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A Conspicuous Connection: Structure Defines Function for the Phosphatidylinositol-Phosphate Kinase Family

Jessica N. Heck, David L. Mellman, Kun Ling, Yue Sun, and Matthew P. Wagoner
Program in Molecular and Cellular Pharmacology, University of Wisconsin-Madison, Department of Pharmacology, University of Wisconsin Medical School, Madison, WI, USA

Nicholas J. Schill
Program in Cellular and Molecular Biology, University of Wisconsin-Madison, Department of Pharmacology, University of Wisconsin Medical School, Madison, WI, USA

Richard A. Anderson
Program in Molecular and Cellular Pharmacology, University of Wisconsin-Madison, Department of Pharmacology, University of Wisconsin Medical School, Madison, WI, USA

Address correspondence to Richard A. Anderson, Professor of Pharmacology, Director, Molecular and Cellular, Pharmacology Program, University of Wisconsin Medical School, Medical Science Center, Room 3750, 1300 University Ave., Madison, WI 53706, USA. E-mail: raanders@wisc.edu

ABSTRACT The phosphatidylinositol phosphate (PIP) kinases are a unique family of enzymes that generate an assortment of lipid messengers, including the pivotal second messenger phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$). While members of the PIP kinase family function by catalyzing a similar phosphorylation reaction, the specificity loop of each PIP kinase subfamily determines substrate preference and partially influences distinct subcellular targeting. Specific protein-protein interactions that are unique to particular isoforms or splice variants play a key role in targeting PIP kinases to appropriate subcellular compartments to facilitate the localized generation of $PI4,5P_2$ proximal to effectors, a mechanism key for the function of $PI4,5P_2$ as a second messenger. This review documents the discovery of the PIP kinases and their signaling products, and summarizes our current understanding of the mechanisms underlying the localized generation of $PI4,5P_2$ by PIP kinases for the regulation of cellular events including actin cytoskeleton dynamics, vesicular trafficking, cell migration, and an assortment of nuclear events.

KEYWORDS phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$), PIP kinase structure, phosphoinositide signaling, lipid messengers

DISCOVERY OF PHOSPHATIDYLINOSITOL-4,5 BISPHOSPHATE ($PI4,5P_2$)

The phosphoinositol cycle was discovered in the 1950s by Lowell and Mabel Hokin (Hokin and Hokin, 1953). At this time, they proposed that phosphatidylinositol (PI) and phosphatidic acid (PA) were major components of the phosphoinositol cycle, however the cycle's intermediates remained unclear (Hokin and Hokin, 1955a, 1955b). Soon after their initial discovery, the Hokins discovered that PI could be sequentially phosphorylated on its myo-inositol ring to generate a phosphoinositide bisphosphate that was likely phosphatidylinositol-4,5 bisphosphate ($PI4,5P_2$) and was thought to be a precursor to other lipid signaling molecules (Dixon and Hokin, 1984; Hokin,

1969, 1985; Hokin and Hokin, 1953, 1954, 1955a, 1955b, 1956, 1964, 1965). While PI4,5P_2 was originally thought of as a signaling intermediate and metabolic precursor of other soluble lipid second messengers such as inositol trisphosphate (IP_3) (Sekar and Hokin, 1986), today PI4,5P_2 is widely recognized as a potent messenger itself, and has taken center stage as a major mediator of biochemical activities and cellular functions (Czech, 2000; Doughman *et al.*, 2003a; McLaughlin *et al.*, 2002; Yin and Janmey, 2003).

A series of discoveries in the 1980s changed the general perception of phosphoinositide signaling as it gained in importance and complexity. The newly defined second messenger PI4,5P_2 directly interacted with and modulated the function of actin modifying proteins, which in turn regulated assembly of the actin cytoskeleton (Anderson and Marchesi, 1985; Janmey and Stossel, 1987; Lassing and Lindberg, 1985). It was also demonstrated that PI4,5P_2 regulated the interactions between cytoskeletal proteins and the plasma membrane (Anderson and Marchesi, 1985). More recently, these initial discoveries surrounding the function of PI4,5P_2 as a second messenger have blossomed into an array of protein interactions and cellular processes that are directly regulated by PI4,5P_2, including vesicular trafficking (Czech, 2000; Downes *et al.*, 2005; Huijbregts *et al.*, 2000; Wenk and De Camilli, 2004), secretion (Hay *et al.*, 1995; Martin, 2001), cell motility/cytoskeletal assembly (Janmey, 1994; Niggli, 2005; Yin and Janmey, 2003), regulation of ion channels (Delmas *et al.*, 2005; Huang *et al.*, 1998; Li *et al.*, 2005; Suh and Hille, 2005), and nuclear signaling pathways (Boronenkov *et al.*, 1998; Cocco *et al.*, 1987; Gonzales and Anderson, 2006; Irvine, 2002).

The last decade has been fruitful in strengthening our biochemical understanding of phosphoinositides, most notably PI4,5P_2. The functional regulation of cellular events by a lipid such as PI4,5P_2 seems to be a convenient and utilitarian biological 'message' and suggests that other phosphoinositide phosphate derivatives may function in a similar manner. It is now understood that the PI head group can be phosphorylated at the 3, 4, and 5 hydroxyl positions in all combinations to generate an assortment of phosphoinosotides that function as second messengers in eukaryotes to regulate specific events and functions (Pertile *et al.*, 1995; Rameh *et al.*, 1997; Tolias *et al.*, 1998).

The vast assortment of lipids present in eukaryotic cells provokes the question, 'why lipid messengers'? The large number of specialized membrane compartments in complex eukaryotes results in a well organized and highly polarized structural arrangement in cells. To regulate intracellular polarization and maintain organization of the plethora of membrane compartments, cells require a pool of highly flexible messages to act as molecular 'address labels' for which soluble messages are not suitable. In this model, phosphoinositide messengers are specifically labeled for delivery to a membrane compartment, where they direct targeting and fusion with other compartments or regulate compartmental assembly of specific cytoskeletal components through direct regulation of their functions. Once a destination has been reached and function carried out, phosphoinositide messengers are easily re-addressed by specific phosphatases that remove previous shipping directions and kinases that stereospecifically apply a new address label to the lipid messenger.

DISCOVERY OF THE PHOSPHATIDYLINOSITOL PHOSPHATE (PIP) KINASES

Phosphatidylinositol-phosphate (PIP) kinase activity was identified by the Hokin's in the early 1960s (Hokin and Hokin, 1964); however, studies surrounding the PIP kinases were not pursued because the insoluble lipids they generated were thought to be less important than their soluble inositol phosphate counterparts (Rana and Hokin, 1990). The PIP kinases were successfully purified for characterization from erythrocytes nearly three decades later (Bazenet *et al.*, 1990; Jenkins *et al.*, 1994; Ling *et al.*, 1989), and it was immediately clear that these enzymes were more complex than originally thought, as erythrocytes had multiple PIP kinases (Bazenet *et al.*, 1990). *In vitro* lipid kinase assays allowed for quantification of PIP kinase activities in cell lysates (Bazenet *et al.*, 1990; Jenkins *et al.*, 1994; Ling *et al.*, 1989) and were originally carried out using PI4P purified from bovine brain and a phosphate donor γ-^{32}P-ATP to generate PI4,5P_2 that could be analyzed by thin layer chromatography (Parker *et al.*, 1998). The first chromatographic step in the isolation of PIP kinase activity from red blood cells required a phosphocellulose ion exchange column, which separated PIP kinase activity into two pools. The

biochemical characterization of these separate pools of activity revealed that they were the result of two distinct enzymes (Bazenet *et al.*, 1990).

The distinct pools of PIP kinase activity were designated the type I and II PIP kinases, for the first and second peaks of activity, and this nomenclature has remained since their discovery. In the red blood cell, the type I PIP kinase (PIPKI) is a peripheral membrane protein whereas the type II PIP kinase (PIPKII) is largely cytosolic (Bazenet *et al.*, 1990). While the type I and II PIP kinases demonstrated very different enzymatic properties, the most striking difference between them was that PIPKI had activity toward red blood cell membranes and generated PI4,5P_2, while PIPKII was unable to use the PIP substrate in these membranes (Bazenet *et al.*, 1990). Additionally, isolation of PIPKI from erythrocytes revealed that this enzyme was stimulated by phosphatidic acid, a property later identified as common to all PIPKI isoforms (Jenkins *et al.*, 1994). Furthermore, antibodies raised to the erythrocyte PIPKI that had an apparent size of 68 kDa by SDS-PAGE, detected multiple immunoreactive proteins in the brain; PIPKI isoforms in the brain had apparent sizes of 90 to 100 kDa by SDS-PAGE and each corresponded to pools of PIP kinase activity. This was the first indication that there were multiple PIPKI isoforms, and as discussed below, these PIPKI immuno-cross-reactive enzymes represent the present day human PIPKIγ isoform (Jenkins *et al.*, 1994).

