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Review

Mammalian phosphatidylinositol 4-kinases as modulators of membrane trafficking and lipid signaling networks

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ABSTRACT

The four mammalian phosphatidylinositol 4-kinases modulate inter-organelle lipid trafficking, phosphoinositide signalling and intracellular vesicle trafficking. In addition to catalytic domains required for the synthesis of PI4P, the phosphatidylinositol 4-kinases also contain isoform-specific structural motifs that mediate interactions with proteins such as AP-3 and the E3 ubiquitin ligase Itch, and such structural differences determine isoform-specific roles in membrane trafficking. Moreover, different permutations of phosphatidylinositol 4-kinase isozymes may be required for a single cellular function such as occurs during distinct stages of GPCR signalling and in Golgi to lysosome trafficking. Phosphatidylinositol 4-kinases have recently been implicated in human disease. Emerging paradigms include increased phosphatidylinositol 4-kinase expression in some cancers, impaired functioning associated with neurological pathologies, the subversion of PI4P trafficking functions in bacterial infection and the activation of lipid kinase activity in viral disease. We discuss how the diverse and sometimes overlapping functions of the phosphatidylinositol 4-kinases present challenges for the design of isoform-specific inhibitors in a therapeutic context.

Contents

1. Introduction

The four enzymes that make up the mammalian phosphatidylinositol (PI) 4-kinase family can be divided into two groups based on primary sequence and biochemical properties: consisting of type II (PI4KIIα and PI4KIIβ) and the type III (PI4KIIIα and PI4KIIIβ) isozymes [1,2]. All members catalyse the phosphorylation of phosphatidylinositol (PI) at the D4 position of the inositol head-group

Abbreviations: ARF, ADP-ribosylation factor; DAG, diacylglycerol; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; FAPP2, Four-phosphate adaptor protein 2; FAAT motif, two phenylalanines in an acidic tract motif; GPCR, G-protein-coupled receptor; HCV, hepatitis C virus; NCS-1, neuronal calcium sensor-1; OSBP, oxysterol binding protein; OSH, oxysterol binding protein homologue; PKD, protein kinase D; PH, pleckstrin homology; PI, phosphatidylinositol; PI4P, phosphatidylinositol 4-phosphate; PI(4,5)P_2, phosphatidylinositol (4,5)-bisphosphate; PI(3,4,5)P_3, phosphatidylinositol (3,4,5)-trisphosphate; PLC, phospholipase C; SARS, Severe acute respiratory syndrome; TGN, *trans* Golgi network; TRPV, transient receptor potential vanilloid..

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to synthesize phosphatidylinositol 4-phosphate (PI4P) which is an essential precursor in the enzymatic pathways that produce PI(4,5)P_2 and PI(3,4,5)P_3 for receptor-activated phospholipase C (PLC) and phosphoinositide 3-kinase signalling [3]. In addition to the generation of phosphoinositides for receptor signalling, PI 4-kinase activity underlies the recruitment a number of PI4P-specific binding proteins such as the pleckstrin homology (PH) domain containing proteins CERT [4] and FAPP2 [5] which regulate lipid synthesis on Golgi membranes, and also the recruitment of the clathrin adaptors AP-1 [6] and AP-3 [7] during Golgi-endosomal trafficking. However, PI 4-kinases do not just produce PI4P. Another level of cellular regulation exists at the non-catalytic, protein-interaction level, where structural binding motifs mediate isoform-specific interactions with molecules such as Rab11 [8], NCS-1 [9,10], AP-3 [7,11,12] and the E3 ubiquitin ligase itch [13]. Recent work has also established that different PI 4-kinase isoform permutations are required during receptor-activated PLC signalling [14–16] and in Golgi-endosomal trafficking [16,17] all of which suggests that there is still much still to be discovered about this, the least well studied mammalian PI kinase family.

2. PI 4-kinases and the intracellular compartmentation of PI4P synthesis

For over 40 years there has been intense interest in mapping out the cellular compartments where PI 4-kinases are active [18–24]. Pioneering work from Michell, Harwood and Hawthorne established an early precedent for PI4P synthesis in plasma membrane enriched fractions [20,25] and the presence of a biochemically distinct PI 4-kinase activity associated with the ER which could be activated by the non-ionic detergent Cutscum [19]. More recently, in the post-cloning era, these original observations have been built on and there has been substantial progress in mapping the subcellular distributions of the four mammalian PI 4-kinase isoforms [26–29] (Fig. 1). Subcellular fractionation and immunocytochemical experiments have established that PI4KIIα (55 kDa) localises to the *trans*-Golgi network (TGN) [6,28–31]) where its lipid kinase activity has been implicated in the recruitment of AP-1 [6] and GGA clathrin adaptors [32]. PI4KIIα also localises to various membranes of the endosomal system [12,26,29,33,34] as well as specialised trafficking intermediates such as synaptic vesicles [35] and Glut4 transport vesicles [36]. In addition, there is biochemical evidence for a minor but highly active pool of PI4KIIα at the endoplasmic reticulum (ER) [29] where it may be activated by interactions with the c-FOS transcription factor [37]. On endosomal membranes both PI4KIIα catalysed PI4P generation and the presence of a dileucine sorting motif are required for optimal binding of AP-3 [7] – a key step in cargo selection and trafficking to late endosomes/lysosomes. Despite its high degree of structural homology to the PI4KIIα isoform, PI4KIIβ has a different subcellular distribution profile and has been observed in both in the cytosol [38,39] and in association with trafficking vesicles [26,40].

