Localization of phosphatidylinositol phosphate kinase II\(\gamma\) in kidney to a membrane trafficking compartment within specialized cells of the nephron

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Submitted 15 May 2008; accepted in final form 24 August 2008

Clarke JH, Emson PC, Irvine RF. Localization of phosphatidylinositol phosphate kinase II\(\gamma\) 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, PIP4K\(\gamma\). Quantitative RT-PCR and in situ hybridization revealed that compared with PIP4K\(\alpha\) and PIP4K\(\beta\), PIP4K\(\gamma\) is expressed at exceptionally high levels in the kidney, especially in the cortex and outer medulla. A specific antibody was raised to PIP4K\(\gamma\), 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, PIP4K\(\gamma\) 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 PIP4K\(\alpha\), bacterially expressed recombinant PIP4K\(\gamma\) was completely inactive but did have the ability to associate with active PIP4K\(\alpha\) in vitro. Overall our data suggest that PIP4K\(\gamma\) may have a function in the regulation of vesicular transport in specialized kidney epithelial cells.

POLYPHOSPHOINOSITIDES are quantitatively minor lipid components of cells and are increasingly being recognized as making diverse and important contributions to many aspects of cell physiology, for example, in ion channel regulation, membrane trafficking, cell proliferation, and cytoskeletal rearrangement (for reviews see Refs. 10, 18, 32, 40, 46). Phosphatidylinositol 5-phosphate (PtdIns5P) is the most recent polyphosphoinositide to be found, first identified as the primary substrate for the least-studied PIP4K isoform, PIP4K\(\gamma\). As a minor component of cell polyphosphoinositides, it is suggested that PtdIns5P either provides a specific lipid pool for a (minor) localized production of PtdIns(4,5)\(P_2\) or that it is a signaling molecule in its own right. Suggested functions include nuclear responses (9, 13), insulin signaling, and protein kinase regulation (5, 22, 31, 38) and phosphatase activation (39).

PtdIns5P can be synthesized from PtdIns by a PtdIns 5-kinase (37) or by dephosphorylation from PtdIns(3,5)\(P_2\) or PtdIns(4,5)\(P_2\) (28, 41–43). The main route of PtdIns5P metabolism is via PIP4Ks, which are represented in vertebrates by three characterized isoforms, \(\alpha\), \(\beta\), and \(\gamma\). PIP4K\(\alpha\) is found predominantly in the cytosol and can be recruited to the plasma membrane (15, 45). PIP4K\(\beta\) is localized to the nucleus (3, 7, 36), where there is evidence that it regulates PtdIns5P levels (21), probably in concert with type I PtdIns(4,5)\(P_2\) 4-phosphatase (47), although it has also been seen to associate with the cytosolic TNF receptor (6). As cellular PtdIns5P levels are modified in response to stress (21, 47), to bacterial infection (28), to receptor-mediated signaling (26, 45), and during cell cycle progression (8), the ability of the PIP4Ks to localize to specific compartments, or to shuttle between them, may be intrinsic to their function.

The third isoform, PIP4K\(\gamma\) (19), has not yet been associated with a cellular function, either related to production of PtdIns(4,5)\(P_2\) or to the attenuation of PtdIns5P. In the present study, we have extensively characterized the tissue distribution of all of the PIP4K isoforms and found an exceptionally high level of PIP4K\(\gamma\) expression in the kidney. We have further investigated the localization within this organ of PIP4K\(\gamma\), using a specific antibody to this isoform, and found that it is remarkably confined to specific cell populations, where it is localized to vesicular structures. Transfection experiments with PIP4K\(\gamma\) suggest that these may be derived from the Golgi apparatus. Our results suggest that PIP4K\(\gamma\) may be involved in vesicle trafficking in specific kidney cells and that this would imply a specialized function for this enzyme.