A NOVEL FAMILY OF KINASES BY SEQUENCE

Purification of the type I and II PIP kinases lead to their protein sequence and isolation of cDNAs encoding the initial representatives of the PIP kinase subfamilies: type I (PIPKI), II (PIPKII) and III (PIPKIII) PIP kinases. The PIPKIIα isoform was the first of the PIP kinases to be cloned from the human erythrocyte and interestingly, its sequence did not share statistically significant homology with any other known kinase. However, phosphoinosotide 3-kinase (PI3K) and phosphoinosotide 4-kinase (PI4K) showed substantial identity and similarity to protein kinases (Boronenkov and Anderson, 1995). Upon further examination, the PIPKIIα sequence was similar to two gene products of *S. cerevisiae*, Mss4p and Fab1p (Boronenkov and Anderson, 1995). The amino acid homology of PIPKIIα with Mss4p and Fab1p is clustered in a set of invariant residues that represent the catalytic core of the PIP kinase family. As shown in Figure 1, when the sequences of the human PIP kinases are aligned against the divergent yeast enzymes, these invariant residues align with perfect identity and are thought to represent the putative catalytic core. The catalytic residues of the PIP kinases are most similar to the conserved catalytic sequences of protein kinases and will be discussed in more detail below (Anderson *et al.*, 1999; Hanks *et al.*, 1988; Loijens and Anderson, 1996). Identification of Mss4p and Fab1p as PIP kinases was an important advancement in the understanding of PIP kinase function, as these kinases are highly divergent members of the PIPKI (Mss4p) and PIPKIII (Fab1p) subfamilies.

MULTIPLE SUBSTRATES AND PRODUCTS

A key feature of the PIP kinase family of enzymes is the unique signaling specificity of each PIP kinase subfamily (Anderson *et al.*, 1999). PIP kinases were originally purified by their activities toward PI4P as it was postulated that this family of kinases phosphorylated the 5-hydroxyl position on the myo-inositol ring to generate PI4,5P_2. Today it is understood that the type I PIP kinase subfamily members (α, β, and γ consistent with human nomenclature) preferentially utilize PI4P as substrate to catalyze this reaction and generate the majority of PI4,5P_2 in the cell, while type II PIP kinases synthesize PI4,5P_2 using PI5P as substrate.

Synthetic forms of PIP kinase substrates including all possible PIP isomers became commercially available in the late 1990's (Prestwich, 2004). Using the new synthetic PIP isomers, it was demonstrated that in addition to PI4P, PIPKI also had activity toward PI3P (Zhang *et al.*, 1997). Type I PIP kinases demonstrate similar K_m values for both PI3P and PI4P, however PI3P has a lower V_m compared to PI4P. When PI3P is used as a substrate, PIPKI sequentially phosphorylates the 4-hydroxyl position of the myo-inositol ring to generate PI3,4P_2, followed by concerted phosphorylation of the 5-hydroxyl to generate PI3,4,5P_3. As shown in Figure 2, the flexibility of the PIPKI to utilize multiple substrates in order to generate distinct second messenger products is significant, however, at that time, *in vivo* PIPKI activity toward PI3P in higher eukaryotes was not well defined. Today, fission yeast provide indirect evidence for the necessity of flexible substrate use *in vivo*; in

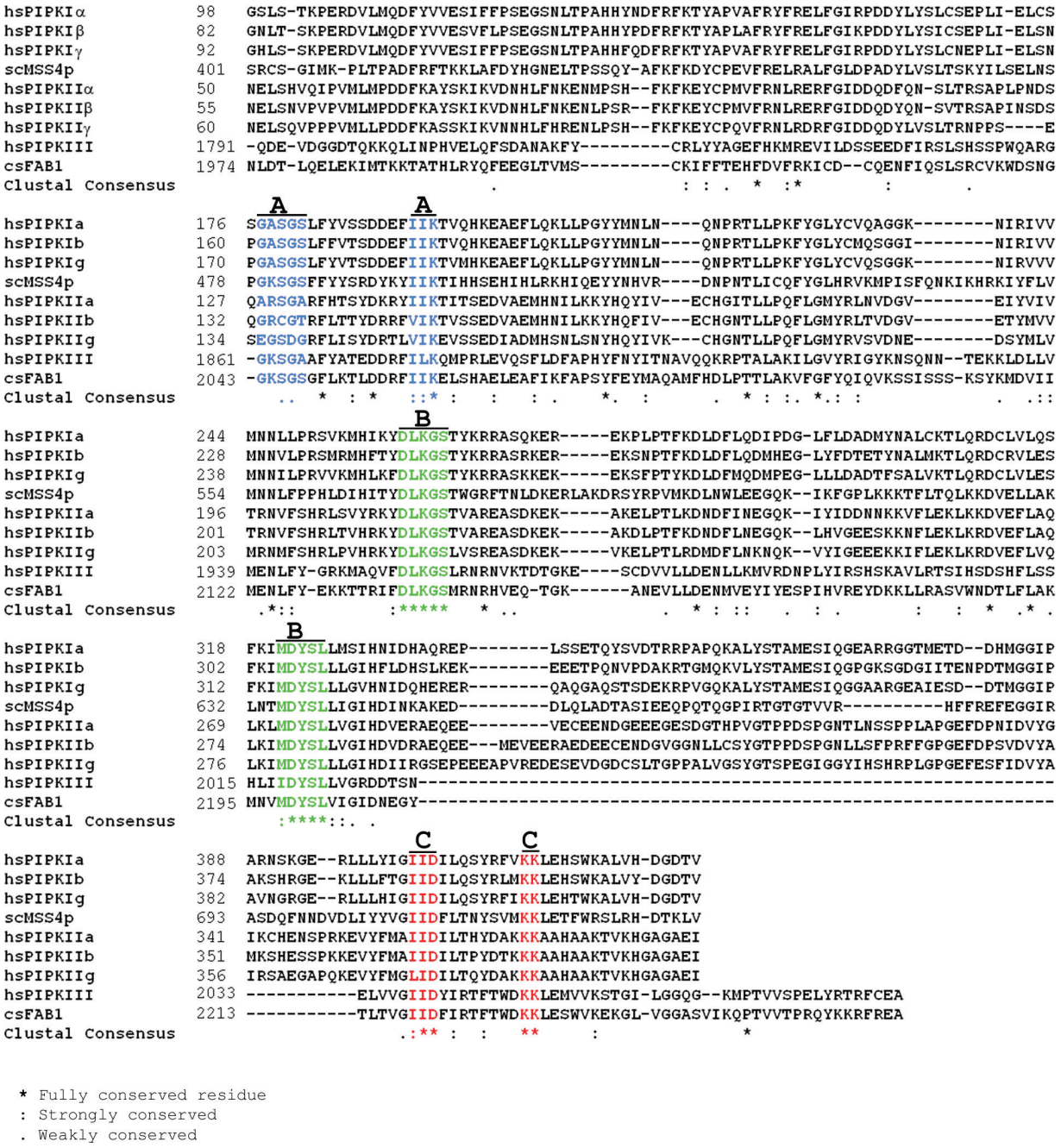

Figure 1 **Sequence comparison of diverse members of the PIP kinase family. When the sequences of human PIP kinases are aligned with divergent yeast enzymes including Fab1p (PIPKI) and Mss4p (PIPKIII), the set of invariant residues which make up catalytic core of the PIP kinase family align with perfect identity. Residues required for ATP binding (A), substrate specificity, (B) and catalysis (C) are highlighted.**

S. pombe the type I PIP kinase its3p acts downstream of the class III PI3 kinase vps34p to generate PI3,4,5P_3 (Mitra *et al.*, 2004). Vps34p requires PI as substrate and can only generate PI3P, suggesting that its3p must sequentially phosphorylate PI3P to generate PI3,4,5P_3. When its3p PIP kinase activity is reduced, cellular PI4,5P_2 is also dramatically reduced, possibly resulting in secondary effects on PI3,4,5P_3 synthesis (Mitra *et al.*, 2004). These results are intriguing and suggest that the flexibility of substrate specificity of type I PIP kinases may still be relevant *in vivo*.

