of signal due to nonspecific binding of primary or secondary antibodies in tissue immunohistochemistry. Spectral separation was also utilized to...
remove significant autofluorescence from the kidney tissue in immunofluorescence experiments (Fig. 4A). Analysis of whole kidney sections confirmed that PIP4Kγ was expressed throughout this organ (data not shown). Detailed examination of representative regions of the kidney at high resolution indicated that the positive signal was seen to be concentrated in the outer medulla (Fig. 4B), confirming the results obtained for transcript expression (Fig. 1B), with much less signal being observed in the cortical labyrinth. This signal was restricted to whole regions of specific tubules, which could be visualized using sequential confocal imaging at 2-µm intervals throughout a 20-µm cryosectioned tissue slice (data not shown). The PIP4Kγ-expressing tubules were present in both the cortical medullary rays and the inner and outer stripe of the outer medulla but were absent from the tubules of the inner medulla (Fig. 4B). However, it was possible to observe single PIP4Kγ-positive cells within tubules in the cortex and inner medulla (Fig. 4B).

Costaining sections with selective markers for different nephron regions allowed accurate determination of the tubules and cells that expressed PIP4Kγ (Fig. 5). The absence of PIP4Kγ in cortical tubules with a significant brush-border lumen lining (Fig. 5, A and B) or coincident with the water channel AQP1 (Fig. 5C), a marker for the proximal convoluted tubule and thin descending limb of the loop of Henle (29), suggested that expression was restricted to the distal part of the nephron. PIP4Kγ-positive tubules also expressed Tamm-Horsfall protein in the outer medulla (Fig. 5, D–F), which suggested

![Fig. 5](image-url)
that PIP4Kγ is localized to cells in the thick ascending limb (TAL) of the loop of Henle (2). The presence of PIP4Kγ-positive tubules in the cortex and outer medulla (Fig. 5, G and H), but not in the inner medulla (Fig. 5I), and distinct from tubules expressing the collecting duct marker AQP2 (24), confirmed that PIP4Kγ was mainly expressed in TAL. Interestingly, isolated PIP4Kγ-positive cells were also localized to the collecting duct but were spatially differentiated from AQP2, which selectively stained principal cells (24). This suggested that PIP4Kγ was also localized to intercalated cells in cortical and medullary collecting ducts (Fig. 5, J–L).

**Fig. 6.** Distribution of PIP4Kγ in kidney thick ascending limb (TAL) cells in situ. Endogenous PIP4Kγ was detected in thin sections of the outer medulla region of adult mouse kidney. Sagittal kidney section shows longitudinal TAL tubule sections (A, C), and horizontal kidney section shows TAL tubule cross-sections (B and D). Scale bars = 10 μm. Statistical analysis of PIP4Kγ distribution across TAL tubule cross-sections (such as in B), by fluorescence intensity, indicated peaks in the luminal region (E; n = 25).

**Discussion**

In this study, we investigated the expression, localization and associated biological activity of PIP4Kγ. We discovered a unique and restricted localization for this PIP4Kγ in kidney tissue and suggest that this has implications for the physiological function of this isoform. We have shown that the comparative mRNA expression levels of all three isoforms are significant in brain, where they have a different spatial distribution (1). PIP4Kα is the most active of the three isoforms in vitro but has a comparably low mRNA expression in most of the tissues that we tested and is the most abundant isoform in the spleen, probably reflecting its role in hematocytes (17, 26). The PIP4Kβ isoform mRNA is highly expressed in heart and skeletal muscle cells, consistent with initial observations (6) and providing a link to insulin resistance (22). We have confirmed the original observation that PIP4Kγ is highly expressed in kidney (19) and also observe high mRNA expression levels in brain, heart, and testis compared with other tissues. Our detection of endogenous PIP4Kγ in tissues is consistent with these transcription levels, also suggesting that PIP4Kγ is abundant in the ovary and may be processed in heart tissue. The distribution of the PIP4Ks across a range of different tissues, and the observed differences in subcellular localization and intrinsic activity (36, 45), would suggest that specialized functions could be attrib-
uted to each and hence to the role of PtdIns(5)P in these locations.

Peptide analysis of the sequence of PIP4Kγ predicts that this protein would not be targeted to the endoplasmic reticulum or plasma membrane due to the absence of a recognized signal peptide, which is consistent with our observation that PIP4Kγ, when overexpressed in cells, is partially colocalized to the structural component of the Golgi apparatus. Roles for PtdIns3P and PtdIns4P in membrane trafficking are established (10, 32), but the recent study (25) of a Golgi-localized phospholipid-inositol phosphatase with a substrate preference for PtdIns5P presents the intriguing possibility that this phosphoinositol is also present in cellular vesicles. PIP4Kγ also has a role in actin remodeling during endocytic transport (30), and PtdIns5P levels have been associated with this and with vesicle translocation to the plasma membrane (38). PIP4Kγ could be recruited to the external surface of a specific microsomal compartment to modify the PtdIns5P signal or to synthesize PtdIns(4,5)P2.

The restricted expression of PIP4Kγ within the kidney may be significant within the context of the specialized function of different regions of the nephron. Our experiments indicate that PIP4Kγ is present in cells constituting the TAL and is also restricted to intercalated cells in the collecting duct and appears...
to have a similar subcellular localization in these cell types as that recently observed for members of the Arf GTPase family (11), which have known roles in membrane trafficking (34). These regions are predominantly concerned with homeostasis by active ion transport and pH regulation and contain a large number of channels and transporters specific to these tasks (for reviews see Refs. 20, 27). Phosphoinositide regulation of both the trafficking to the plasma membrane and the activity of various collecting duct-localized channels has been reported (14, 44), but a specific role for PIP4Kγ in this process has yet to be established.

PIP4Kγ has been shown to have PtdIns5P 4-kinase activity when immunoprecipitated from mammalian cells (19), and the recombinant protein, purified from E. coli, is inactive, suggesting that a eukaryotic modification to PIP4Kγ is required for kinase activation. However, due to the ability of the PIP4Ks to dimerize (4, 16, 35) in vivo, we also cannot rule out the kinase activation. However, due to the ability of the PIP4Ks to dimerize (4, 16, 35) in vivo, we also cannot rule out the possibility that the observed activity associated to PIP4K and its heterodimers. This raises the possibility that PIP4Kγ is able to recruit PtdIns5P 4-kinase activity (in the form of PIP4Kα) to specific compartments, based on the potential ability to act as a scaffolding protein.

The roles of PtdIns5P, PtdIns(4,5)P2, and hence the PIP4Ks, in kidney function are unknown. We have shown that PIP4Kγ is the predominant PIP4K in the kidney, and we suggest that its role may be related to its specific localization in this organ.

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