MATERIALS AND METHODS

**PIP4K cloning.** PIP5K2 genes were amplified from a whole human brain mRNA library (Clontech Laboratories, Mountain View, CA) using gene-specific primers (PIP5K2A forward: 5’-ATGGCGACCCCCCGCAACCTAGGTC-3’ and reverse: 5’-TACAGTGCAAGATGTTGGCAATAAAGTC-3’; PIP5K2B forward: 5’-ATGTCGTCACCCCTGACCCACCAAC-3’ and reverse: 5’-CTACGTCAAGATGTTGGCAATAAACAC-3’; and PIP5K2C forward: 5’-ATGGCGGCTCCTCCTGGTCCACCGC-3’ and reverse: 5’-TATAGGCAAGATGTTGGTAAATAAC-3’). PCR products were cloned, via incorporated HindIII and BamHI restriction sites, into appropriate plasmid vectors. Recombinant protein expressed from *Escherichia coli* harboring PIP5K2s in pET-32a (Novagen, Madison, WI) was purified using TALON metal affinity resin (Clontech) and cleaved with enterokinase (New England Biolabs, Ipswich, MA). Endotoxin-free plasmid from bacterial clones harboring PIP5K2s in pEGFP-C1 (Clontech) was prepared (DNA extraction kit; Qiagen, Huntsville, AL). All constructs were confirmed by sequencing.

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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. 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 ratioing curves for each primer set indicated a single product, and cDNA libraries were constructed by 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. 1. PIP5K2C is differentially expressed in adult mouse tissues. A: expression of PIP5K2A, PIP5K2B, and PIP5K2C determined by quantitative PCR using the comparative threshold cycle (Ct) method. Normalized expression is presented as relative fold increase above PIP5K2A in testis (n = 9). B: in situ hybridization using PIP5K2C-specific probes on thin sections of adult mouse brain (a), liver (b), and kidney (c; not to scale). Control incubations using an excess of cold probe indicate nonspecific binding (d-f). C: autoradiographic emulsion staining of mouse kidney cortex tubules, silver grains (arrows) indicating PIP5K2C mRNA expression (tu, tubule; gl, glomerulus). Scale bar = 20 μm.

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 lysis buffer (PBS with 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, and 100 μM/l Sigma P8340 protease inhibitor cocktail) and centrifuging at 10,000 g for 10 min at 4°C.

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Fig. 2. Specificity of anti-PIP4Kγ antibody. Detection of PIP4Kγ, but not the PIP4Kα or PIP4Kβ isoforms, was established in various procedures. A: equivalent amounts of purified recombinant protein of each isoform were visualized on SDS-PAGE (i) and detected by Western blotting (ii). B: HeLa cells overexpressing green fluorescent protein (GFP)-tagged PIP4Kγ or PIP4Kα were fixed and stained with anti-PIP4Kγ antibody. Scale bar = 10 μm. C: Surface plasmon resonance analysis using PIP4Kα, PIP4Kβ, and PIP4Kγ adsorbed to a nitrocellulose acid biosensor. Anti-PIP4Kγ antibody was passed over the biosensor at 0 s and exchanged for wash buffer at 45 s. Data were normalized to 6XHis tag control protein binding.
**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).
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 Histocontrol 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

**PIPK2 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 isoform-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 PIP5K2 probes confirmed that transcription 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).

Silver grain labeling of PIP5K2C mRNA in the kidney suggested that expression was confined to segments of the nephron within the cortical and medullary regions and was not present in the kidney vasculature (Fig. 1C).

**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 NTa sensor chip by 6xHis 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. Endogenous PIP4Kγ is differentially expressed in the adult mouse nephron. Actin (red) staining of cortical regions (A) identified luminal brush borders (arrow) in proximal tubules (d; distal tubule, p; proximal tubule), and localization of PIP4Kγ (green) in distal tubules (B). PIP4Kγ was not coincident with AQP1 (red) in the cortex (C) but was present in Tamm-Horsfall protein (THP; red) positive tubules in the outer medulla (D–F). PIP4Kγ was also seen in THP-negative tubules in this region (F; arrows). Double staining for PIP4Kγ (green) and AQP2 (red) in different kidney regions (G–L) revealed tubules positive for PIP4Kγ but negative for AQP2 in cortex and outer medulla (G, H; arrows). Isolated cells in AQP2-positive tubules also expressed PIP4Kγ (I–L; arrows). A, B, D–F, and K: images from horizontal sections. C, G–J, and L: images from sagittal sections. Scale bars = 20 μm.
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).