PIPKIIα and PIPKIIβ were first identified and characterized by their activity toward PI4P substrate purified from bovine brain (Bazenet *et al.*, 1990; Boronenkov and Anderson, 1995; Ling *et al.*, 1989). The PIPKII isoforms demonstrated poor activity toward synthetic PI3P and PI4P compared to the PIPKI enzymes, suggesting that this subfamily may utilize an alternate

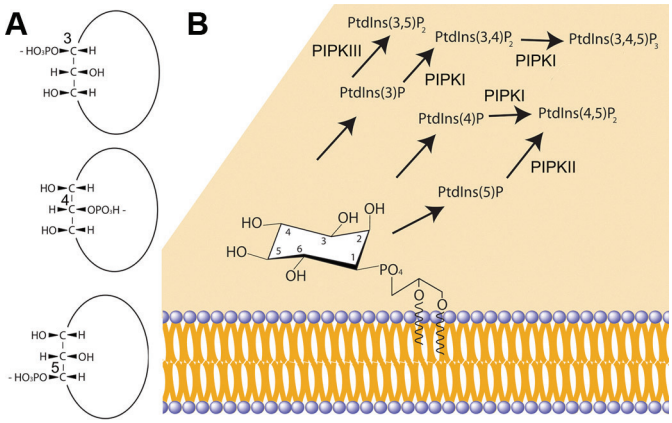

Figure 2 **PIP kinases use multiple substrates to generate multiple products. (*A*) A key distinguishing feature of the PIP kinase family is their ability to utilize multiple substrates to generate products while at the same time demonstrating increased affinity for a preferred substrate. The major substrate for each PIP kinase subfamily as well as the stereochemistry around the required monophosphate is shown. (*B*) PIP is synthesized by PI kinases from phosphatidylinositol and is used by PIP kinases to generate a number of lipid products including PI4,5P_2. The potential substrates of each PIP kinase subfamily and products they generate are shown.**

PIP substrate more efficiently. PI5P was identified as the *in vivo* substrate for type II enzymes, and PIPKIIβ utilized synthetic PI5P most efficiently *in vitro* (Rameh *et al.*, 1997). PIPKIIβ phosphorylates PI5P on the 4-hydroxyl of the myo-inositol ring to generate PI4,5P_2 by an alternative pathway (Rameh *et al.*, 1997); PIPKIIα and PIPKIIγ also preferentially use PI5P as substrate (Itoh *et al.*, 1998). While enzymatically type I and II PIP kinases catalyze a basic reaction where a phosphate group is added to a directly adjacent hydroxyl of a PIP substrate as depicted in Figure 2, these kinases are not functionally redundant. In *S. cerevisiae*, deletion of the type I PIP kinase Mss4p can be rescued by expressing the type I PIP kinase human homolog, but cannot be rescued by the expression of the type II PIP kinase (Homma *et al.*, 1998) suggesting that type I and type II PIP kinases modulate distinct cellular functions.

While the cellular functions of the type I PIP kinases are well documented, recent major breakthroughs have helped begin to define biological functions for the type II PIP kinases. It has been demonstrated that PIPKIIβ knock out mice exhibit insulin hypersensitivity and have reduced body weights compared to their wild-type litter mates. Additionally, enhanced *in vivo* AKT activation in skeletal muscle and liver from mice lacking PIPKIIβ suggest increased insulin signaling. These knockout mice demonstrated that PIPKIIβ plays a role in determining insulin sensitivity and adiposity *in vivo*, and as the authors suggest, from a pharmacological perspective PIPKIIβ could be an enzyme to target for inhibition in the clinical treatment of type II diabetes (Lamia *et al.*, 2004).

Nuclear localized PIPKIIβ has also been demonstrated to mediate stress response by modulating levels of PI5P. PI5P is an essential cofactor for the PHD finger-containing tumor suppressor protein ING2 (Inhibitor of Growth protein 2) and this interaction is regulated by the lipid kinase activity of PIPKIIβ (Gozani *et al.*, 2003). Under non-stressful conditions, nuclear PIPKIIβ is active and converts PI5P to PI4,5P_2 to maintain basal levels of PI4,5P_2. However, in response to cellular stress such as oxidative stress or UV irradiation, PIPKIIβ is phosphorylated downstream of activated p38-MAPK signaling resulting in inhibition of its lipid kinase activity and subsequently, a rise in nuclear PI5P. Increased nuclear PI5P promotes the translocation and activation of ING2, which in turn enhances the acetylation of p53 to promote p53-dependent processes such as apoptosis (Jones *et al.*, 2006).

While the type III PIP kinase subfamily (PIPKIII) is the most sequence divergent of the PIP kinases (Anderson *et al.*, 1999), these enzymes still contain all of the conserved catalytic residues characteristic of the larger family (Figures 1 and 3). The type III PIPK are large proteins with an elaborate domain structure; in addition to the kinase domain located at the far C-terminus, the type III PIP kinases also contain an N terminal FYVE domain, a DEP domain, a chaperonin-like domain, a cysteine-rich domain and appear to be conserved from yeast to humans. PIPKIII was first discovered in *S. cerevisiae* as a homolog of PIPKIIα and the yeast enzyme Fab1p (Boronenkov and Anderson, 1995). Its mammalian homolog was named PIKfyve, an acronym for phosphoinositide kinase with a specificity for the 5′-hydroxyl position on the myo-inositol ring, and contains a fyve finger domain (Sbrissa *et al.*, 1999). The activities of Fab1p and PIKfyve *in vitro* suggest that the yeast and mammalian homologs show specificity for phosphorylation of the 5-hydroxyl on the myo-inositol ring. *In vitro*, these enzymes preferentially use PI3P as substrate to generate PI3,5P_2 (McEwen *et al.*, 1999) and weakly phosphorylate the 5-hydroxyl position of PI, however are unable to synthesize PI4,5P_2 from PI4P. Consistent with their activity *in vitro*, PIPKIIIs favor PI3P as substrate to generate PI3,5P_2 *in vivo* (Dove *et al.*, 1997).

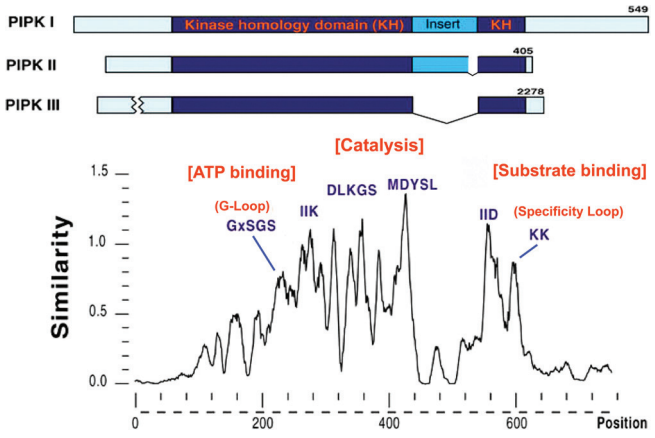

Figure 3 **Core catalytic residues are conserved within the PIP kinase family. The PIP kinase subfamilies are represented schematically to illustrate the homology within their kinase domains. The sequenced regions shown in the homology plot represent the invariant catalytic regions of the PIP kinases required for ATP binding, substrate binding, and catalysis and demonstrate that while the stereochemical recognition of PIP substrates by PIP kinase subfamilies is variable, the core residues required for catalysis are conserved. The specificity loop lies within the far C-terminal region of the kinase domain. A detailed depiction of the specificity loop is illustrated in Figure 5C.**

While the function of PIPKIII appears to be multifold (Cooke *et al.*, 1998; Dove *et al.*, 1997, 2002; Efe *et al.*, 2005; Mitra *et al.*, 2004; Sbrissa *et al.*, 1999; Yamamoto *et al.*, 1995) these functions all appear to be dependent on the generation of PI3P by the yeast PI3-kinase, vps34p (Dove *et al.*, 1997). In yeast, PIPKIII enzymes function in vesicular trafficking of membrane proteins, morphogenesis of the central vacuole, and Fab1p is important for the generation of $PI3,5P_2$ after hyperosmotic stimulation (Dove *et al.*, 1997). This supports a model where the PI kinase, in this case vps34p, generates substrate for the PIP kinase in a compartment specific fashion (discussed further below).

