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Two novel phosphatidylinositol-4-phosphate 5-kinase type Iγ splice variants expressed in human cells display distinctive cellular targeting

Nicholas J. SCHILL*†[2] and Richard A. ANDERSON†[1,2]

*Program in Cellular and Molecular Biology, University of Wisconsin–Madison, 1525 Linden Drive, Madison, WI 53706, U.S.A., and †Department of Pharmacology, University of Wisconsin School of Medicine and Public Health, 1300 University Avenue, Madison, WI 53706, U.S.A.

The generation of various phosphoinositide messenger molecules at distinct locations within the cell is mediated via the specific targeting of different isoforms and splice variants of phosphoinositide kinases. The lipid messenger PtdIns(4,5)P_2 is generated by several of these enzymes when targeted to distinct cellular compartments. Several splice variants of the type Iγ isoform of PIPK (PtdIns4*P* 5-kinase), which generate PtdIns(4,5)P_2, have been identified, and each splice variant is thought to serve a unique functional role within cells. Here, we have identified two novel C-terminal splice variants of PIPKIγ in human cells consisting of 700 and 707 amino acids. These two splice variants are expressed in multiple tissue types and display PIPK activity *in vitro*. Interestingly, both of these novel splice variants display distinct subcellular targeting. With the addition of these two new splice isoforms, there are minimally five PIPKIγ splice variants that have been identified in mammals. Therefore, we propose the use of the HUGO (Human Genome Organization) nomenclature in the naming of the splice isoforms. PIPKIγ_i4 (700 amino acids) is present in the nucleus, a targeting pattern that has not been previously observed in any PIPKIγ splice variant. PIPKIγ_i5 (707 amino acids) is targeted to intracellular vesicle-like structures, where it co-localizes with markers of several types of endosomal compartments. As occurs with other PIPKIγ splice variants, the distinctive C-terminal sequences of PIPKIγ_i4 and PIPKIγ_i5 may facilitate association with unique protein targeting factors, thereby localizing the kinases to their appropriate cellular subdomains for the site-specific generation of PtdIns(4,5)P_2.

Key words: cadherin, endosomal trafficking, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], phosphatidylinositol-4-phosphate 5-kinase type Iγ (PIPKIγ), splice variant.

INTRODUCTION

The signalling pathways that utilize members of the PtdInsP_n family of lipids to transduce messages from one functional complex to another are as distinct as they are complex. PtdIns(4,5)P_2 is positioned at the crossroads of many of these signalling cascades, as it may be metabolized by phospholipase C to generate Ins(1,4,5)P_3 and 1,2-diacylglycerol, further phosphorylated at the 3-position of the inositol ring by phosphatidylinositol 3-kinase to generate PtdIns(3,4,5)P_3 [1] or used directly as a messenger molecule by binding to proteins containing PH (pleckstrin homology), PX (phox homology), FERM (band 4.1, ezrin, radixin, moesin) or comparable domains [1,2]. However, the method of PtdIns(4,5)P_2 generation utilized in these pathways results in its availability being far from ubiquitous. Rather, PtdIns(4,5)P_2 seems to be synthesized in a highly site-specific manner at distinct subcellular locales where it is directly utilized as a signalling molecule, thereby modulating the activity, conformation, assembly or disassembly of proteins at these sites [1,3,4]. The spatial and temporal metabolism of PtdIns(4,5)P_2 has emerged as a crucial regulator of multiple cellular processes, including actin reorganization [1,5,6], focal-adhesion dynamics [1,7–9], endocytosis and exocytosis [4,10–19], nuclear signalling pathways [20,21] and gene expression [22].

Most cellular PtdIns(4,5)P_2 generation is fulfilled by the α, β and γ isoforms of the type I PIPKs (PtdIns4*P* 5-kinases). Although retaining high homology within the lipid kinase domain, each isoform exhibits a distinct subcellular localization pattern and functional specificity [23]. It is believed that the N- and C-terminal sequence divergence of each PIPKI isoform contributes to this diversity in targeting and function. PIPKIα participates in both nuclear and cytoplasmic PtdIns(4,5)P_2 generation, where it has been implicated in the regulation of RNA polyadenlyation machinery and growth-factor-induced reorganization of the cytoskeletal superstructure [3,24]. The cellular roles of PIPKIβ are less defined, but a function for PIPKIβ in actin assembly and endocytosis has been suggested [1,3,23].

The newest member of the type I family, PIPKIγ (type Iγ PIPK), is a workhorse for site-specific PtdIns(4,5)P_2 generation in a plethora of cytoplasmic processes. PIPKIγ is a fundamental regulator of the assembly and disassembly of sites of cell-matrix [1,8,9] and cell–cell interaction [17,25] termed focal adhesions and adherens junctions respectively. Importantly, the human PIPKIγ is known to encode at least two alternative splice variants, PIPKIγ640 and PIPKIγ668 [26]. These splice variants differ by the inclusion of exon 17, which encodes a 28-amino-acid C-terminal extension specific to PIPKIγ668 [26]. This 28-amino-acid extension has been demonstrated to

Abbreviations used: AP, adaptor protein; DTT, dithiothreitol; EEA1, early endosome antigen 1; EGF, epidermal growth factor; HA, haemagglutinin; HGVS, Human Genome Variation Society; His_6, hexahistidine; HUGO, Human Genome Organization; LAMP1, lysosomal-associated membrane protein 1; PIPKIγ, type Iγ isoform of PtdIns4*P* 5-kinase; RACE, rapid amplification of cDNA ends; SC-35, splicing factor, arginine/serine-rich 2; siRNA, small interfering RNA; TfnR, transferrin receptor; UTR, untranslated region.

[1] To whom correspondence should be addressed (email raanders@wisc.edu).

[2] The authors declare they have no competing financial interests.

The mRNA and peptide sequences of PIPKIγ_v4 and PIPKIγ_v5, including the sequenced region of each splice variant's 3′-UTR (untranslated region) sequence, will appear in the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession numbers FJ965536 and FJ965537 respectively.

Table 1 Summary of proposed revisions to PIP5K1C splice variant nomenclature

Human nomenclature	Mouse nomenclature	Corresponding exons (human)	HUGO mRNA nomenclature	HGVS‡ protein nomenclature
PIPKIγ640, PIPKIγ87, PIPkinIγb	PIPKIγ635, PIPKIγ87, PIPkinIγb	1–16a, 18	PIPKIγ_v1	PIPKIγ_i1
PIPKIγ668, PIPKIγ90, PIPkinIγa	PIPKIγ661/662, PIPKIγ90, PIPkinIγa	1–16a, 17, 18	PIPKIγ_v2	PIPKIγ_i2
*	PIPKIγ93, PIPkinIγc	1–16a, 16c†, 17, 18	PIPKIγ_v3	PIPKIγ_i3
PIPKIγ700	*	1–16a, 16b, 16c	PIPKIγ_v4	PIPKIγ_i4
PIPKIγ707	*	1–16a, 16c	PIPKIγ_v5	PIPKIγ_i5

* Splice variant not defined.
† Partial exon.
‡HGVS, Human Genome Variation Society.

confer specific subcellular targeting and function on PIPKIγ668, where it consequently regulates focal adhesion dynamics, EGF (epidermal growth factor)-stimulated directional migration, basolateral targeting of E-cadherin, and endocytosis of the TfnR (transferrin receptor) [8,9,13,14,16–18,27]. Aside from mediating specific protein–protein interactions, this extension also affords several methods of regulating PIPKIγ668 activity, as it is tyrosine- and serine-phosphorylated by Src [28] and cyclin-dependent kinase [29] respectively. In addition, PIPKIγ can be directly phosphorylated by EGFR (EGF receptor) [27]. These phosphorylation events are in turn antagonized by specific phosphatases [29,30]. In the absence of a specific C-terminal extension, PIPKIγ640 also plays a specific cellular role, as it is the major contributor of the PtdIns(4,5)P_2 utilized in G-protein-coupled-receptor-mediated Ins(1,4,5)P_3 generation [31].