As for the two wortmannin-sensitive PI4KIIIs [41–43], PI4KIIIα [42,44–46] (230 kDA) has been localised by immunostaining with isoform specific-antibodies to cytosplasmic membranes [47–49], and more specifically to early *cis*-Golgi compartments [50] and the nucleolus [47]. PI4KIIIα has also been identified the main isozyme responsible for PI4P generation at the plasma membrane [14,27,51]. Recently, Nakatsu et al. [52] demonstrated that PI4KIIIα visits the plasma membrane, in a dynamic process mediated by interactions with the palmitoylated, membrane-resident protein EFR3B and TTC7B – a protein recruited from the cytosol [52]. Most strikingly, the generation of PI4P hotspots at the plasma membrane in yeast requires similar molecular components indicating a very high degree of evolutionary conservation for this process [53]. These recent insights also revealed a major function for mammalian PI4KIIIα in maintaining the normal proteomic and lipid composition of the plasma membrane as evidenced by the mistrafficking of GPCRs and cholesterol to intracellular compartments in PI4KIIIα knock-out cells [52]. In a separate study, Hammond and colleagues have established multiple, non-precursor, roles for PI4P at the plasma membrane [54,55]. The first such function could be considered a general one, whereby PI4P in combination with other negatively charged phosphoinositide species, electrostatically defines the cytoplasmic-facing plasma membrane layer as a negatively charged lipid landscape with the capacity to recruit a range of proteins containing polybasic lipid binding domains such as those found in MARCKS and K-Ras. Secondly they observed that PI4P regulates particular ion channels such as the transient receptor potential vanilloid 1 (TRPV1) cation channel, the activity of which was found to be inhibited through either selective depletion of PI4P or PI(4,5)P_2 [54]. Additionally, PI4P, at physiologically relevant concentrations as low as 2 mol%, can induce curvature in model membranes [56]. Therefore, PI4P can contribute to the biochemical and biophysical identity of the plasma membrane, and this multifunctional pool of PI4P is generated principally by PI4KIIIα. The other wortmannin-sensitive isoform, PI4KIIIβ [57–60] (92 kDa) does not have a major role in the generation of plasma membrane PI4P but instead seems to mainly function in the generation of Golgi-derived carriers [8,31,61–63]. In line with these trafficking functions, PI4KIIIβ can interact with Arf1 [63–65], neuronal calcium sensor-1 (NCS-1) [9,10,65–67] and the Rab11 GTPase [8]. In addition to the Golgi apparatus, PI4KIIIβ has also been visualised on lysosomes [68] where it functions to maintain lysosomal membrane integrity and in a PKD-phosphorylated from in the nucleus [69] where it's physiological function has yet to be determined.

In summary, the markedly different subcellular distributions of the four PI 4-kinases give rise to highly compartmentalised PI4P synthesis and thus organelle-specific functions for this phosphoinositide species [5,70,71].

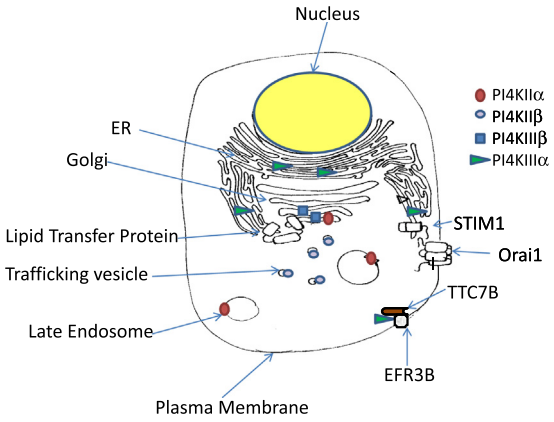

Fig. 1. Organisation and subcellular compartmentation of PI4P synthesis. The four mammalian PI 4-kinases have different subcellular localisations. The main pool of plasma membrane PI4P is maintained by PI4KIIIα which has been imaged in separate studies either in association with the ER or recruited to the plasma membrane by EFR3B and TTC7B. PI4KIIIβ and PI4KIIα generate PI4P at the trans-Golgi network and PI 4-kinase activity on these membranes is required for the formation of constitutive plasma membrane vesicular carriers, secretory vesicles and in trafficking to late endosomes. Additionally, PI4P at the TGN targets lipid transfer proteins such a FAPP2 and CERT which effect non-vesicular transfer of glucosylceramide and ceramide to late Golgi compartments. PI4KIIβ has been observed in the cytosol and in association with membranes, here we show PI4KIIβ in association with intracellular endosomal-like vesicles. PI4KIIα also localises to late endosomes where it modulates the trafficking and degradation of internalised ligand-activated receptors and cargo sorting during TGN to late endosome trafficking.

3. Cell signalling

The requirement for PI4P synthesis and resupply during receptor-activated phosphoinositide signalling was one of the principal spurs for early studies aimed at identifying and purifying the mammalian PI 4-kinases. Early work demonstrated the presence of an EGF-activated PI 4-kinase activity at in membrane fractions that was associated with a decreased apparent K_m for PI substrate but with no increase in the V_{max} for PI phosphorylation activated EGFR [72,73]. Subsequent investigations demonstrated that activated EGFR could be co-immunoprecipitated in complex with a PI4KII activity, PLCγ and phosphatidylinositol transfer protein [74,75] (which was hypothesized to transfer PI substrate to the lipid kinases during ligand-activated signalling). Further detailed analyses of phosphoinositide turnover kinetics during receptor-stimulated PLC signalling, demonstrated a requirement for a wortmannin-inhibited PI4KIII activity in the generation of a signaling pool of PI4P [76,77]. A subsequent study that used RNA interference to abrogate the expression of individual PI 4-kinase isozymes discovered that wortmannin-sensitive PI4KIIIα was primarily responsible for maintenance of the plasma membrane phosphoinositide pool which is turned over rapidly during GPCR signalling [14,27].