As a family, the PIP kinases demonstrate flexibility in substrate specificity *in vitro* and as a result, the second messengers they generate. This fundamental characteristic was originally discovered in the PIPKI family members and has now been attributed to all three subfamilies (Anderson *et al.*, 1999). Each subfamily demonstrates a relatively high specific activity for a particular PIPn isomer, and also has activity toward other PIPn species with lower efficacy. This demonstrates that the catalytic site of these kinases is sufficiently flexible to accommodate multiple substrates differing in the stereochemistry around the required mono-phosphate of the PIP substrate. PIP substrates are illustrated in Figure 2. In order for the PIP kinase active site to accommodate multiple substrates, there must be a substantial change in the orientation of the three substrates within the active site (Figure 2). In addition, the specificity of PIPKIII is different from the PIPKI and PIPKII subfamilies, as the 5-hydroxyl position it phosphorylates is not directly adjacent to the PI3P mono-phosphate. As shown in Figure 3, while the stereochemical recognition of the PIP substrate is variable, the core catalytic residues are conserved within the PIP kinase family.

It is important to note that the availability of PIP substrates in physiological membranes is significantly different than substrates used *in vitro*. *In vitro* lipid kinase assays performed in the absence of detergents use lipid micelles with 100 mole percent PIP substrate to measure the relative activity of PIP kinases. However, in cells PI4P is the most abundant PIP and is approximately 1 mole percent of the total phospholipids on the cytoplasmic face of membranes. The concentration of PI3P and PI5P *in vivo* are more than 10-fold less than PI4P, however these substrates at likely concentrated in specific subcellular compartments. As a result, *in vitro* kinase activities are measured in an environment that is very different than the *in vivo* milieu of a cellular membrane. For this reason, the behavior of PIP kinases needs to be understood in the context of their interaction with membranes where their substrate is a minor component.

STRUCTURE DEFINES A MECHANISM FOR MEMBRANE ASSEMBLY AND KINASE ACTIVITY

The best understanding of how PIP kinases function at the molecular level comes from the resolved structure of PIPKIIβ. The quaternary structure of PIPKIIβ consists of two relatively globular monomers that associate to form a dimer in solution (Rao *et al.*, 1998). As depicted in Figure 4A, the globular monomers form a dimer interface by a subunit-spanning anti-parallel N-terminal β sheet that joins the two β strands by packing between two α helices to create a clasp-like interface. While it is not clear whether the equilibrium between the dimeric and monomeric state is dynamic or regulated, dimer formation is a potential mechanism to modulate assembly onto membranes *in vivo*.

The PIPKIIβ dimer is an elongated disc approximately 100 × 40 × 30 Å in dimension. Figure 4C illustrates that the globular monomers join to form the

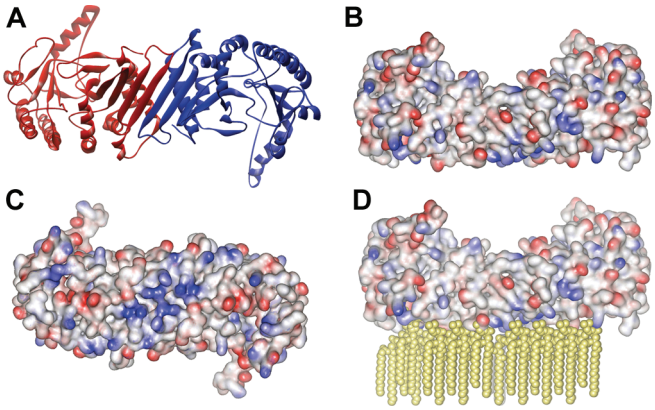

Figure 4 **The structure of PIPKIIβ is a model for the kinase domain of all PIP kinases. (*A*) A ribbon structure of PIPKIIβ depicts the dimerization of PIPKIIβ globular monomers to form a clasp-like interface. (*B*) A side view of the space filling structure of PIPKIIβ shows the flat interface available for membrane association. (*C*) A space filling model of the PIPKIIβ dimer looking up from the membrane highlights the basic patch (*blue*) key for interactions with the negatively charged membrane. (*D*) The space filling model of PIPKIIβ as depicted in (B) docked on one leaflet of a phospholipid bilayer shows that while PIPKIIβ does not penetrate the lipid bilayer, nearly the entire basic patch is in contact with the membrane.**

central face of the disc, which contains 14 lysine (K), 4 histidine (H), and 4 arginine (R) residues, which form a highly basic flat patch (shown in blue) peripherally decorated with acidic residues (shown in red). As shown in Figures 4A and B, this relatively flat 30 × 50 Å basic patch with a net charge of +14 extends across the dimer interface suggesting this region functions as the major site for association with the negatively charged membrane interface (Rao *et al.*, 1998).

A triple lysine (K) to glutamate (E) mutation reduced the charge on the dimer face from +14 to +2 and disrupted membrane association, demonstrating that the basic interface described is key for simple association with a negatively charged membrane (Burden *et al.*, 1999). While these mutations do not affect PIPKIIβ dimerization, there was a 16-fold decrease in the apparent K_a for mixed liposomes containing 25% phosphatidylserine/75% phosphatidylcholine vesicles, indicating that reducing the positive charge of the interface reduces membrane association *in vitro*. Figure 4D is a model of the PIPKIIβ docked at a lipid bilayer where PIPKIIβ interacts with the phospholipid surface in a manner by which nearly the entire basic patch is in contact with phospholipid head groups, however the PIP kinase does not penetrate the lipid bilayer. From these data it is evident that PIPKIIβ interacts with model membranes through electrostatic interactions that occur between its basic patch and the negatively charged lipid headgroups (Burden *et al.*, 1999).

In a reaction analogous to that of protein kinases, PIPKIIβ transfers the high-energy γ-phosphate from ATP (or GTP) to the 4-hydroxyl of PI5P substrate. While there is no statistically significant sequence homology between protein kinases and the PIP kinase family, there is significant structural homology between PIPKIIβ and protein kinases. The structural homology described lies within the ATP binding site of the PIP kinase catalytic core and additional conserved residues within the PIP kinase invariant regions spatially align with the catalytic residues of protein kinases. Approximately 20% of PIPKII isoform residues conform to the protein kinase fold and compose the most divergent structural homolog of protein kinases.

There are three residues that are absolutely conserved among protein kinases or PIP kinases; K150, D278, and D369 in PIPKIIβ correspond to K72, D166, and D184 of protein kinase A (PKA). K150 of the II**K** motif in PIPKIIβ spatially corresponds to the invariant K72 in protein kinases that in the presence of Mg^{2+} ion pairs with the non-transferable α- and β-phosphates of ATP and mutation of this residue in the protein kinase and the analogous residue in the PIP kinase destroys kinase activity (Knighton *et al.*, 1991a, 1991b; Rao *et al.*, 1998; Taylor *et al.*, 1992, 1993). In PKA, D166 of the HR**D**^{166}LK motif is conserved in most protein kinases and functions as a weak base in protein kinase catalysis. While there are variations in the residues that surround D278 of PIPKIIβ (Knighton *et al.*, 1991a, 1991b; Rao *et al.*, 1998; Taylor *et al.*, 1992, 1993), D278 is conserved in all PIP kinases, lies in the Y/F**D**LKGS motif and appears to be analogous to D166 of the protein kinase. Protein kinases also contain a **D**FG motif, where the aspartate is absolutely conserved and functions in the binding the Mg^{2+} or Mn^{2+} of the Mn^{2+}-ATP complex. D184 of PKA corresponds to D369 of PIPKIIβ in the M**D**YSL sequence motif and is also conserved within the PIP kinase family. These combined motifs and invariant residues are required for the phosphotransferase reaction for both protein kinase and PIP kinase families. Most importantly, mutation of K150, D278, and D369 of PIPKIIβ or the analogous residues in other PIP kinases results in loss of catalytic activity (Rao *et al.*, 1998). The PIP kinases also contain a glycine-containing loop positioned similar to that of protein kinases which serve as a phosphate anchor

and are situated so their amide groups interact with the triphosphate backbone of ATP (Knighton *et al.*, 1991a, 1991b; Rao *et al.*, 1998; Taylor *et al.*, 1992, 1993).