A third PIPKIγ splice variant consisting of 688 amino acids was described in mouse and rat neuronal tissue by Giudici et al. [32, 33]. In addition to containing the 28-amino-acid C-terminal extension of PIPKIγ668, this novel splice variant also contained a unique 26-amino-acid sequence inserted prior to the extension first defined in PIPKIγ668 and appears to be neuronal-specific [32]. Although Giudici et al. uncovered the presence of a sequence homologous with this insertion in the human genome, they did not confirm its existence in human tissues [32]. Here, we provide evidence that two unique PIPKIγ C-terminal splice variants do indeed exist in human cells. The two novel splice variants consist of 700 and 707 amino acids, possess PIPK activity, and are expressed in a multitude of cell types and tissues. Importantly, these splice variants display subcellular localization patterns that are unique from PIPKIγ640 or PIPKIγ668, suggesting that each splice variant likely fills a distinct functional role within cells. Since the discovery of these two new splice variants further complicates the PIPKIγ nomenclature, all further instances of PIP5K1C gene products mentioned in the present paper have been assigned nomenclature based on the guidelines established by the Genetic Nomenclature Committee of HUGO (Human Genome Organization) (Table 1). Following this convention, the unique PIPKIγ mRNAs described herein are referred to as PIPKIγ_v4 and PIPKIγ_v5, whereas their protein products are termed PIPKIγ_i4 and PIPKIγ_i5.

EXPERIMENTAL

Cloning of PIPKIγ splice variants

mRNA from mammary epithelial cell line MCF10A was isolated using the Micro-FastTrack™ 2.0 mRNA Isolation Kit (Invitrogen). 3′-RACE (3′ rapid amplification of cDNA ends) was performed with the GeneRacer system (Invitrogen) using primers specific to a portion of PIPKIγ exon 16 (5′-GCCTCTGCTGCTG-TTGAAGTAGAAA-3′) and the supplied 3′ adaptor primer according to the manufacturer's instructions. PCR products were run on agarose gels, and individual DNA bands were excised, purified and ligated into the pGEM-T Easy Vector (Promega). Full-length PIPKIγ_v4 and PIPKIγ_v5 were amplified from MCF10A cDNA using the 5′ primer (5′-ATGGAGCTGGAGGT-ACCGGA-3′) and 3′ primer (5′-TTACCCAAAGCCCTTCTGG-AAA-3′).

Expression constructs

Human PIPKIγ splice variants were amplified via PCR for insertion into the pCMV-HA vector (Clontech). Upon insertion into expression vectors, the 3′-UTR (untranslated region) of each PIPKIγ splice variant was removed. For expression in *Escherichia coli*, PIPKI coding sequences were subcloned into pET28 (Novagen). PIPKIγ point mutations were generated using PCR primer overlap extension with primers containing the desired mutations.

Antibodies

Polyclonal antibodies towards the PIPKIγ splice variants were created as previously described [8]. Anti-HA (haemagglutinin) monoclonal antibody HA.11 was obtained from Covance. Rabbit polyclonal anti-HA and anti-(lamin β1) were purchased from Santa Cruz Biotechnology. Anti-β-tubulin, anti-N-cadherin, anti-E-cadherin, anti-α-adaptin, anti-TfnR, anti-EEA1 (early endosome antigen 1) and anti-SC-35 (splicing factor, arginine/serine-rich 2) antibodies were purchased from BD Biosciences, and anti-actin antibody was obtained from MP Biomedicals. Anti-nucleolin and anti-CD63 antibodies were obtained from Millipore. Anti-LAMP1 (lysosomal-associated membrane protein 1) monoclonal antibody was from Abcam, and anti-talin was from Sigma–Aldrich. Alexa 350-, Alexa 488-, Alexa 555-, Alexa 647- and Pacific Blue-conjugated secondary antibodies were purchased from Molecular Probes. Secondary horseradish-peroxidase-conjugated antibodies for Western blotting were obtained from Jackson Immunoresearch Laboratories.

Purification of recombinant protein

PIPKI coding regions subcloned into the pET28 vector were transformed into *E. coli* Rosetta™ 2(DE3) competent cells from Novagen. Overnight starter cultures were expanded in 0.5 litre cultures in Luria Broth to an attenuance (D_{600}) of $\leqslant 0.6$ and were then induced with 1 mM isopropyl β-D-thiogalactoside for 3 h at 37 °C with agitation. His_6 (hexahistidine)-tagged fusion proteins were then purified from *E. coli* lysates with His-Bind™ resin (Novagen) according to the manufacturer's instructions.

PIPKI lipid kinase activity assay

The lipid kinase activity of PIPKI was assayed against 25 μM PtdIns4*P* micelles or Folch Brain Extract as previously described [17,34].

Subcellular fractionation

HeLa cells were plated at 1.3×10^6/10-cm-diameter plate and grown overnight. Cells were lifted with a non-enzymatic cell dissociation buffer (Sigma–Aldrich), collected by centrifugation (1000 ***g*** for 5 min at 4 °C), and washed twice in cold PBS. One half of the cell pellet was lysed directly in 2 × loading buffer [1 × loading buffer is 10 mM Tris, 5 % (v/v) glycerol, 1 % (w/v) SDS and 1 % (v/v) 2-mercaptoethanol, pH 6.8] as a whole-cell lysate control. The remainder of the cells were resuspended in 300 μl Buffer A [10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT (dithiothreitol)], vortex-mixed briefly and incubated on ice for 30 min. After the addition of 0.2 % (v/v) Nonidet P40, cell lysis was verified by Trypan Blue exclusion and cells were then centrifuged at 300 ***g*** for 15 min at 4 °C. The cytoplasmic fraction was removed and the pellet containing nuclei was lysed for 30 min at 4 °C in 100 μl of buffer C [20 mM Hepes, 25 % (v/v) glycerol, 450 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 0.5 mM DTT]. The nuclear lysate was then cleared by centrifugation at 16000 ***g*** for 15 min at 4 °C. The cytoplasmic, nuclear and whole-cell lysates were then quantified with Bradford's reagent (25 ml of phosphoric acid, 12.5 ml of ethanol and 25 mg of Coomassie Brilliant Blue; Bio-Rad Laboratories) before Western blotting.

Cell culture and transfection

HeLa cells plated at 5.5×10^5 cells/60-mm-diameter plate in 10 % (v/v) fetal bovine serum + Dulbecco's modified Eagle's medium were incubated overnight before transfection with 8 μg of total DNA and 9 μl of Lipofectamine™ 2000 (Invitrogen). Cells were harvested for analysis at about 18 h post-transfection. For siRNA (small interfering RNA) knockdown of PIPKIγ, HeLa cells were transfected with Lipofectamine™ 2000 and either a non-targeting or pan-PIPKIγ siRNA duplex (GCCACCUU-CUUUCGAAGAA) and harvested at either 48 or 72 h post-transfection.