A possible disconnection between sites of PI4P synthesis and utilisation was revealed in elegant studies that used organelle-targeted, recombinant phosphoinositide phosphatases such as Sac1, to deplete PI4P levels at particular subcellular membranes [16,54]. Using this strategy Golgi-compartmentalised PI4P synthesis was shown to be required during the substrate replenishment phase of the angiotensin-stimulated PLC response [16]. At first glance it seems counterintuitive that PI4P synthesis at distal sites can supply the plasma membrane during ligand-activated signalling. However, the recent discovery of stable inter-organelle membrane contacts which facilitate lipid transfer between different organelles [78,79] is worth considering in this context. Most strikingly, a precedent for such inter-organelle regulation of phosphoinositide concentrations has been demonstrated in yeast where the ER-associated PI4P phosphatase Sac1, can act in *trans* at inter-membrane contact sites to dephosphorylate PI4P at the plasma membrane [80]. This process requires the PI4P-binding Osh3 (oxysterol homology 3) protein that appears to simultaneously function both as a plasma membrane PI4P sensor and activator of the Sac1 PI4P phosphatase activity at intermembrane contact sites. Additionally, 6 proteins have been identified that mediate ER-plasma membrane tethering and regulate plasma membrane PI4P levels. These proteins are the vesicle-associated membrane protein-associated proteins (VAP) Scs2 and Scs22 which are similar to mammalian VAP proteins that target FFAT (two phenylalanines in an Acidic Tract [81,82]) motif-containing proteins to the ER; 3 tricalbin protein isoforms, Tcb1, Tcb2 and Tcb3 that contain membrane spanning, lipid binding and multiple C2 domains and are related to the mammalian synaptotagmins [83,84]; and Ist2, which contains multiple transmembrane spanning domains and is orthologous to the mammalian TMEM16 family of Ca^{2+}-activated chloride channels [85–88]. Interestingly, Ist2 has been shown in an unconnected study to mediate interactions between the plasma membrane and ER [89].

While mammalian cells express orthologues of the yeast proteins required for the formation of ER-plasma membrane contacts and the control of plasma membrane PI4P concentrations, it is not yet known if an exactly analogous regulatory system exists in higher eukaryotes. Nonetheless, it is noteworthy that in mammalian cells, transient ER plasma membrane contacts are known to form during agonist-stimulated PLC and Ca^{2+} signalling in the capacitive re-entry phase when extracellular Ca^{2+} replenishes depleted ER stores [79,90–92]. This store refilling event is mediated by direct interactions between an ER protein STIM1 and a plasma membrane Ca^{2+} channel Orai1 [91,92]. Therefore, this Ca^{2+}-triggered membrane contact event demonstrates that it may be feasible for an ER-associated PI 4-kinase to supply phosphoinositide substrate to receptors during signalling. Of relevance to this proposal are the findings that PI4KIIIα inhibition results in decreased store-operated Ca^{2+} entry [90] and that PI4KIIIα expression is required for the formation of STIM1-Orai1 intermembrane contacts [52]. Intriguingly, we have recently discovered that CDP-DAG synthase and phosphatidylinositol synthase – two enzymes required to produce the PI 4-kinase substrate PI, also localise to ER membrane microdomains that are in close contact with the plasma membrane [93]. Hence, it seems more and more likely that inter-organelle membrane contact sites play a pivotal role in the cellular organization of receptor-evoked phosphoinositide signalling (Fig. 1).

Since non-vesicular lipid transfer between contacting membrane sites tends to be high flux and energy independent [94,95] such a mechanism would be particularly well suited to supporting large scale and rapid turnover of PI4P pools following GPCR activation. These insights may mean that apparent spatial restrictions on PI4P generation due to the differential organelle targetting of the PI 4-kinases may not be as restrictive for signalling as previously envisaged. Furthermore, the ability for lipids to be transferred between closely apposed membranes suggests that the dynamics of organelle contact site formation and dissolution may impact on the spatiotemporal control of cellular PI4P metabolism.

While PI4KIIIα has a prominent role in GPCR stimulated PLC signalling which tends to be a very dramatic and robust response mediated by G-protein activated PLCβ, the situation differs with the two PI4KII isoforms which seem to have at best minor roles in the agonist-stimulated Ca^{2+} response [14,90]. However there are specific instances of PI4KIIα being important for the regulation of receptor tyrosine kinase [15,33] and Wnt signalling [13,96,97]. In particular, TGN/endosome localised PI4KIIα regulates the endosomal traffic of activated EGFR [33] and knockdown of this enzyme retards the degradation of internalised EGFR. Further evidence for this isozyme modulating receptor signalling has emerged from a study which demonstrated a role for PI4KIIα in regulating Wnt3a signalling through Frizzled seven transmembrane receptors [96,97]. This particular mode of canonical Wnt signalling is associated with β-catenin and T cell factor-induced gene transcription rather than Wnt-activated PLC and Ca^{2+} signalling. These studies found that PI4KIIα is required for $PI(4,5)P_2$ formation and Wnt3a-dependent phosphorylation of the low-density lipoprotein receptor-related protein 6 (LRP6) [96]. Furthermore, Dishevelled (Dvl) – a cytosolic protein recruited to Wnt3a–bound Frizzled receptors, forms a complex with PI4KIIα resulting in a doubling of lipid kinase activity [97]. Conversely it was later discovered that Itch, a HECT-type E3 ubiquitin ligase that regulates the endosomal trafficking of Wnt-activated Frizzled receptors, inhibits PI4KIIα activity and that this effect is mediated by direct association of the Itch WW domains with a PPxY motif of PI4KIIα (residues $_{15}PPDY_{18}$ in the amino terminus of PI4KIIα) [13]. Whilst PI4P synthesis is inhibited by the PI4KIIα Itch complex formation, the ubiquitin ligase activity of Itch is enhanced [13]. Hence, the opposing effects of Dvl and Itch are consistent with activated PI4P synthesis being important in the early events of Wnt signalling, particularly for phosphorylation of the LRP6 co-receptor, but with a non-catalytic role for PI4KIIα becoming predominant during the later stages of Wnt-receptor trafficking and sorting via the ubiquitin pathway. Interestingly while RNAi studies have not implicated the PI4KIIIs in Wnt signalling, there is evidence that both PI4KIIIα and PI4KIIIβ are required for activation of Hedgehog signalling in mammalian cells – a process which in *Drosophila* is thought to involve PI4P upregulating

vesicular trafficking of the Smoothened receptor to the plasma membrane [98].