Based on the structural homology between the catalytic domains of PIPKIIβ and protein kinases, as well as the defined structures of protein kinases bound to ATP, a model of PIPKIIβ bound to ATP was constructed. This PIPKIIβ · ATP structural model revealed that the catalytic site of PIPKIIβ accommodates ATP so that the γ-phosphate of ATP is spatially oriented toward the putative membrane association interface of the PIPKIIβ, consistent for PIP kinase function. To assess if this model would accommodate an endogenous PIP substrate, PI5P, the preferred substrate for PIPKIIβ was docked onto the PIPKIIβ · ATP structural model using the structure of the PKI inhibitor peptide bound to PKA · ATP as a guide. By this approach, the PI5P was docked such that the 4-hydroxyl of the myo-inositol ring is positioned for nucleophilic substitution of the γ-phosphate of ATP (Knighton *et al.*, 1991a, 1991b; Rao *et al.*, 1998; Taylor *et al.*, 1992, 1993).

Consistent with the PIP kinase preference of PIP as substrate, there are four basic residues in the PIP kinase (R134, K218, R224, and K239) that are clustered adjacent to the 5-phosphate of PI5P in the structural representation of PIPKIIβ · ATP · PI5P (Figure 5). Basic residues analogous to K218 and R224 are invariant in all known PIP kinases, residues analogous to R134 are conserved in most PIP kinases, and K239 analogs are conserved in PIPKI and PIPKII subfamilies, but not in PIPKIII. While the modeled PI5P binding site contains a putative 5-phosphate binding pocket, the remainder of the binding site is open and shallow. The openness of the PIP binding site suggests an explanation for the multiple substrate specificities of the PIP kinases; in this structure, the PIP substrates are free to rotate such that the 3-, 4-, or 5-phosphate could occupy the highly basic and conserved phosphate-binding pocket. In addition, the substrate-binding site is sufficiently shallow allowing for movement of the 2-hydroxyl, as there do not appear to be any interactions with the 2-hydroxyl that would hinder this movement. The structural model of the PIPKIIβ · ATP · PI5P is internally consistent with the orientation of the ATP binding site and the positioning of the PIPKIIβ on membranes (Knighton *et al.*, 1991a, 1991b; Rao *et al.*, 1998; Taylor *et al.*, 1992, 1993) and as a result, appears

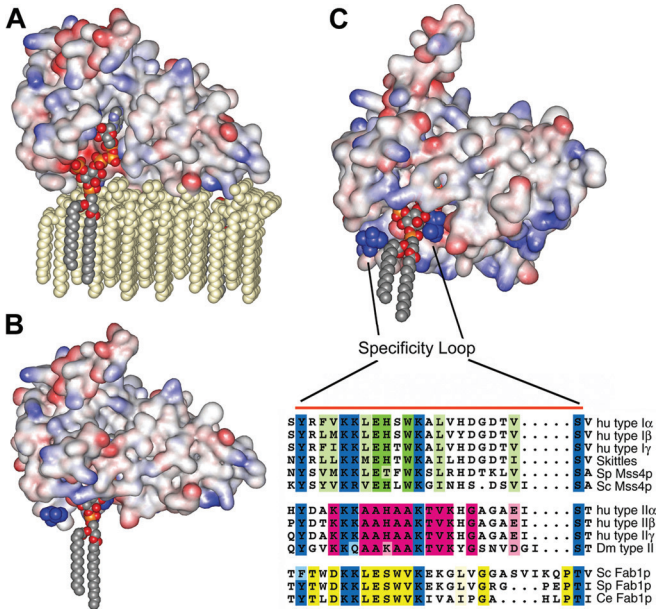

Figure 5 **Structural representation of PIPKIIβ bound to PI5P substrate and ATP. (*A*) Monomeric PIPKIIβ bound to substrate and ATP is docked on a phospholipid interface. Some residues have been removed from the PIPKIIβ structure to reveal the binding pocket where ATP and PIP substrate bind. (*B*) Monomeric PIPKIIβ bound to PIP substrate highlights the resolved residues in the PIPKIIβ structure that mark the beginning and end of the specificity loop. (*C*) The position of the specificity loop in the PIPKIIβ structure as well as a sequence pileup of type I, II, and III PIP kinase specificity loops are shown. The KK motif is important for membrane targeting, while the glutamate of type I (E362) and the alanine of type II (A381) PIP kinases is required for substrate recognition.**

to accommodate all major substrates of the PIP kinase family.

The PIPKIIβ model, shown in Figure 5, does not take into account key regions of the PIP kinase that are disordered. Three regions within PIPKIIβ crystal structure were unresolved including the N-terminal 33 residues, residues 304–342 from the highly variable insert sequence that is found in the type I and II PIP kinases, and of particular interest, a loop from 373–390 that spans the PIP binding site and coincides with the specificity loop of protein kinases as illustrated in Figure 5. The position of this loop suggests a key role in generation of PI4,5P_2 by the PIP kinases and these regions of PIPKIIβ are likely to be critical to PIP kinase function (Rao *et al.*, 1998).

SIGNALING SPECIFICITY IS DEFINED BY THE SPECIFICITY LOOP

The specificity loop of protein kinases is positioned to modulate kinase activity as well as interactions with and specificity toward protein substrates. The

position of the region that spans the PIP binding site in PIPKIIβ is analogous to that of the specificity loop in protein kinases and is positioned at the interface with the membrane. The sequence of this loop is conserved within each subfamily but is divergent between PIP kinase subfamilies. Since the defining difference between the PIP kinase subfamilies is their specificity toward PIP substrates this suggests this loop plays a role in substrate specificity (Kunz *et al.*, 2000; Rao *et al.*, 1998).

To assess this possibility, the loops of the PIPKIβ and the PIPKIIβ were swapped, and their activity toward the PI4P and PI5P were compared with the parental enzymes (Kunz *et al.*, 2000). As shown in Figure 6, each loop swap mutant completely changed its substrate specificity to generate PI4,5P_2, demonstrating that the specificity loop defines the substrate specificity. The specificity loop must specifically bind and position the substrate for stereospecific phosphotransfer of the γ-phosphate of ATP to the appropriate hydroxyl. These data suggest that the catalytic core of the PIP kinases provides a platform for the phosphotransferase reaction, but not specificity. From a structural standpoint, the manner in which the loop interacts with substrate is not well understood; however, evidence suggests that conserved and invariant residues within the specificity loop facilitate this interaction.

There are three lysine residues within the specificity loop that are conserved within the PIP kinase family. For PIPKIIβ these are K378, K379, and K385. When the pair of lysine residues (K378, K379) in the PIPKIβ loop were mutated to N378/N379 the specificity toward PI4P was unchanged; however, the K_m for PI4P and PI3P was increased by 30-fold, indicating that the affinity for these substrates was reduced. When these same lysine residues are mutated to arginine (R378/R379) the substrate specificity was unchanged, nevertheless the affinity for PI4P and PI3P increased. These observations are consistent with reports that arginine residues bind more strongly to acidic phospholipids than lysine residues (Mosior and McLaughlin, 1992). These data suggest that the invariant lysine residues in PIP kinase may interact with an acid component of the substrate that is also invariant such as the phosphodiester moiety, but this does not provide insight into the residues that define the specificity of the loop toward different substrates (Kunz *et al.*, 2000).

To further define substrate specificity, the N- and C-terminal halves of the specificity loop were swapped between PIPKIα and PIPKIIβ and provided evidence that the N-terminal half of the loop was the primary determinant of substrate specificity (Kunz *et al.*, 2002). Comprehensive mutagenesis of these sequences further demonstrated that substrate preferences of type I and type II PIP kinases could be modified by a single amino acid substitution in the specificity loop. An A $\rightarrow$ E point mutation at A381 in PIPKIIβ is sufficient to switch substrate specificity and generate PI4,5P_2, whereas the corresponding change of E $\rightarrow$ A at E362 in PIPKIβ increased affinity for PI5P, but retained activity toward PI4P. This demonstrated that the positioning of the invariant glutamate residue within the PIPKI loop is required for substrate specificity toward PI4P (Kunz *et al.*, 2002).