Immunofluorescence and confocal microscopy

MCF10A and HeLa cells were grown on glass coverslips placed inside six-well plates 24 h prior to transfection. Coverslips containing cells were washed in PBS at 37 °C, and then fixed with chilled methanol or 4 % (w/v) paraformaldehyde, followed by permeabilization with 0.5 % (v/v) Triton X-100 in PBS. The cells were then blocked for 1 h at room temperature (25 °C) in 3 % (w/v) BSA (Jackson Immunoresearch Laboratories) in PBS. Primary-antibody incubation was performed at 37 °C for 2 h or 4 °C for 16 h, whereas incubation with fluorophore-conjugated secondary antibodies was performed at 37 °C for 30 min. Cells were washed in between incubation steps with 0.1 % (v/v) Triton X-100 in PBS. Indirect immunofluorescence microscopy was performed on a Nikon Eclipse TE2000U instrument equipped with a Photometrics CoolSNAP CCD (charged coupled device) camera. Images were captured and further processed using MetaMorph (Molecular Devices) or AutoQuant (Media Cybernetics) cellular imaging software. Images were exported to Photoshop CS2 (Adobe) for final processing and assembly.

Tissue and cell-line immunoblotting

Mouse tissue was excised from a CO_2-asphyxiated C57BL/6 female mouse and flash-frozen in liquid nitrogen. Proteins were extracted from tissues by grinding with a tissue homogenizer into a buffer consisting of 20 mM Tris/HCl, pH 7.6, 1 % (v/v) Triton X-100, 137 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM DTT and protease inhibitors. Tissue homogenates were rotated for 2 h at 4 °C to complete lysis. Lysates were cleared of tissue debris by centrifugation at 15000 ***g*** for 20 min at 4 °C. Protein concentrations were calculated using the BCA (bicinchoninic acid) protein quantification assay (Bio-Rad Laboratories) according to the manufacturer's instructions. For Western blotting of mouse tissue lysates, 20 μg of each tissue lysate was subjected to SDS/7.5%-(w/v)-PAGE. Cell line lysates were generated by scraping a 100-mm-diameter plate of each cell type into 1 ml of RIPA buffer [50 mM Tris/HCl, 150 mM NaCl, 1.0 % (v/v) Nonidet P40, 0.5 % (w/v) sodium deoxycholate, 1.0 mM EDTA and 0.1 % (w/v) SDS, pH 7.4), supplemented with protease inhibitors, followed by incubation for 1 h at 4 °C. Lysates were cleared, quantified and 15 μg of each cell line lysate was analysed by Western blot as described above. A PageRuler Prestained Protein Ladder (Fermentas) or a Benchmark Prestained Protein Ladder (Invitrogen) was used as the molecular-mass standard for Western blotting.

RESULTS

Identification of PIPKIγ mRNAs in MCF10A human epithelial cells

The human PIPKIγ mRNA that encodes PIPKIγ_i1 or PIPKIγ_i2 contains 17 or 18 exons respectively, with exon 18 consisting of the translational stop codon for both proteins as well as a long 3′-UTR region (~3 kb) (Figure 1B). When Western-blotting cell lysates from MCF10A cells were incubated with a pan-PIPKIγ antibody, an immunoreactive band was observed at a slightly greater molecular size than that attributable to PIPKIγ_i2. This provided evidence that there may be other PIPKIγ splice variants that have yet to be identified. In order to identify the putative novel PIPKIγ species in MCF10A epithelial cells, mRNA was extracted from cells and subjected to reverse transcription–PCR. Initially, any unidentified splice variant was expected to share exon 18 as a 3′-UTR. In subsequent PCRs, primers were used that targeted internal exons, along with a primer targeting the 3′-UTR, to amplify PIPKIγ mRNAs. These amplicons were subcloned into a T/A cloning vector and ~50 clones per region were screened via restriction-enzyme digest and DNA sequencing for variations in exon structure. Although some variation of exon structure was observed (Figure 1B and results not shown), each alternative splicing event that was detected by this method resulted in a transcript that was shorter than PIPKIγ_v2. Because the immunoreactive band we had observed in MCF10A lysates was of greater molecular size, these smaller splice variants were not characterized further.

Since the previously described splice variants of PIPKIγ display alternative splicing of the 3′ end of their mRNA to encode a unique C-terminal tail region, we hypothesized that the unknown splice variant may contain a longer unique C-terminus distinct from that of PIPKIγ_i2. The PIPKIγ gene is quite long, being over 70 kb in length (chromosome 19; 3 581 182–3 651 445); however, the mRNA which encodes PIPKIγ_v2 is only ~5 kb. Although this is not uncommon, it leaves the possibility that other splicing events can incorporate sequences that are assumed to be intronic. Interestingly, introns 16–17 of the human PIPKIγ sequence, which in PIPKIγ pre-mRNA lies between the majority