Unexpectedly, non-catalytic functions of the PI 4-kinases may also be important in regulating receptor-stimulated phosphoinositide signalling. This can particularly be seen in the case of the PI4KIIα isoform where recombinant overexpression of a lipid kinase-inactive mutant impairs EGF-stimulated Akt activation [15]. Overexpression of kinase-inactive PI4KIIα does not inhibit endogenous PI4P synthesis but induces defective TGN-endosomal trafficking as evidenced by enlarged late endosomes and reduced transferrin receptor trafficking to recycling endosomes [7,26,33]. Therefore, the effect on Akt activation can be attributed to defective intracellular vesicle trafficking rather than reduced PI4P substrate supply and subsequent $PI(3,4,5)P_3$ generation [15]. PI4KIIα expression levels determine the time course of EGFR trafficking [33] to the lysosome and are important for the recruitment of clathrin adaptors during Golgi-endosomal trafficking [6,7,11,32,34]. Moreover, these trafficking functions are mediated through both lipid kinase and modular protein binding properties of the enzyme. Therefore, non-lipid kinase functions may regulate phosphoinositide signalling pathways, albeit indirectly through effects on the intracellular trafficking dynamics of signalling proteins.

Although it only accounts for a small fraction of overall cellular PI4P production, the PI4KIIβ isoform has been reported to undergo membrane recruitment following receptor activation [38]. The mechanism ascribed for this regulatory event is unique amongst the PI kinases and it involves in the absence of agonist, PI4KIIβ being sequestered in a cytosolic complex with Hsp90 where it is catalytically inactive but at the same time protected from ubiquitination and consequent proteolytic degradation. Following receptor tyrosine kinase activation, the Hsp90 interaction is interrupted and PI4KIIβ undergoes membrane translocation [38] and possible activation by the Rac GTPase. [99] PI4KIIβ palmitoylation is associated with membrane recruitment and catalytic activity but unlike the PI4KIIα isoform it is not constitutively palmitoylated [38,100]. Membrane-associated PI4KIIβ is subject to agonist-dependent phosphorylation in its structurally unique amino terminal domain but this post-translational modification does not alter its lipid kinase activity [38]. Biochemical evidence suggests that this isozyme can also associate with crosslinked T cell receptor CD3 zeta chains following tyrosine phosphorylation of the receptor [101]. PI4KII activity has been observed by several groups in complex with tetraspanins (transmembrane-4 superfamily proteins) [40,102–107] which are cell surface, plasma membrane spanning proteins with important roles in adhesion and signalling [108–110]. In the case of the tetraspanin CD81, PI4KIIβ has been shown to be the co-immunoprecipitated isoform [40]. However, the molecular mechanisms that determine PI4KIIβ interactions with either tetraspanins or tetraspanin-enriched membrane domains have yet to be elucidated.

4. Interactions with other lipid pathways

There are now multiple reports that PI4P generation on Golgi membranes is intrinsically linked with the synthesis of other lipids specifically glycosphingolipids and sphingomyelin, and that this is mediated by lipid transfer proteins that associate with Golgi membranes via PI4P-binding PH domains [5]. In addition to its role in promoting non-phosphoinositide lipid production there is also evidence that PI 4-kinase activity is itself modulated by membrane composition [111–113] and thus PI4P synthesis plays a key role in sensing and controlling the concentrations of a variety of lipid classes on late Golgi membranes.

4.1. Sterol regulation of PI4KIIα

PI4KIIα localises mainly to the TGN and endosomes and is only PI kinase enzyme that constitutively associates with membranes [28,29,100,114–116]. Different to all the other PI kinases, the biochemistry and enzymology of PI4KIIα is largely defined by its targeting to cholesterol and glycosphingolipid-enriched microdomains of the TGN and the modulation of its lipid kinase activity by membrane sterol and oxysterol concentrations [28,100,111–113,116,117]. Work from our laboratory has shown that manipulating the sterol concentration of intracellular membranes with methyl-β-cyclodextrin results in changes to the morphology and size of these membranes which affects both the diffusion rate and mobile fraction of the enzyme [112]. There is also evidence that enhanced sterol concentrations augment the catalytic activity of PI4KIIα [111–113]. Targeting of PI4KIIα to TGN rafts is achieved through dual-palmitoylation of cysteines within a CCPCC motif located in the catalytic region of the protein by Golgi-associated palmitoyl transferases [100,115–117]. Interestingly, tight membrane association of PI4KIIα does not necessitate its prior palmitoylation since palmitoyl-mutants remain tightly membrane bound. However, palmitolylation is indispensible for PI phosphorylation and for the correct subcellular targeting of the enzyme [100,115]. Most recent work has revealed that PI4KIIα is in fact palmitoylated by DHHC3 and DHHC7 which are two Golgi-associated palmitoyl acyltransferases [117]. PI4KIIα can be co-immunoprecipitated from cholesterol-rich lipid raft-like domains of the TGN in complex with these palmitoyl acyltransferases and these interactions are lost following sterol depletion with methyl-β-cyclodextrin [117]. In this way reversible palmitoylation represents a unique cholesterol-sensitive regulatory mechanism that simultaneously links the lipid kinase activity of PI4KIIα with its targeting to raft-like microdomains of TGN-endosomal membranes [117]. Another way of viewing these biochemical relationships is to consider PI4KIIα localisation and activity at this subcellular locus as reporters for the sterol content and lipid microdomain organization of the tubulovesicular TGN compartment [111,112].