A mechanism for this specificity was proposed to be the electrostatic repulsion between the negatively charged glutamate and the phosphate residue present at the 5-hydroxyl of the PI5P head group. This hypothesis is supported by the substitution of E362 in PIPKIβ to an uncharged alanine residue that eliminates the potential for electrostatic repulsion resulting in the utilization of both PI4P and PI5P. Alternatively, E362 could interact with a positively charged residue of an ion pair to stabilize a specific confirmation of the specificity loop such that it specifically interacts with PI4P but not PI5P. Since only the loop is required for stereo-specific substrate recognition, this glutamate may interact with one or more of the invariant lysine residues in the loop. Thus, a glutamate in this position of the specificity loop may prevent access of phosphoinositide substrates that are phosphorylated at the 5-hydroxyl position of the inositol head group. To support this, all PIP kinases that phosphorylate the 5-hydroxyl on the inositol head group have a glutamate at this position. Kinetic studies further indicate that specificity loops have promiscuous substrate binding specificity *in vitro*, however it remains that residues within the loop are key for orienting the substrates in the kinase core for efficient and specific phosphotransfer. In addition, it is clear that the different subfamilies of PIP kinase are functionally non-redundant, and the promiscuity of *in vitro* specificity is not sufficient to compensate *in vivo*.

The *in vivo* function of the PIPKIIβ-PIPKIβ loop chimera was evaluated using a genetic approach in yeast (Kunz *et al.*, 2000) *S. cerevisiae* does not have type II enzymes but contains a type I PIP kinase, Mss4p which is essential for viability, actin polymerization, and cell

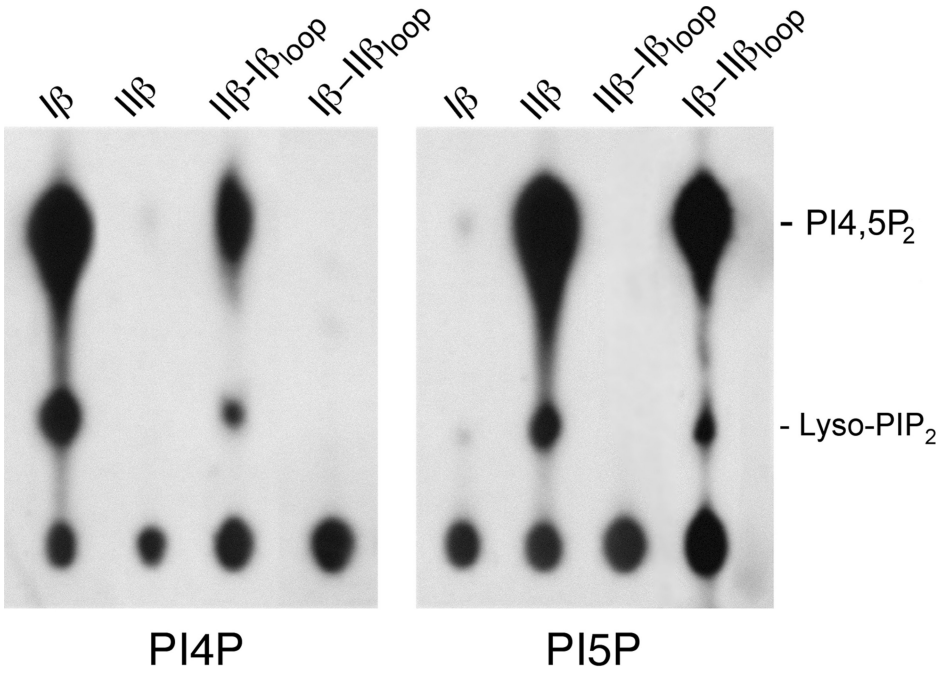

Figure 6 **Signaling specificity of the PIP kinases is dependent on the specificity loop. Loop swap mutants demonstrate that kinase activity can be completely changed when the specificity loops of PIPKIβ and PIPKIIβ are exchanged. An autoradiogram of the ^{32}P-labeled kinase products is shown and illustrates the altered substrate usage by loop swap mutants is dependent on the specificity loop.**

wall integrity (Desrivieres *et al.*, 1998; Homma *et al.*, 1998). The rescue of the *mss4p* phenotype provided a system to analyze *in vivo* specificity of PIP kinases and the loop chimeras. Using this approach, mammalian type I but not type II PIP kinases restore growth to an *mss4p* mutant (Homma *et al.*, 1998). While PIPKIIβ does not rescue the *mss4p* mutant phenotype, the PIPKIIβ-PIPKIβ loop chimera fully rescued the phenotype (Kunz *et al.*, 2000) demonstrating that the PIPKIIβ-PIPKIβ loop chimera functions as type I PIP kinase *in vivo*. Further, since PIPKIIβ also produced PI3,4P_2, but was unable to facilitate actin reorganization, it was clear that the ability of the PIPKIIβ-PIPKIβ loop chimera to rescue these defects depends entirely on its production of PI4,5P_2 (Kunz *et al.*, 2000).

SPECIFICITY LOOP CONTRIBUTES TO SUBCELLULAR TARGETING

In addition to substrate specificity, type I and II PIP kinases also demonstrate differential subcellular targeting driven by their specificity loops. As shown in Figure 7, when epitope tagged PIPKIβ is overexpressed in cells it specifically targets to the plasma membrane, while PIPKIIβ localizes to the nucleus and cytoplasm. Specificity loop swap mutants were generated to demonstrate the importance of the specificity loop in kinase targeting. Loop swap mutants targeted identically to their loop donor demonstrating that the specificity loop is both necessary and sufficient for specific targeting of the type I and II PIP kinases. Since the specificity loop defines substrate preference, this discovery suggested that substrate specificity may drive the subcellular targeting of the PIP kinases (Kunz *et al.*, 2000).

To test this hypothesis, the invariant lysine pair in the PIPKIβ specificity loop required for membrane targeting was mutated to asparagine residues (N/N) resulting in an apparent 30-fold decrease in affinity for PI4P and a dramatic reduction in plasma membrane targeting. In contrast, mutation of the lysine pair to arginine (R/R) resulted in an increased affinity for PI4P and strong plasma membrane targeting. In addition, mutating E381 (E $\rightarrow$ A) in the PIPKIβ specificity loop resulted in diminished affinity for PI4P, reduced activity toward both PI4P and PI5P, and inefficient targeting to the plasma membrane while the reverse mutation (A $\rightarrow$ E) in the PIPKIIβ specificity loop increased affinity toward PI4P and resulted in a partial targeting to the plasma membrane. These data are summarized in Figure 7 and support the hypothesis that targeting of the PIP kinases to the plasma membrane upon ectopic expression is dependent upon their interaction with PI4P (Kunz *et al.*, 2000, 2002).