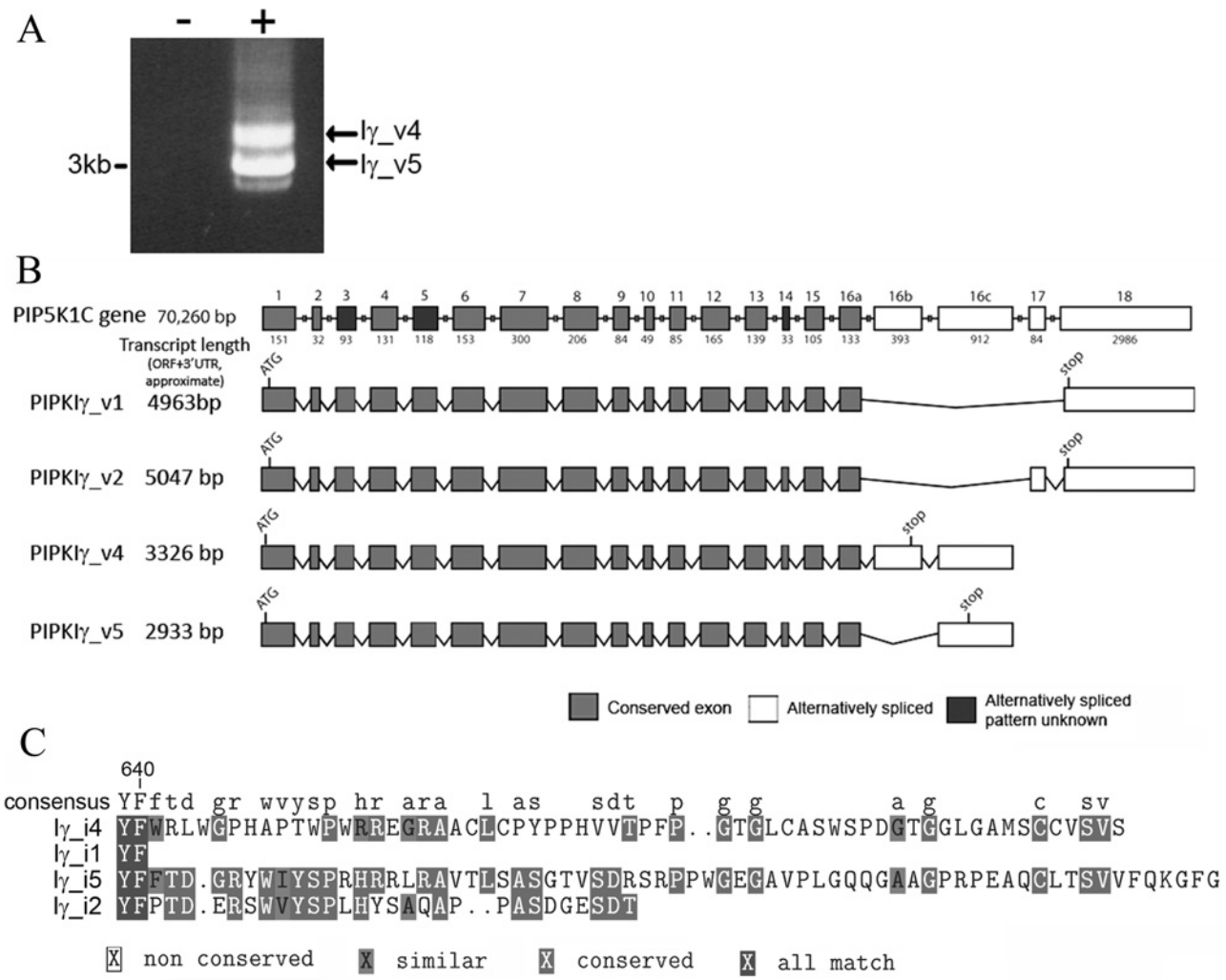

Figure 1 The human PIPKIγ gene encodes at least four C-terminal splice variants

(**A**) The full-length PIPKIγ_v4 and PIPKIγ_v5 transcripts were amplified from MCF10A-epithelial-cell cDNA using a primer targeted to the conserved 5′-end of the PIPKIγ transcript and a second primer matching the putative 3′ alternatively spliced exon identified via 3′-RACE. The less prominent band running immediately below PIPKIγ_v5 was also sequenced and found to be non-specific. (**B**) A revised exon map of the human PIPKIγ gene illustrates the four major C-terminal splice variants that have been identified. The overall lengths of PIPKIγ transcripts listed are approximate estimates based on the defined open reading frames plus the 3′ untranslated region. Two novel exons, which have been termed exon 16b and 16c, make up the alternatively spliced C-termini of the PIPKIγ_v4 and PIPKIγ_v5 transcripts. Interestingly, the PIPKIγ_v4 transcript is longer than that of PIPKIγ_v5, but PIPKIγ_v4 mRNA encodes a shorter protein, owing to a stop codon in exon 16b. PIPKIγ transcripts were identified in our initial experiments that lacked portions of exons 3 or 5, or all of exon 14, but these splice variants were not further characterized. (**C**) CLUSTALW alignment of the C-terminus of PIPKIγ_i5 (W^{647}IYSPRH^{653}) is similar to the C-terminus of PIPKIγ_i2 (W^{647}VYSPLH^{653}). The presence of a full stop (period) indicates the lack of a corresponding amino acid residue at the indicated position.

of the PIPKIγ coding sequence and the 84 bp which encode the unique C-terminus of PIPKIγ_i2, is also quite large ($\sim$5.3 kb). If another PIPKIγ C-terminal splice variant were encoded by the PIPKIγ gene, it is likely that this intron would contain the source sequence for the unique extension. With this in mind, we performed 3′-RACE on MCF10A cDNA using a forward primer directed towards exon 16. The amplicons were then analysed by agarose-gel electrophoresis, individual bands were extracted for ligation into a T/A vector, and the ligated inserts were sequenced. The sequence information obtained from 3′-RACE indicated the presence of a previously unidentified exon mapping to intron 16–17 (3 589 519–3 589 881) of the PIPKIγ gene. PCR was then performed to amplify the full-length splice variant using a primer directed towards the known start codon of PIPKIγ and the predicted stop codon and its 5′ flanking sequence. This resulted in the identification of two splice variants containing the novel exon, which we have named according to HUGO guidelines as PIPKIγ_v4 and PIPKIγ_v5 (Table 1; Figure 1A). PIPKIγ_v4 was predicted to contain a reading frame of 2103 nucleotides, whereas the reading frame of PIPKIγ_v5 was expected to contain 2124 nucleotides (Figure 1B). Interestingly, both splice variants utilize the novel exon 16c. In the case of PIPKIγ_v5, this exon encodes a unique 67-amino-acid C-terminus. By contrast, the unique C-terminus of PIPKIγ_v4 is encoded by a second novel exon, 16b, whereas exon 16c makes up a portion of its 3′-UTR. Upon identification of the splice variant mRNAs in MCF10A cells, we then sought to determine whether these messages were evolutionarily conserved.

The coding regions of PIPKIγ_v4 and PIPKIγ_v5 are evolutionarily conserved

The PIPKIγ_v2 splice variant has been conserved through evolution, as the DNA sequence encoding its distinctive

Figure 2 PIPKIγ_i4 and PIPKIγ_i5 are evolutionarily conserved

A CLUSTALW alignment of putative orthologues of PIPKIγ_i4 and PIPKIγ_i5 was created using sequence information in the ENSEMBL database. The C-terminal amino acid sequences of PIPKIγ_i4 and PIPKIγ_i5 were used to search the ENSEMBL sequence database for potential matches in other species. Key to species not already identified: *C. familiaris*, *Canis familiaris* (dog); *G. gallus*, *Gallus gallus* (chicken); *H. sapiens*, *Homo sapiens* (man); *M. musculus*, *Mus musculus*, house mouse; *R. norvegicus*, *Rattus norvegicus*, Norway rat.

28-amino-acid C-terminal extension is present in most vertebrates, with a high level of conservation within mammals [1,26,32]. This conservation underlines the importance of this splice variant in multiple cellular functions. Using the unique C-terminal amino acid sequences of human PIPKIγ_i4 and PIPKIγ_i5, a thorough BLASTN search was performed of both the complete and incomplete genome assemblies available on ENSEMBL. Putative orthologues of PIPKIγ_i5 were identified in multiple vertebrate species, indicating that the function of PIPKIγ_i5 is most likely conserved (Figure 2). Interestingly, PIPKIγ_i4 orthologues were identified in *Macaca mulatta* (rhesus macaque monkey) and *Pan troglodytes* (chimpanzee), but our search of translated sequence databases did not yield PIPKIγ_i4 orthologues in the other species where sequence information was available. In contrast with this finding, the presence of the PIPKIγ_i4 protein was observed in canine, mouse and rat cell lines via Western blot (see below; Figure 3D). Since a full assembly of the mouse genome has recently been completed [35], we searched the mouse PIPKIγ gene for the PIPKIγ_v4 coding sequence. We did observe a nucleotide sequence in introns 16–17 of the mouse PIPKIγ gene that partially matches the human PIPKIγ_v4 coding sequence. However, we have not been able to translate this sequence into a putative version of the mouse PIPKIγ_i4 protein, owing to apparent gaps in the genomic sequence. Our unsuccessful attempts to identify the mouse PIPKIγ_i4 orthologue reinforce the notion that the mouse genome assembly may require localized review and revision [35].

PIPKIγ_v4 and PIPKIγ_v5 are expressed as human cellular proteins

To verify that these unique messages were expressed in human cells as proteins, polyclonal antibodies were made to specifically detect each of the novel splice variants. Recombinant peptides corresponding to each C-terminus of the novel splice variants (Figure 1C) were used to immunize rabbits, and antibodies were affinity-purified from bulk sera over an antigen column. These antibodies were analysed by Western blotting HeLa cell lysates transfected with each of the PIPKIγ splice variant constructs (Figure 3A). The antibodies specifically detected their target splice variant, with no evident cross-reactivity with other PIPKIγ splice variants. The specificity of these antibodies was further confirmed by siRNA knockdown of total cellular PIPKIγ and Western blotting (Figure 3B). The results of these Western blots confirm that the band detected by each splice variant antibody is the intended target protein.

With functional and specific polyclonal antibodies towards PIPKIγ_i4 and PIPKIγ_i5, we were then able to Western-blot epithelial and fibroblast cell line lysates from human, mouse, rat and canine cells. As shown in Figure 3(C), each cell line tested via Western blot expressed both the PIPKIγ_i4 and PIPKIγ_i5 splice variants, with an apparent molecular mass of approx. 100 kDa. Since these splice variants are detectable in human, canine, mouse and rat cell lines, this confirms that the expression of PIPKIγ_i4 and PIPKIγ_i5 is evolutionarily conserved within mammals. Interestingly, PIPKIγ_i4 appears as a tight doublet in some cell lines. PIPKIγ_i2 also appears as several discrete bands via Western blotting, and this may be due to the phosphorylation events that occur on its C-terminus [27–29]. Therefore, PIPKIγ_i4 may undergo phosphorylation or other post-translational modifications that affects its apparent molecular mass.