4.2. PI 4-kinases & sphingomyelin synthesis

Sphingomyelin is generated from phosphatidylcholine and ceramide at the TGN in a reaction catalysed by sphingomyelin synthase 1 which also produces diacylglycerol (DAG). However, ceramide is synthesized in the ER and therefore must be transported to the TGN in order for sphingomyelin synthesis to take place. The transfer of ceramide to the Golgi is effected by CERT – a lipid transfer protein [118–120]. Structurally, in addition to carboxy-terminal StART lipid-binding domain, CERT contains a FAAT motif [81,82,121–123] which can bind to ER-localised VAP proteins and in its amino terminus a PH domain which specifically binds PI4P at the Golgi. The positioning of a Golgi-interacting PH domain and ER interacting FFAT motif at opposite ends of CERT facilitates the simultaneous binding of two compositionally distinct membranes and the non-vesicular transfer of ceramide across this protein-mediated inter-organelle contact site [118,120]. Through pharmacological inhibition and RNA interference studies the Balla laboratory established that the Golgi/TGN-localised PI4KIIIβ isoform is required for CERT-mediated non-vesicular trafficking of ceramide to the TGN [4]. In this way, PI4KIIIβ controls the supply of substrate to sphingomyelin synthase and thereby the rate of sphingomyelin production at the TGN. More recently, a study from Banerji and colleagues has detailed how OSBP – another PH domain containing protein, regulates sterol levels at the TGN which affects PI4KIIα activity, PI4P-dependent CERT recruitment and consequently sphingomyelin production [111]. This places PI4KIIα downstream of OSBP and of critical importance in

integrating changes to cholesterol concentration with sphingomyelin synthesis. Since cholesterol and sphingomyelin are proposed to be concentrated within lipid rafts, one inference from these studies is that PI4KIIα is a key enzyme in driving lipid raft formation at the TGN. While this is an attractive model it should be noted that the Balla laboratory only found a minor role for PI4KIIα in CERT recruitment to the TGN [4], hence there is some debate over which PI 4-kinase isoform is most important for maintaining sphingomyelin levels.

The reaction catalyzed by sphingomyelin synthase also produces DAG [124] which is required for activation [125] and targeting Protein Kinase D (PKD) to TGN membranes [126,127]. Furthermore, PI4KIIIβ catalytic activity is activated through PKD catalysed phosphorylation of serine 294 [62,128]. This suggests the existence of an integrated lipid-based regulatory mechanism where the generation of DAG by sphingomyelin synthase leads to increased PI4P production through the DAG-PKD-PI4KIIIβ axis [129]. PKD-induced phosphorylation of PI4KIIIβ upregulates the formation of plasma-membrane destined vesicular carriers in a process that involves the formation of a complex between PI4KIIIβ, 14-3-3γ adaptin dimers and CtBP1-S/BARS [130]. Whilst membrane trafficking to the plasma membrane driven by PI4KIIIβ is increased, ceramide delivery to the TGN is decreased as PKD phosphorylation of CERT on serine 132 inhibits interaction of the CERT PH-domain with PI4P [131]. PKD also serine phosphorylates oxysterol-binding protein (OSBP) another protein implicated in PI4P-dependent sphingomyelin synthesis at the TGN resulting in impaired sterol and oxysterol-dependent recruitment of OSBP [132]. Together these recent findings illustrate the central importance of PKD in the homeostatic control of the TGN lipid composition.

4.3. PI 4-kinases & glycosphingolipid synthesis

The synthesis of complex glycosphinglipids at the Golgi is critical for maintaining the characteristic lipid compositions of the both the TGN and plasma membrane, and depends absolutely on the activity of PI4KIIIβ and to a lesser extent PI4KIIIα [133–135]. Four-phosphate adaptor protein 2 (FAPP2) [119,120,136] like CERT, is a member of the family of lipid transfer proteins which contains an N-terminal PI4P-binding PH domain [119,120,137]. However, unlike CERT, FAPP2 contains a C-terminal glycolipid transfer protein homology domain which is required for efficient transfer of glucosylceramide from *cis*- to *trans*-Golgi compartments, or as laid out in alternative model, the retrograde transfer of glucosylceramide to the ER followed by subsequent transport back to the Golgi for additional enzymatic glycosylation [138]. CERT also contains a FFAT-like motif which may mediate interaction with ER associated VAP proteins [139]. PI4P production by PI4KIIIβ and to a lesser extent PI4KIIα has been implicated in the recruitment of FAPP2 to late Golgi membranes [134]. Furthermore, FAPP2 is a component of the recently described PKD- and PI4KIIIβ-regulated molecular complex that facilitates the formation of plasma membrane-destined vesicular carriers [5,130,140].

Since PI4KIIIβ activity is under the control of DAG activated PKD and PI4KIIα is responsive to membrane sterol concentrations, it could be said that the Golgi/TGN PI 4-kinases co-ordinate a membrane composition and sensing function that can transduce changes in membrane environment into altered PI4P generation thereby modulating the synthesis of the lipid raft–defining sphingomyelin and glycosphingolipids (Fig. 2). As both PI 4-kinases also mediate vesicle trafficking from the TGN, augmented PI4P synthesis has the potential to modify the lipid composition of distal membranes such as the plasma membrane. Therefore, PI 4-kinases occupy a key role in co-ordinating and integrating the lipid composition of the various post-Golgi membranes with enormous consequences for signalling, trafficking and membrane organization at extra-Golgi loci [5,141,142].