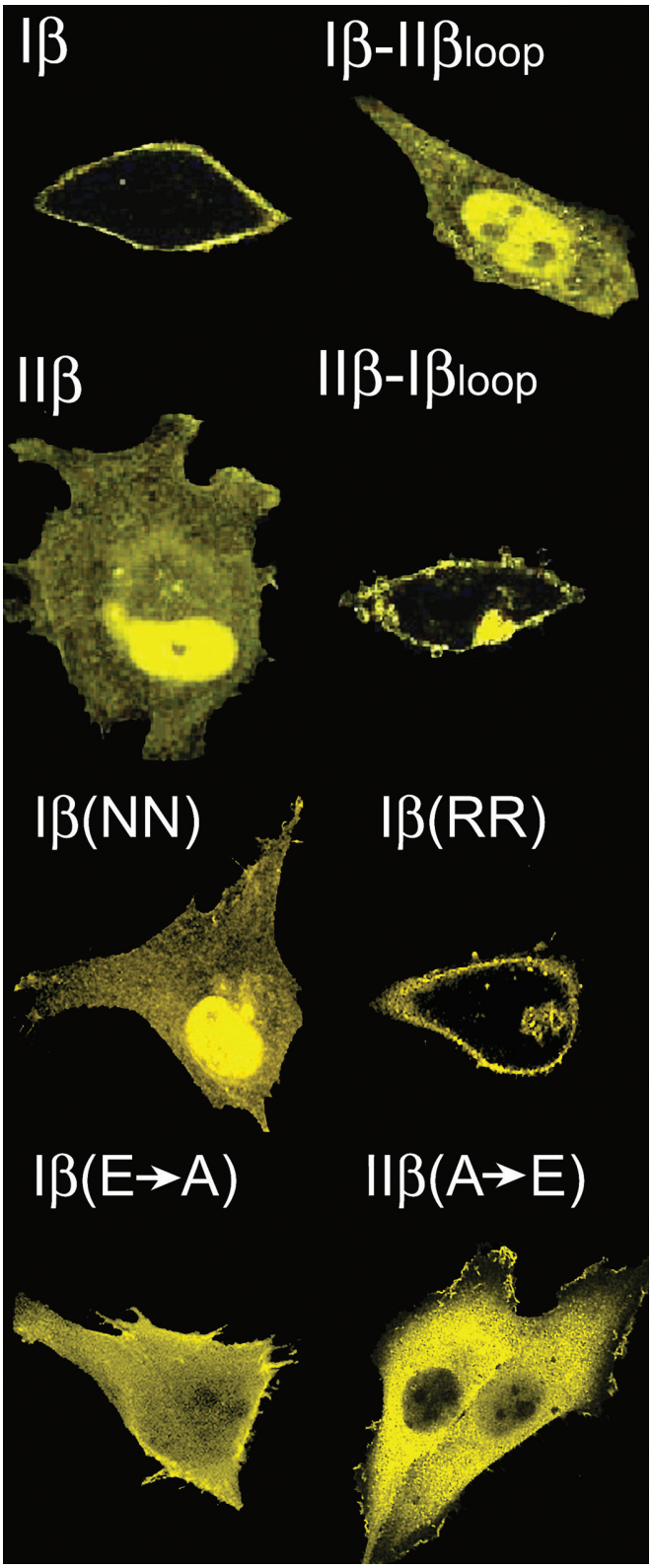

Figure 7 **PIPKIβ is targeted to the plasma membrane by its specificity loop. The specificity loop determines signaling specificity based on substrate preference. When PIPKIβ is ectopically expressed it is targeted to the plasma membrane, which suggests that subcellular targeting is in part dependent on the availability of substrate. Mutations in key residues that determine substrate specificity result in altered substrate preference and reduced plasma membrane targeting.**

The specificity loop is important for substrate specificity and resulting spatial targeting of the PIP kinase. Since PI kinases generate the substrates for PIP kinases, some functional synergism is likely. In this synergistic model, PI kinases generate products that recruit the PIP kinase that uses this product as substrate (Anderson *et al.*, 1999; Kunz *et al.*, 2000, 2002). The synergism between Vps34p and Fab1p in yeast is a physiological example that supports this model; in yeast, the PI3K vps34p generates PI3P and when stimulated by osmotic stress Fab1 generates $PI3,5P_2$ from PI3P (Dove *et al.*, 1997; Odorizzi *et al.*, 1998). Consequently, in *vps34p* knock outs there is a loss of $PI3,5P_2$ production (Dove *et al.*, 1997). While the targeted recruitment mechanism of Vps34p and Fab1p has not been explored, these data suggest that Fab1p uses the PI3P generated by Vps34p as substrate, supporting a model for compartmentalized generation of PI3P which may in part recruit Fab1p to generate $PI3,5P_2$ at specific sites (Michell *et al.*, 2006; Odorizzi *et al.*, 1998). While the subcellular targeting of ectopically expressed PIP kinases can be very different from that of endogenous type I PIP kinases, this demonstrates that additional factors beyond substrate specificity are important for the physiologically relevant targeting of the PIP kinases.

PROTEIN INTERACTIONS ARE KEY FOR SPATIAL TARGETING

The above discussions support the concept that PIP kinases are targeted to specific intracellular sites at least in part by specificity loop-mediated substrate interactions. As illustrated in Figure 8A, evidence suggests that within the type I PIPK subfamily, individual isoforms (α, β, γ consistent with human nomenclature) exhibit distinct subcellular targeting patterns. Since the specificity loops of the PIPKI isoforms are highly homologous, these data suggest the specificity loop is not the major factor that determines intracellular targeting. Recent evidence suggests that isoform and splice variant specific protein-protein interactions are key for specific subcellular targeting of the PIPKI isoforms. As shown in Figure 8A, while the specificity loop for each is nearly identical PIPKIα targets to membrane ruffles, PIPKIβ targets to a perinuclear region, and PIPKIγ targets to focal adhesions. Figure 8B shows the 26 amino acid C-terminal extension of PIPKIγ661 and the differential targeting of PIPKIγ635

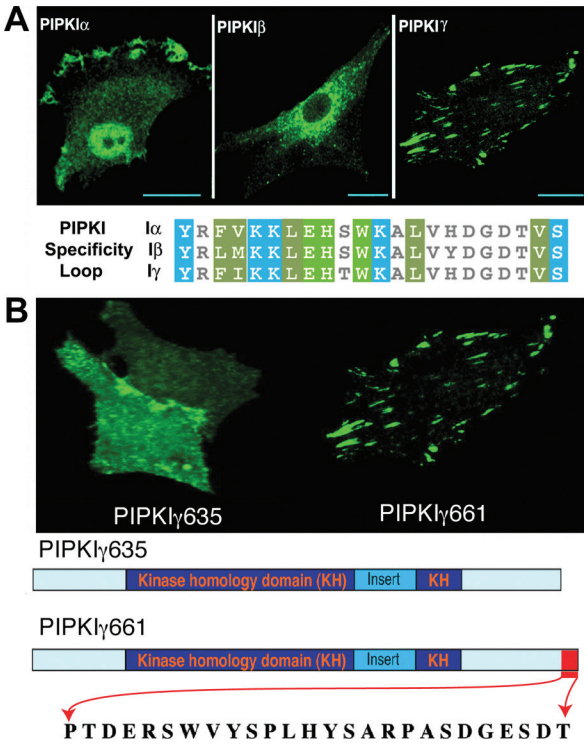

Figure 8 **Additional sequences influence subcellular localization of PIPKI isoforms. (*A*) While the specificity loops of PIPKIα, β, and γ are almost identical, their subcellular targeting is distinct; PIPKIα targets to membrane ruffles and nuclear speckles, PIPKIβ targets to a perinuclear region, and PIPKIγ targets to focal adhesions. PIPKIα, β, and γ are named according to their human nomenclature. (*B*) A schematic comparison of two PIPKIγ splice variants illustrates a C-terminal extension required for specific protein-protein interactions. Unique protein-protein interactions that occur within the C-terminal extension result in distinct subcellular targeting such as the interaction with talin which targets PIPKIγ to focal adhesions.**

(diffuse cytosolic) and PIPKIγ661 (focal adhesions) when expressed in cells (Di Paolo *et al.*, 2002; Ling *et al.*, 2002). Endogenous PIPKIγ661 partially targets to focal adhesions (Di Paolo *et al.*, 2002; Ling *et al.*, 2002) and since focal adhesions are modulated by the generation of PI4,5P_2, this suggests a functional role for the PIPKIγ661 in focal adhesion dynamics. Specific functional roles for PIP kinases and PI4,5P_2 in cell migration and vesicular trafficking will be discussed in more detail below.

CELL MIGRATION

Cell migration is critical to many biological and pathological processes, including embryonic development, the inflammatory immune response, wound repair, tumor formation, and metastasis (Lauffenburger and Horwitz, 1996; Le Roy and Wrana, 2005; Ling *et al.*, 2006; Saunders *et al.*, 2006; Yamaguchi *et al.*, 2005). By spatially and temporally generating PI4,5P_2, PIP kinases regulate actin reorganization and focal adhesion dynamics, both of which play key roles in cell migration (Lauffenburger and Horwitz, 1996; Ling *et al.*, 2006; Niggli, 2005; Yamada and Araki, 2001; Yamaguchi *et al.*, 2005).