To determine the tissue distribution of these splice variants, 20 μg of C57BL/6 mouse tissue lysates was Western-blotted with each of the anti-(splice-variant) antibodies as well as the anti-pan-PIPKIγ antibody. As shown in Figure 3(D), each of the PIPKIγ splice variants displays a distinct expression pattern in mouse tissue. Consistent with previous reports, PIPKIγ_i2 is most strongly expressed in brain tissue [26,32,36], but is also expressed in greater quantities in the heart and lungs. PIPKIγ_i4 is strongly expressed in the pancreas and liver, but is also present in lesser quantities in the brain, heart, lung and kidney. PIPKIγ_i5 is present in large amounts in the heart and large intestine, but is also present in the lung, pancreas and thyroid, and, to a lesser extent, brain, stomach and kidney. Interestingly, the apparent molecular mass of the PIPKIγ_i5 mouse orthologue is approx. 5–10 kDa lower than that of the human form. This size shift could be the result of a second alternative splicing event that has removed a portion of the mouse PIPKIγ_i5 mRNA. In mouse brain tissue lysates, two bands appear that are immunoreactive to the anti-PIPKIγ_i5 polyclonal antibody, but neither corresponds to the major species of PIPKIγ_i5 observed in other mouse tissues (Figure 3D). It is possible that the lower band is the

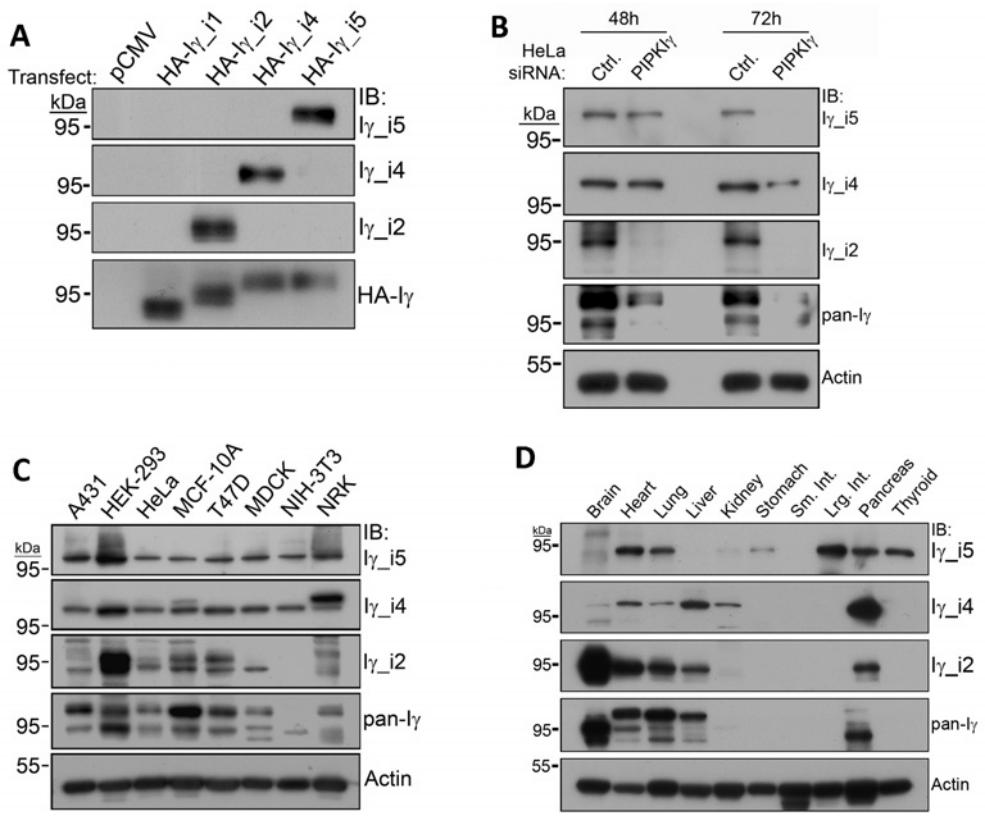

Figure 3 PIPKIγ_v4 and PIPKIγ_v5 transcripts are expressed as proteins

(**A**) The specificity of purified polyclonal antibodies toward the unique C-terminal splice variants of PIPKIγ was tested via Western blot. HA-tagged PIPKIγ constructs were transfected into HeLa cells, and Western blots of whole-cell lysates were probed with splice variant-specific anti-PIPKIγ polyclonal antibodies. (**B**) To verify that the anti-PIPKIγ polyclonal antibodies can recognize endogenous protein and are specific towards their intended splice variant, total PIPKIγ was knocked down in HeLa cells using siRNA for 48 or 72 h. Cell lysates were Western-blotted using each of the anti-PIPKIγ polyclonal antibodies, and anti-actin antibody was used as a loading control. (**C**) Expression of PIPKIγ splice variants in several mammalian cell lines was determined by Western-blotting cell line lysates with anti-PIPKIγ antibodies. Anti-actin antibody was used as a loading control. (**D**) Fresh tissue was extracted from a C57BL/6 mouse, lysed, and total soluble protein was quantified. A 20 μg portion of lysate was subjected to Western blotting with the anti-PIPKIγ polyclonal antibodies to determine the tissue distribution of splice variants. Abbreviations: Ctrl., control; HA-Iγ, HA-tagged PIPKIγ; IB:, immunoblot; Iγ_i5 (etc.), PIPKIγ_i5; Lrg. Int., lareg intestine; pan-Iγ, pan-PIPKIγ; Transfect., transfection. i_5 etc. designates the protein, whereas v_5 designates the mRNA.

brain-specific PIPKIγ splice variant (PIPKIγ_i3), identified by Giudici et al. [32,33], which shares partial sequence homology with the human PIPKIγ_i5 variant described here [32,33]. The upper band is approx. 100 kDa, and, in view of its molecular mass, is potentially the full mouse orthologue of PIPKIγ_i5. Importantly, the expression profiles of each of the PIPKIγ splice variants are indicative of a specialized role for each of these proteins in a particular tissue.

PIPKIγ_i4 and PIPKIγ_i5 possess lipid kinase activity towards PtdIns4*P*

The type-I PIPKs synthesize PtdIns(4,5)P_2 using the cellular pool of PtdIns4*P* as substrate [1,3,23]. This reaction can be reproduced *in vitro* using recombinant or immunoprecipitated PIPKI enzyme and PtdIns4*P*-containing micelles or liposomes [34]. The kinase domains of PIPKIγ_i4 and PIPKIγ_i5 are identical in sequence composition with that of the previously characterized PIPKIγ splice variants. However, we wanted to explore whether the additional amino acids present at the C-terminus of PIPKIγ_i4 and PIPKIγ_i5 affected the activity of the enzymes towards PtdIns4*P*. To test this, an *in vitro* kinase activity assay was done using His_6-tagged recombinant PIPKIγ splice variants or PIPKIα (positive control), PtdIns4*P* micelles, and [γ-^{32}P]ATP. As shown

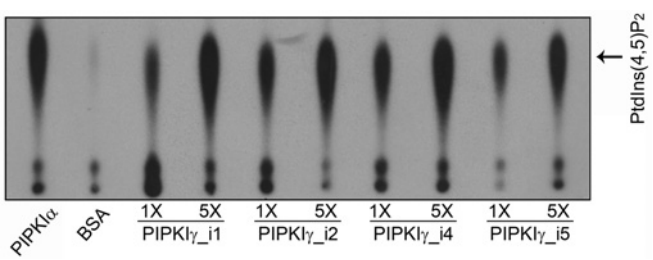

Figure 4 PIPKIγ splice variants display PtdIns(4)P 5-kinase activity

Either 1 μg (1X) or 5 μg (5X) of His_6-tagged recombinant PIPKIα or PIPKIγ splice variants were added to PtdIns4*P* micelles and [γ-^{32}P]ATP for 5 min at room temperature to test *in vitro* PIPK activity, and lipids extracted from the reaction mixtures were separated by TLC. Purified BSA was incubated under the same conditions as a control.

in Figure 4, no apparent differences in the *in vitro* kinase activity towards PtdIns4*P* exist between the four enzymes. This result was confirmed by substituting Folch Brain Extract as substrate in these assays (results not shown) [9]. These results suggested that, at least *in vitro*, the C-terminal extensions of PIPKIγ splice variants do not directly have an impact on the kinase activity of the enzymes.