5. Emerging roles for PI 4-kinases in disease

In this section we explore the emerging roles for the PI 4-kinases across a wide range of human disease but particularly in cancer, neurological disease and infections caused by both bacteria and viruses.

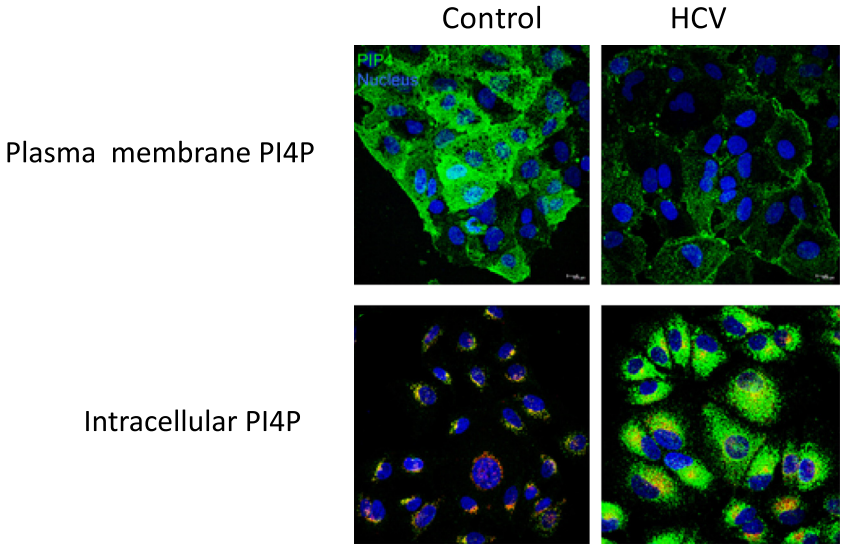

Fig. 2. Redistribution of PI4P from the plasma membrane to a membranous web during HCV infection. PI4P (green) was imaged by immunostaining with anti-PI4P antibody and the plasma membrane and intracellular pools of this lipid were visualised by microscopy. Nuclei are stained blue with the Hoescht dye and the Golgi protein giantin appears in red. In control Huh7.5 hepatoma cells, there is a sizeable plasma membrane-associated pool of PI4P. However, in cells replicating subgenomic replicons of HCV (genotype 2a) there is a reduction in PI4P at the plasma membrane and a concomitant large increase in intracellular PI4P in the HCV-induced membranous web which originates from the ER. PI4KIIIα is the PI 4-kinase implicated in producing both the plasma membrane and membranous web pools of PI4P. This figure is adapted and reproduced from the work of Bianco and co-workers [51].

5.1. Cancer

The indications so far from cell based studies are that alterations to individual PI 4-kinase isozyme expression levels can modulate receptor tyrosine kinase, Wnt, integrin and tetraspanin signalling – all of which when deregulated can contribute towards the development of neoplastic disease. However, in the absence of disease causing mutations in the PI 4-kinases, it is not yet established whether alterations to catalytic or non-catalytic functions of the enzymes are most important in cancer (reviewed in [143]). So far, increased expression of PI4KIIα and PI4KIIβ has been reported for a range of cancers with increased PI4KIIα levels associated with augmented activation of the HER2 receptor kinase pathway, HIF production and angiogenesis [144]. In a separate study, a potential anti-metastatic role for PI4KIIβ has emerged which involves this isozyme promoting the trafficking of CD81 tetraspanin proteins away from the plasma membrane into a population of intracellular trafficking vesicles that also contain actinin thereby inducing anti-migratory remodelling of the actin cytoskeleton [40]. Meanwhile PI4KIII has been identified in non-biased screens as contributing towards a more aggressive metastatic phenotype of pancreatic ductal carcinoma cells [145] and was identified as one of several proteins that mediate resistance to the chemotherapeutic agents gemcitabine [146] and cisplatin [147]. However, unlike the well established case of constitutively active phosphoinositide 3-kinase mutations as found in PI3KCA or phosphatase PTEN deletions which can both elevate PI(3,4,5)P_3 levels and drive oncogenic signaling [148,149], it is significant that neither activating mutations of the PI 4-kinases nor deletions of PI4P phosphatases have yet been discovered in cancer. While there is some evidence that stimulation of PI4KIIIβ activity by the oncogenic eukaryotic protein elongation factor eEF1A2 may have a role in the development of metastatic breast cancer [150–152], it is not yet clear whether increased catalytic or non-catalytic functionality of the PI 4-kinases, or even a combination of both properties, are important for oncogenesis. However, the balance of evidence so far suggests that defective vesicular trafficking, an important parameter in mediating the spatiotemporal control of receptor signalling, may be an important contributory factor in malignancies associated with augmented PI 4-kinase expression.

5.2. Neurological disease

Decreases in PI 4-kinase expression levels have also been associated with neuronal dysfunction and in particular the impaired survival of specific cell populations within the CNS. Reduced PI4KIIIα expression in the CA1 region of the hippocampus following surgically induced-transient ischemia [153], while PI4KIIIβ expression is reduced in the brain of rodents subject to chronic ethanol consumption [154]. There is also some evidence that polymorphisms in the PIK4CA gene which encodes for PI4KIIIα may be associated with genetic predisposition to schizophrenia [155] and psychiatric disorders associated with chromosome 22q.11 [156,157], although this may be confined to particular, restricted populations [158].