**PIP4Kγ has a distinct subcellular compartmentalization.**

Tubule sections of medullary TAL showed a distinct concentration of PIP4Kγ around the lumen (Fig. 6, A–D). Analysis of a pool of cross-sectioned tubules with a diameter of 23–25 μm gave an average fluorescence profile that indicated a sixfold increase of signal within 3 μm of the apical membrane of tubule cells, compared with the basolateral membrane (Fig. 6E). Higher magnification of these cells suggested that PIP4Kγ might be present in a vesicular compartment (Fig. 6, C and D). To further investigate this compartmentalization, PIP4Kγ expression was studied in kidney-derived cell lines, but because endogenous levels of enzyme were not visible by immunocytochemistry in these cells, overexpressed protein levels were required for colocalization experiments. Cells expressing green fluorescent protein-tagged PIP4Kγ were stained with antibodies against different cellular markers (Fig. 7). PIP4Kγ was not seen to associate with markers for defined vesicular compartments such as peroxisomes (catalase), endosomes (EEA1, mannose 6-phosphate receptor), or lysosomes (lglp110) or with markers for structural components such as tubulin or actin (data not shown). In our experiments, PIP4Kγ was also not seen to associate with endoplasmic reticulum markers [calreticulin (Fig. 7A) and BiP] in contrast with the suggested endoplasmic reticulum localization seen by Itoh et al. (19).

**Lipid kinase activity of PIP4Kγ, PIP4Kκ, overexpressed in E. coli cells and purified by metal-affinity resin chromatography, was used as a source of enzyme for in vitro kinase assays.** PIP4K activity was only observed using the PIP4Kκ isoform as the recombinant PIP4Kγ was inactive (Fig. 8A). Using immunoprecipitation experiments with recombinant protein, we were able to show that PIP4Kγ can associate with PIP4Kκ strongly enough to be selectively purified with a PIP4Kκ-specific antibody in vitro (Fig. 8B).

**Discussion**

In this study, we investigated the expression, localization and associated biological activity of PIP4Kγ. We discovered a unique and restricted localization for this PIP4K 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). PIP4Kκ 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 protein 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

Fig. 7. Overexpressed PIP4Kγ localizes to a specific cellular compartment. Transiently transfected cells, expressing GFP-tagged PIP4Kγ (green), were costained with antibodies to specific markers for different cellular compartments (red). No specific colocalization was observed with the endoplasmic reticulum marker calreticulin (A). Partial colocalization was observed with the cis-Golgi marker GM130 (B), and this juxtaposed labeling was maintained during Golgi dissociation by treatment with Brefeldin A (C). Scale bars = 10 μm.

Fig. 8. Activity and binding characteristics of recombinant PIP4Ks. PIP4Kα and PIP4Kγ recombinant protein was purified from a bacterial source. A: PIP4K assay using equivalent amounts of recombinant protein from each PIP4K, showing generation of PtdIns(4,5)P2. B: immunoprecipitation (IP) of recombinant PIP4K protein using PIP4Kα-specific antibody. IPs are from equivalent amounts of PIP4Kα, PIP4Kγ, or a mixture of the 2 isoforms, in buffer. Control IP contains no recombinant protein. Western blots (WB) were probed with either anti-PIP4Kα (i) or anti-PIP4Kγ (ii). Recombinant protein markers (rec) indicate PIP4K-positive bands (65 kDa).
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 PIP4K in this process has yet to be established.

PIP4K 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 PIP4K 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 possibility that the observed activity associated to PIP4K is attributable to PIP4K heterodimers. This raises the possibility that PIP4K 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 PIP4K is the predominant PIP4K in the kidney, and we suggest that its potential ability to act as a scaffolding protein.

ACKNOWLEDGMENTS

We thank Dr. Patrick Lynch for practical advice, Søren Nielsen for helpful suggestions, and Dr. Mihriban Tuna for technical expertise and analysis of biacore experiments.

GRANTS

This study was supported by a Programme Grant (WT063581) from the Wellcome Trust and the Biotechnology and Biological Sciences Research Council.

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