PIPKIγ_i4 is a nuclear-targeted splice variant

The 28-amino-acid C-terminal extension that is present on PIPKIγ_i2 is directly responsible for the targeting of this splice variant to focal adhesions in mesenchymal cells and to cell–cell contacts in polarized epithelial cells. This occurs via the association of this unique C-terminus with talin or E-cadherin and AP (adaptor protein) complexes [8,9,17,27,28]. Therefore, it is likely that the unique C-terminal PIPKIγ variants described here also facilitate protein–protein interactions which target each splice variant to a discrete location within the cell.

To explore the subcellular targeting of these new splice variants, we stained cells with polyclonal antibodies to pan-PIPKIγ, PIPKIγ_i4 and PIPKIγ_i5. Unfortunately, the PIPKIγ_i5 polyclonal antibody resulted in very poor staining in all cell lines tested. When MCF10A cells were stained with a anti-pan-PIPKIγ polyclonal antibody, we observed PIPKIγ localization largely at cell–cell contacts where it co-localizes with the adhesion molecule E-cadherin (Figure 5A). A portion of this staining is likely to be indicative of PIPKIγ_i2, the functional contribution of which to E-cadherin biology is well established [4,17]. However, the anti-pan-PIPKIγ polyclonal antibody also shows some reactivity towards punctuate nuclear structures (Figure 5A). Interestingly, our anti-PIPKIγ_i4 polyclonal antibody indicated a localization of this variant to subnuclear structures, and, to a lesser extent, the cytoplasm, in MCF10A (Figure 5A), HeLa (Figure 5B), and NRK (normal rat kidney) cells (results not shown). Another type-I PIPK, namely PIPKIα, targets to subnuclear sites known as 'nuclear speckles'. where it associates with splicing factors to regulate mRNA processing [22,37]. PIPKIγ_i4 staining co-localized with SC-35, a marker of nuclear speckles, but not nucleolin (Figures 5A and 5B). This result is striking, as PIPKIγ has not been previously identified as a nuclear PIPKI. To further assess the targeting of PIPKIγ_i4, HeLa cells were fractionated into their nuclear and cytosolic components, and these lysates were subjected to Western blotting with anti-PIPKIγ polyclonal antibodies. Blotting with the anti-pan-PIPKIγ antibody indicated that PIPKIγ is mainly located in the cytosolic fraction, but a discernable amount of PIPKIγ was present in the nuclear fraction as well (Figure 5C). In agreement with the results from blotting with anti-pan-PIPKIγ antibody, PIPKIγ_i4 was identified in both the cytoplasmic and nuclear fractions (Figure 5C). Interestingly, PIPKIγ_i4 appeared as a doublet in the nuclear, but not cytoplasmic, fraction. This probably indicates post-translational modification of PIPKIγ_i4, which is consistent with our observations of this splice variant in Figure 3(C). Taken together, these results support the presence of PIPKIγ_i4 in the nucleus and suggest that PIPKIγ_i4 could be functionally active in cytoplasmic as well as nuclear processes.

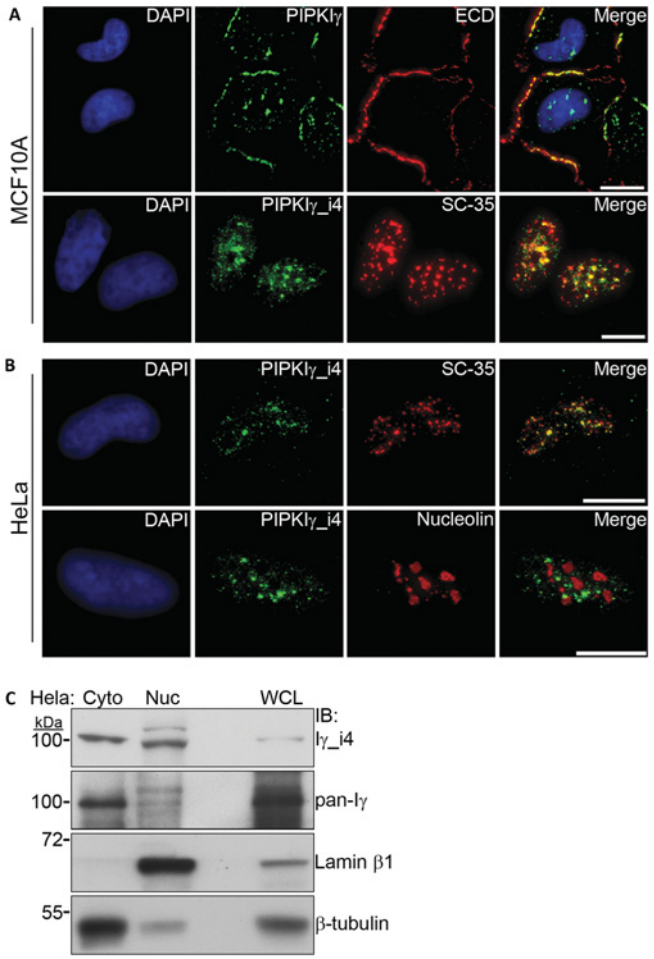

Figure 5 PIPKIγ_i4 is a nuclear-targeted phosphoinositide kinase

(**A**) MCF10A or (**B**) HeLa cells were grown on coverslips, fixed in methanol, and probed with antibodies for pan-PIPKIγ (green), PIPKIγ_i4 (Iγ_i4; green), E-cadherin (ECD) (red), and SC-35 (red). Co-localization of red and green immunofluorescence channels is indicated in yellow. DAPI (4′,6-diamidino-2-phenylindole) staining was omitted from merged images (Merge) of labelled nuclei. The scale bar represents 10 μm. (**C**) The cytosolic (Cyto) and nuclear (Nuc) protein fractions of HeLa cells were separated as described in the Experimental section, then subject to Western blotting with anti-PIPKIγ_i4 and pan-PIPKIγ antibodies. Lamin β1 and β-tubulin were Western-blotted as controls for the nuclear and cytosolic fractions respectively. Abbreviation: WCL, whole-cell lysate.

PIPKIγ_i5 targets to discrete cytoplasmic domains

As our attempts to utilize the anti-PIPKIγ_i5 polyclonal antibody for immunofluorescenve staining were unsuccessful, HA-tagged PIPKIγ_i5 was expressed in HeLa cells and its localization was observed. PIPKγ_i5 was found to target to the plasma membrane as well as to punctuate and enlarged cytoplasmic vesicle-like structures (Figure 6). To confirm the nature of these structures, HeLa cells transfected with PIPKIγ_i5 were stained for various markers of endosomal compartments. PIPKIγ_i5 was found to partially co-localize with a subset of vesicles that stained positive for TfnR (recycling endosomes), EEA1 (early endosomes), CD63 (multi-vesicular bodies/late endosomes), and, to a lesser extent, LAMP1 (lysosomes) (Figure 6). As we observed some co-localization of PIPKIγ_i5 with LAMP1, this could indicate active degradation of PIPKIγ_i5. However, degradation of PIPKIγ_i5 was not observed, as treatment of these cells with chloroquine did not alter PIPKIγ_i5 expression levels (results not shown). As shown in Figure 6, only a subset of PIPKIγ_i5-positive vesicles co-localized with any of these endosomal markers. However, these results suggest that PIPKIγ_i5 may be an active participant in endosomal trafficking events at multiple locations within the endosomal system.