While cell-based studies have revealed important roles for the PI 4-kinases in neuronal vesicular trafficking [11] it is not yet clear which PI 4-kinase dependent functions lead to aberrant neuronal survival. However, Genetrap mice which do not express PI4KIIα are subject to selective cerebellar cell loss and progression to cerebellar spinal degeneration which is ultimately fatal [159]. Interestingly, transgenic animals with knocked-out PI4KIIIα expression or those with a conditional knock-in of a lipid kinase inactive version of this isozyme do not exhibit any neurological abnormalities [160]. This may be an indication that the non-catalytic functions of PI4KIIα may be important for neuronal survival since unlike PI4P generation, these isoform-specific functions are less likely to be compensated for by other isozymes. Cell based studies have shown that PI4KIIα is recruited to clathrin-coated vesicles through interactions with the clathrin adaptor AP-3 and is part of the protein complex that transports proteins such as dysbindin [11] and calcyon [161]. These observations intimate that at least for PI4KIIα, alterations to clathrin-dependent intracellular trafficking could possibly be important for maintaining neuronal viability.

5.3. Bacterial infection

All of the mammalian PI 4-kinases have roles in either bacterial entry or replication. In the case of *Listeria monocytogenes*, which enters the cell through initial binding of the bacterial protein InlB to the Met hepatocyte growth factor receptor in membrane sites that also contain the tetraspanin CD81 [162]. Additionally both PI4KIIα and PI4KIIβ are required for bacterial internalisation and this may be in part mediated by these isozymes regulating cell surface levels of CD81 through affects on the intracellular trafficking dynamics of this protein [40]. This is an interesting parallel with the proposed role for PI4KIIβ in hepatocellular carcinoma where alterations to cell adhesion and motility are due to this isoform regulating cell surface levels of CD81 [40]. PI4KIIα has also been localised along with the PI4P producing PI(4,5)P_2 5-phosphatase OCRL to the surface of *Chlamydia trachomatis* inclusions [163]. These bacterial inclusions are non-acidified vacuoles that fuse with the Golgi and multivesicular bodies during the cytosolic remodelling phase of their development. Protected within inclusions, *Chlamydiae* can survive and replicate whilst avoiding trafficking to the lysosomes. Further evidence for PI 4-kinase involvement in this disease mechanism stems from the observation that the PI4P-binding and ceramide transporting protein CERT is recruited to the inclusions at sites of interorganelle contact with the ER [164,165]. Therefore, PI4P synthesis on *Chlamydial* inclusions sustains the lipid identity of this replicative membrane compartment and thus the trafficking of this structure away from lysosomes but towards the perinuclear region of the cell.

The PI4KIIIβ isoform has been implicated in the replication of *Legionella pneumophila* [166], a process which occurs inside intracellular vacuoles and is concomitant with the subversion and interference of host cell lipid signalling and trafficking pathways [167]. Amongst the bacterial proteins translocated into the host cytosol are two proteins called SidC and SidM/DrrA [167–169] that compete for a limited pool of PI4KIIIβ-synthesised PI4P on the cytosolic-facing vacuolar membrane. DrrA is of particular note since the carboxy terminal helical domain of this protein mediates the highest yet recorded binding affinity for PI4P [169]. These PI4P-anchored proteins can redirect membrane trafficking by an unusual mechanism involving the recruitment and activation of Rab1b by DrrA-catalyzed adenosine monophosphorylation (AMPylation) of the small GTPase on tyrosine 77 [170]. This PI4P-dependent, covalent modification of Rab1b causes ER-derived vesicles to be recruited to the replicative vacuole resulting in a major diversion of intracellular membrane trafficking pathways [170–172] Therefore, bacterial entry is dependent on PI 4-kinases to transport the bacterium into the cell in association with endogenous cell surface binding partners while replication depends on silencing and masking of phagosomal PI4P functions which would otherwise result in host-mediated elimination.

Finally, PI4KIIIα has been identified in an RNA interference screen as an endogenous host factor along with an ubiquitin hydrolase USP22, and the ubiquitin ligase CDC27, which are required for the intracellular replication of *Francisella tularensis* [173]. Unlike the scenarios with *Chlamydia* and *Legionella* where PI4P production maintains a pro-replicative membrane environ-

ment through subversion of host membrane trafficking, PI4KIIIα is required during the later cytosolic phase of *Francisella* proliferation. Hence these recent findings demonstrate that PI 4-kinases can regulate multiple steps in different phases of infection by pathogenic bacteria and with the possible exception of PI4KIIIα this occurs by molecular exploitation and modulation of host endosomal trafficking pathways that are dependent on PI4P generation.

5.4. Viral disease

In the past three years there has been huge progress in identifying PI 4-kinases as essential host factors required for the propagation of a number of RNA viruses including those responsible for human diseases such as Hepatitis C [49,160,174–184], polio [185] and severe acute respiratory syndrome (SARS) [186]. Similar to the emerging functions for PI 4-kianses in bacterial infection, PI4P production has been implicated in the generation of intracellular membranous web-like structures that provide an environment supportive of pathogen replication. This could be viewed as further evidence for the role of PI 4-kinases and particularly the PI4KIIIs in promoting organelle biogenesis at the level of lipid synthesis. One important difference with all other pathologies involving the PI 4-kinases is that viral proteins actively stimulate PI4P synthesis through direct binding [49]. To date, PI4KIIIβ has been implicated in the intracellular RNA replication of several enteroviruses [185,187,188] and in the endosomal fusion phase of SARS coronoavirus [186] infection. While in the case of the Hepatitis C virus (HCV), PI4KIIIα has emerged from RNA interference screens and pharmacological studies as a host protein that is bound and activated by the non-structural viral protein NS5A [49,181] to produce large amounts of PI4P and thus an ER-derived membranous web that promotes viral replication [51,174,189] (Fig. 2). This represents the first example of activated PI 4-kinase activity having a pathological role. Unlike the scenario with PI4P on the surface of bacterial vacuoles which is exploited by bacterial proteins to redirect intracellular trafficking there is as yet little evidence that formation of the large ER-derived PI4P rich web structure impairs vesicular trafficking along the secretory pathway. Indeed the presence of the Golgi-localised PI4KIIIβ isoform is required for the propagation of Hepatitis C virus [182,183] and enteroviruses [185,188].