PIPKIγ_i5 is functionally distinct from PIPKIγ_i2

Although the C-terminus of PIPKIγ_i4 is quite distinct from that of the other human splice variants, the C-terminus of PIPKIγ_i5 shows partial similarity to that of PIPKIγ_i2 (Figure 1C). In particular, the $W^{647}VYSPLH^{653}$ (one-letter amino acid code) motif present in PIPKIγ_i2 shows a high level of similarity to the sequence $W^{647}IYSPRH^{653}$ in the C-terminus of PIPKIγ_i5. In PIPKIγ_i2, this sequence modulates the association of

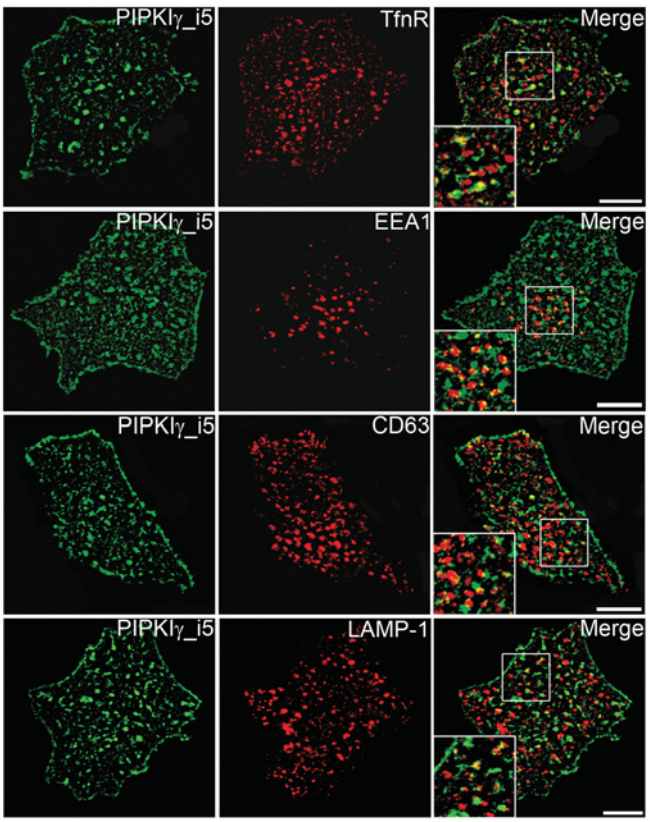

Figure 6 PIPKIγ_i5 localizes to endosomal compartments

HeLa cells expressing HA-tagged PIPKIγ_i5 were fixed in paraformaldehyde and stained with anti-HA (green) and antibodies towards TfnR (recycling endosomes), EEA1 (early endosomes), CD63 (multi-vesicular bodies/late endosomes) and LAMP-1 (lysosomes) (all in red). Yellow areas indicate co-localization of red and green immunofluorescence signals. The inset figures are 175 % zooms of the outlined area. The scale bar represents 10 μm.

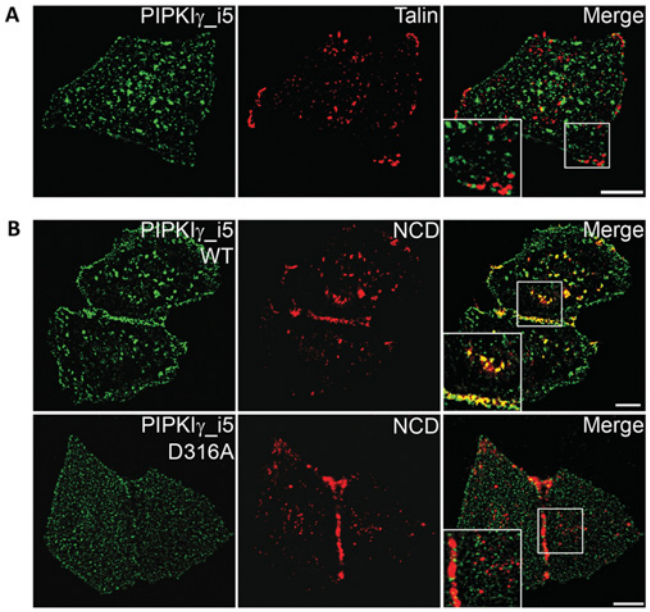

Figure 7 Localization of PIPKIγ_i5 is distinct from that of PIPKIγ_i2

HeLa cells expressing HA-tagged PIPKIγ_i5 were fixed in paraformaldehyde and stained with (**A**) anti-HA (green) and anti-talin (red) or (**B**) anti-HA (green) and anti-N-cadherin (NCD) (red) antibodies. Yellow areas indicate co-localization of red and green immunofluorescence signals. The inset figures are 175 % zooms of the outlined area. The scale bar represents 10 μm.

PIPKIγ_i2 with talin [1] and AP complexes [13,14,16–18,38]. To determine potential PIPKIγ_i5 functional overlap with PIPKIγ_i2, HeLa cells expressing PIPKIγ_i5 were stained for endogenous talin or α-adaptin. PIPKIγ_i5 did not co-localize with talin (Figure 7A) or α-adaptin (results not shown) in HeLa cells. In addition, PIPKIγ_i5 did not co-immunoprecipitate with talin1, talin2 or α-adaptin (results not shown), confirming that, although the C-terminus of PIPKIγ_i5 contains this similar sequence, it does not target to the same cellular domains and probably cannot functionally compensate for PIPKIγ_i2.

PIPKIγ_i2 directly associates with E-cadherin in polarized epithelial cells, both at cell–cell contacts and in TfnR-positive recycling endosomes, and the targeting of E-cadherin to the plasma membrane by PIPKIγ_i2 requires both PtdIns(4,5)P_2 generation and the association of PIPKIγ_i2 with AP1B via its unique C-terminus [17]. HeLa cells do not express E-cadherin; instead, cell–cell contacts in HeLa cells are mediated by N-cadherin, another member of the classical cadherin family. N-cadherin also directly associates with PIPKIγ via its conserved kinase domain [17,39] and, as the kinase domain of PIPKIγ_i5 is identical with that of PIPKIγ_i2, we tested to see whether PIPKIγ_i5 could associate with N-cadherin *in vivo*. In HeLa cells co-stained for PIPKIγ_i5 and N-cadherin, we observed co-localization of PIPKIγ_i5 and N-cadherin at both cell–cell contacts and within cytoplasmic vesicles (Figure 7B). In addition, this targeting of PIPKIγ_i5 required kinase activity, as a kinase-dead mutant (D316A) of PIPKIγ_i5 [17] localized diffusely within cells and did not co-localize with N-cadherin (Figure 7B). However, N-cadherin-based cell–cell junctions were not disrupted in HeLa cells expressing PIPKIγ_i5 D316A, indicating that the generation of PtdIns(4,5)P_2 by PIPKIγ_i5 is not required for the trafficking of N-cadherin to the plasma membrane. Taken together, these results are consistent with a functionally distinct role for PIPKIγ_i5 in cadherin biology.

DISCUSSION

The popular nomenclature used for splice variants of PIPKIγ, including those based on amino acid composition or apparent molecular mass, suffers from ambiguity. Splice variants derived from different species may contain similar exons and serve identical biological functions, but often differ in amino acid number and consequently their molecular mass. In addition, PIPKIγ splice variants are post-translationally modified, which alters their apparent molecular mass on gel electrophoresis. In the present paper we have proposed and implemented a standardization of nomenclature which conforms to the HUGO Genetic Nomenclature Committee (http://www.genenames.org) and HGVS (Human Genome Variation Society) (http://www.hgvs.org) guidelines for transcripts and protein products respectively of the PIP5K1C gene [40]. Continued use of this standardized nomenclature will greatly simplify communication between investigators who study PIPKIγ biology.

Alternative splicing of RNA transcripts is an efficient cellular mechanism that increases the diversity of its protein products, thereby enhancing the overall functional specificity of a particular protein family. Much recent work has been devoted to delineating the mechanisms by which the unique C-terminal domain of PIPKIγ_i2 is able to confer functional specificity on this splice variant [1,3,4,41]. PIPKIγ_i2 functions by its C-terminus associating with protein-targeting factors (i.e., talin, AP complexes), which then target the kinase to focal adhesions or

cell–cell contacts respectively. Once targeted to its site of function, PIPKIγ_i2 then generates PtdIns(4,5)P_2, which regulates the activities of proteins in the vicinity. Importantly, several proteins that directly associate with PIPKIγ_i2 are also PtdIns(4,5)P_2 effectors [1,3,4,41]. In the present paper we have described two previously undefined splice variants of the PIP5KIC gene, PIPKI_i4 and PIPKIγ_i5, each of which contains a unique C-terminal domain. It is very probable that these unique C-termini direct the specific functions of PIPKIγ_i4 and PIPKIγ_i5 via the association of each kinase with distinct protein-targeting factors in a manner that parallels PIPKIγ_i2. By this mechanism, the results presented here suggest that PIPKIγ_i4 and PIPKIγ_i5 may fill specific functional roles within the nucleus and endosomal transport system respectively.

The existence of a nuclear phosphoinositide signalling pathway that is independent of the cytoplasmic phosphoinositide cycle has been established, albeit that it remains relatively poorly defined [24]. Several nuclear phosphoinositide kinases have been identified, including another type-I PtdIns4*P* 5-kinase, PIPKIα [20,24]. Recently, the PtdIns(4,5)P_2 generated by PIPKIα at sites of concentrated pre-mRNA processing factors known as nuclear speckles was shown to regulate the activity of the nuclear poly(A) polymerase Star-PAP [22,37]. Interestingly, our data indicates several striking parallels between PIPKIα and our newly discovered PIPKIγ_i4 splice variant. First, endogenous PIPKIγ_i4 was found in both the nuclear and cytoplasmic fractions of HeLa cells and also co-localizes with nuclear-speckle markers, both in a manner similar to PIPKIα [20]. However, endogenous PIPKIα, but not PIPKIγ, was detectable in Star-PAP immunoprecipitates [22]. Moreover, another PtdIns(4,5)P_2-generating enzyme, PIPKIIβ, targets to nuclear speckles and also does not associate with Star-PAP [22], which supports the hypothesis that there are several discrete pools of nuclear PtdIns(4,5)P_2 that are generated by PIPKIγ_i4, PIPKIα or PIPKIIβ. In other words, the specific targeting of these kinases and their association with a unique subset of proteins allows each to fill a distinct functional niche in nuclear phosphoinositide signalling pathways.

Secondly, it is important to note that PIPKIγ_i4 is not visible in the nucleus upon overexpression, a localization phenotype that is identical with that of overexpressed PIPKIα [20,22]. However, endogenous PIPKIα and PIPKIγ_i4 have been observed in both the cytoplasm and the nucleus, which leads us to speculate that the nuclear targeting of these kinases must be regulated in some manner. It is possible that post-translational modification of the unique C-terminus of PIPKIγ_i4, or interaction of a nuclear-targeted protein with this sequence, could modulate its nuclear entry. As observed in Figure 5(C), PIPKIγ_i4 appears as a doublet in the nuclear, but not in the cytosolic, fraction of HeLa cells. Therefore, nuclear PIPKIγ_i4 could be modified as a signal for nuclear retention or functional specificity within a nuclear subdomain. However, evidence supporting these speculations is lacking, and further investigation is required to determine the method of import as well as the potential nuclear functions of PIPKIγ_i4.

Interestingly, the PIPKIγ_i3 splice variant identified by Giudici et al. [33] shares 75 % sequence identity with the first 20 amino acids of the PIPKIγ_i5 C-terminus. In agreement with our data, Guidici et al. reported that PIPKIγ_i3 localized to vesicle-like and punctuate cytoplasmic structures when expressed in non-neuronal cells, where they also observed modest co-localization with vesicular markers [33]. However, as the splice variant identified by Giudici et al. seems to be limited to mouse neurons, we propose that the much more ubiquitous human PIPKIγ_i5 could serve to perform similar biological functions in non-neuronal cells.

The role of phosphoinositides in the regulation of the endosomal network has been well defined for 3′-phosphorylated polyphosphoinositides [42–44], but less is known about the role of PtdIns(4,5)P_2 in these signalling pathways. However, it is probable that PtdIns(4,5)P_2 may also be a potent regulator of endosomal transport [45,46], and the targeting of PIPKIγ_i5 to endosomal compartments suggests that the generation of PtdIns(4,5)P_2 could regulate endosomal system function or even transport between endosomal subdomains. As the type-I PIPKs have been shown to phosphorylate 3-phosphoinositides *in vitro* [3], an alternative explanation is that, given a certain subcellular condition or protein interaction partner, the substrate preference of PIPKIγ_i5 could be changed to utilize 3-phosphoinositides, thereby generating lipid messengers in addition to PtdIns(4,5)P_2. However, this shift in PIPK substrate preference has not yet been shown to occur *in vivo* in organisms other than *Schizosaccharomyces pombe* [3].

The significance of the sequence similarity between the talin and AP complex binding/regulatory site that is present in the C-terminus of PIPKIγ_i2, and its 'sister' sequence that is present in the C-terminus of PIPKIγ_i5 cannot be overlooked. Our data indicate that, in the light of this similarity, PIPKIγ_i5 does not associate with talin or APs and is not targeted in a manner similar to PIPKIγ_i2. Although both PIPKIγ_i2 and PIPKIγ_i5 co-localize with cadherins, the mechanism by which PIPKIγ_i5 is involved in N-cadherin function likely differs from that of PIPKIγ_i2. When kinase inactive PIPKIγ_i2 or PIPKIγ_i1 (which lacks a C-terminal tail) was expressed in polarized epithelial cells, trafficking of E-cadherin to the plasma membrane was hindered [17], indicating that both kinase activity as well as the unique C-terminus of PIPKIγ_i2 is required for efficient basolateral targeting of E-cadherin. HeLa cells expressing PIPKIγ_i5 D316A showed no apparent inhibition of N-cadherin trafficking to cell–cell contacts, but co-localization of PIPKIγ_i5 and N-cadherin was lost. These data suggest that PIPKIγ_i5 potentially regulates the post-endocytic trafficking of N-cadherin rather than its exocytosis. Since PIPKIγ_i5 is partially localized at several types of endosomal compartments, this splice variant is positioned to regulate the endosomal trafficking of N-cadherin, E-cadherin or other proteins at multiple steps within the endosomal system. However, further study is required to resolve the nature of the endosomal compartment at which PIPKIγ_i5 and N-cadherin co-localize, and to determine the extent of regulation by PIPKIγ_i5.

AUTHOR CONTRIBUTION

Nicholas J. Schill performed the experiments, analysed and interpreted the data and wrote the manuscript. Richard A. Anderson provided scientific guidance and edited the manuscript prior to submission.

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