There is a possibility that the PI4P enriched surface of the membranous web may act as a docking surface for host, as opposed to viral proteins that contain apposite binding domains. In concordance with this hypothesis both OSBP [190] and CERT [191] – proteins with key roles in non-phosphoinostide lipid transfer and sensing at the TGN, have been identified as additional host factors required for HCV replication. This raises another issue as to whether such a massive enrichment of PI4P molecularly defines the identity of this virally induced organelle in terms of its surface electrostatics and also in the recruitment and homeostasis of other components of this membrane compartment [192]. Of relevance to this idea is the observation that host sphingolipid synthesis, a process known to be under the control of PI 4-kinases, is known to be greatly upregulated during HCV infection [193,194]. In addition, the PI4P-binding protein OSBP, which may control CERT-mediated ceramide transfer to sphingomyelin synthase, interacts with the amino terminal domain I of the HCV protein NS5A protein at the TGN and is required for the process of HCV maturation [190,191].

Another possible outcome of virally-induced PI4KIIIβ activation is greatly enhanced signalling due to elevated PI4P substrate levels. Consistent with this proposal, a number of reports have demonstrated HCV-induced alterations to pro-oncogenic signalling pathways [195–199]. It remains to be established though, if elevated PI4P at the virally-induced compartment constitutes a signalling competent pool capable of supplying substrate to upstream phosphoinositide kinases and phospholipases at the plasma membrane and endosomes. Another proviso is that alterations to signalling and trafficking could be accounted for by the interactions of viral proteins with multiple host proteins. As an example, in addition to PI4KIIIβ, NS5A can bind other host proteins including the cellular retinoic acid binding protein 1, centaurindelta2 [174] and other lipid metabolizing enzymes such as calcium-dependent, group IVA phospholipase A2 [200]. In terms of PI signalling, Cenaturindelta2, also known as ARAP1, is especially interesting as it is a multi-domain protein that contains phosphoinositide binding PH domains, ankyrin repeats as well as GAP domains specific for Arf and Rho GTPases [201,202]. Furthermore, the related protein ARAP2 has been implicated in the internalization of *Listeria monocytogenes* which suggests the engagement of similar host factors for bacterial and viral infections [203]. Therefore it seems likely that alterations to PI4KIIIα activity may contribute to an overall systems level change in signalling outputs during HCV infection [192,200].

6. Pharmacological targeting of the PI 4-kinases and future perspectives

Due to their emerging and important role in several diseases there has been some interest in developing small molecule, isoform-selective inhibitors of the mammalian PI 4-kinases. There has been some progress in identifying reasonably specific inhibitors of the PI4KIIIs. Particularly noteworthy in this regard are PIK93, the anti-enteroviral T-00127-HEV1 and some aminoimidazole inhibitors developed by Novartis [183] all of which selectively inhibit PI4KIIIβ. Although there is now one report demonstrating that some coxsackievirus mutants can overcome their dependence on PI4P for their intracellular replication [187], suggesting that the acquisition of resistance may become a problem in targeting host PI4KIIIβ in viral disease. As regards PI4KIIIα the anti-viral molecules AL-9 – a 4-anilino quinazoline molecule [51], and the Boehringer Ingelheim compounds A and B [160] are all newly identified, selective inhibitors of this isoform. This contrasts with the current situation with the PI4KIIs where with the exception of less specific molecules such as resveratrol [204,205], and epigallocatechin gallate [206] there has been little progress. Nevertheless, the pharmacological targeting of individual isoforms may not be straightforward and this may in part due to the multiplicity of cell functions controlled by the PI 4-kinases coupled with compensation particularly at the level of PI4P synthesis – a common function shared by all isoforms. Hence to effectively silence PI 4-kinase-dependent trafficking, it may also be necessary to inhibit their modular protein-binding functions. A good example of such a scenario is the role of PI4KIIα in the recruitment of AP-3 on late endosomal membranes as this function requires both PI4P synthesis and a dileucine AP-3 interaction motif [7]. Therefore, co-incident targeting of more than one aspect of PI 4-kinase structure may be required to silence a single biological function. To extend this idea, there are now documented instances where more than one PI 4-kinase controls distinct steps along the same trafficking pathway. This has been observed in yeast in the sequential recruitment of AP-1 and clathrin adaptors at the TGN [207], and in mammalian cells in the trafficking of β–glucocerebrosidase enzyme from the TGN to lysosomes in distinct steps requiring PI4KIIIβ and PI4KIIα consecutively [17]. These new insights suggest that it may be necessary to inhibit the activity of more than one PI 4-kinase isoform to comprehensively suppress a particular biological pathway.

A further challenge in targeting the PI 4-kinases has emerged from more recent studies which have revealed differential cell and tissue dependencies on the PI 4-kinase isoforms that are not always predictable. As an example, animal studies have shown that

genetic knockdown of PI4KIIIα leads to severe changes in the gastrointestinal mucosal epithelium [160] whereas loss of PI4KIIα leads to selective loss of specific neuronal cell populations such as cerebellar Purkinje cells [159]. Therefore, it is not yet clear if inhibiting individual PI 4-kinase isoforms in a therapeutic setting represents a feasible strategy. However, a more comprehensive knowledge of the roles of different PI 4-kinase permutations in modulating lipid metabolism, signalling and trafficking, may illuminate which PI4P pathways to target in human disease.

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