Specific changes in the actin cytoskeleton including polymerization and depolymerization of actin filaments in lamellipodia or membrane ruffles are required for cell migration (Lauffenburger and Horwitz, 1996). The functions of many proteins involved in actin cytoskeleton rearrangement are regulated by PI4,5P_2 including α-actinin, WASP/N-WASP, gelsolin, cofilin, profilin, and villin (Niggli, 2005). For example, cooperative binding of PI4,5P_2 and Cdc42 to WASP/N-WASP exposes the VCA region of WASP/N-WASP, activating the Arp2/3 complex and leading to rapid actin polymerization (Miki and Takenawa, 2003). Conversely, binding of PI4,5P_2 leads to a conformational change in the actin capping protein gelsolin, causing its dissociation from the barbed ends of growing actin filaments to promote actin assembly (Janmey *et al.*, 1987; Janmey and Stossel, 1987).

Recent evidence demonstrates that PIPKIα is present at membrane ruffles where localized pools of PI4,5P_2 have been reported. When added to permeabilized platelets, PIPKIα induced actin filament uncapping and assembly, while a kinase dead PIPKIα mutant failed to promote actin assembly and blocked actin assembly stimulated by thrombin or Rac (Tolias *et al.*, 2000). Furthermore, PIPKIα is crucial to PDGF-induced membrane ruffling; upon PDGF stimulation, wild type PIPKIα is recruited to membrane ruffles where it generates PI4,5P_2, however, expression of kinase-dead PIPKIα inhibits PDGF-stimulated membrane ruffling (Doughman *et al.*, 2003b). In agreement with the function of PIPKIα at membrane ruffles, knockdown of PIPKIα in mouse embryonic fibroblasts (MEFs) blocked cell migration, indicating that PIPKIα is required for cell motility (Kisseleva *et al.*, 2005). The subcellular translocation and kinase activity of PIPKIα can be regulated by the Rho family small G-proteins since Rac1 activity is necessary for the translocation of PIPKIα to membrane ruffles downstream of PDGF stimulation (Doughman *et al.*, 2003b). In addition, a number of PIPKIα interacting proteins such as the LIM protein Ajuba contribute to regulation of PIPKIα. Ajuba recruits PIPKIα to membranes and enhances its enzymatic activity (Kisseleva *et al.*, 2005).

In addition to reorganization of the actin cytoskeleton, focal adhesion dynamics are key to cell migration (Webb *et al.*, 2002). As shown in Figure 9, PIPKIγ661 specifically targets to focal adhesions where it regulates focal adhesion dynamics through a direct interaction with the FERM domain of talin, a ubiquitous component of focal adhesion complexes (Di Paolo *et al.*, 2002; Ling *et al.*, 2002). Talin forms homodimers and scaffolds F-actin, β-integrin, and vinculin at focal adhesions, providing a link between actin filaments and the plasma membrane. Binding of talin to the cytoplasmic tail of the β integrin subunit induces a conformational change in the integrin extracellular domain which activates integrins by increasing their affinity for ligands (Tadokoro *et al.*, 2003). Through its direct interaction with talin, PIPKIγ661 has a dual effect on regulating the affinity of talin for integrin. By locally generating $PI45P_2$, PIPKIγ661 may enhance the affinity of integrin to bind talin, or PIPKIγ661 may compete with integrin for talin binding since their binding sites overlap.

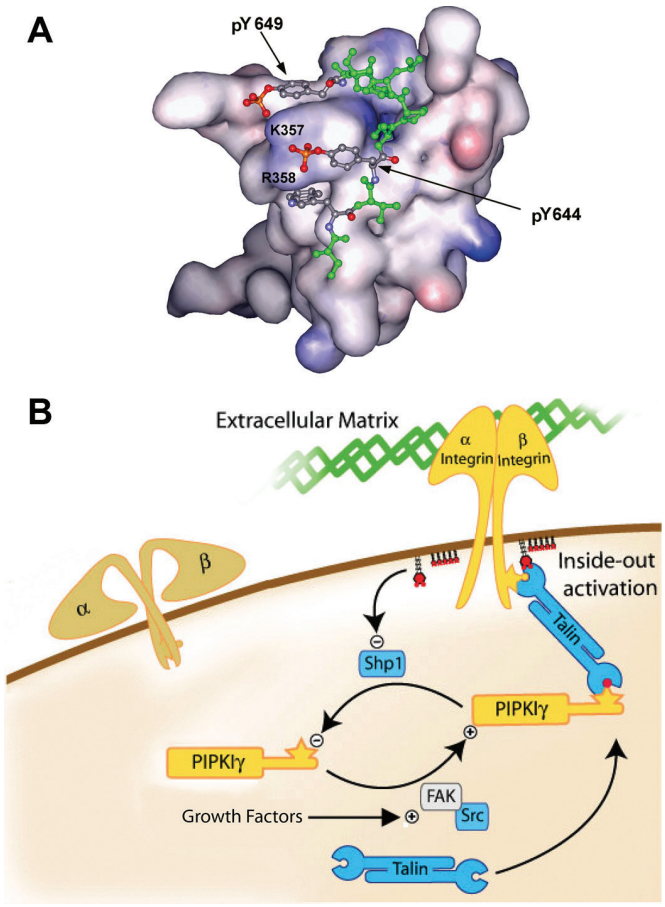

Figure 9 **PIPKIγ661 interacts with the talin FERM domain and is regulated by tyrosine phosphorylation. (*A*) A structural model of talin bound to the C-terminal extension of PIPKIγ661 highlights key residues which facilitate this interaction including K357 and R358 on talin and Y644 and Y649 on PIPKIγ661. (*B*) Tyrosine phosphorylation of PIPKIγ661 is regulated by Src and Shp-1 and works as a molecular switch to regulate talin association with PIPKIγ661 and integrins. Phosphorylation of PIPKIγ661 enhances its association with talin and increases localized generation of $PI4,5P_2$ at focal adhesions.**

Tyrosine phosphorylation of PIPKIγ661 at Y644 works as a 'molecular switch' for talin binding between PIPKIγ661 and integrin, where increased tyrosine phosphorylation enhanced talin binding. Cell adhesion and focal adhesion kinase (FAK) signaling at focal adhesions (Baukrowitz *et al.*, 1998) leads to Src-dependent tyrosine phosphorylation of PIPKIγ661, which corresponds to increased lipid kinase activity as well as an enhanced PIPKIγ-talin interaction (Ling *et al.*, 2002). When Y644 is phosphorylated, PIPKIγ661 binds with high affinity to the F3 lobe of talin's FERM domain, where K357 and R358 are key residues on talin that increase talin's affinity for phosphorylated Y644 on PIPKIγ661 (Kong *et al.*, 2006; Ling *et al.*, 2003). In contrast, Shp-1, a SH2 domain-containing phosphatase, directly interacts with PIPKIγ661 to dephosphorylate the Src-phosphorylated Y644 (Bairstow *et al.*, 2005). Y644 phosphorylation on PIPKIγ661 dramatically enhances talin interaction and potentially facilitates focal adhesion targeting, enabling the generation of $PI4,5P_2$ at focal adhesions to improve the integrin–talin interaction. The enhancement of the integrin-talin association by $PI4,5P_2$ and the tyrosine dephosphorylation by Shp-1 may ultimately displace PIPKIγ661 from talin. Thus, phosphorylation of PIPKIγ661 regulates focal adhesion dynamics by modulating the integrin-talin association. In addition, PIPKIγ661 S645 may also be involved in the regulation of the PIPKIγ-talin interaction. S645 can be phosphorylated by proline-directed protein kinases as well as MAPK, and dephosphorylated by calcineurin (Lee *et al.*, 2005). S645 phosphorylation prevents the interaction between PIPKIγ and talin without affecting PIPKIγ kinase activity (Lee *et al.*, 2005).

VESICULAR TRAFFICKING

Vesicle Formation

In general, membrane trafficking events can be classified as either exocytic or endocytic based on their destination directed toward or away from the plasma membrane. These events include biosynthetic transport from the ER-Golgi pathway and receptor recycling, endosomal-lysosomal recycling and degradation, as well as clathrin dependent and independent

endocytosis. Phosphoinositides have been shown play a critical role in most of these processes including clathrin dependent and independent events including macropinocytosis, phagocytosis, as well as fusion of secretory granules with the plasma membrane. Clathrin-mediated endocytosis is the major pathway for the selective internalization of plasma membrane receptors and this internalization is initiated by the formation of the clathrin lattice followed by incorporation of other components to form clathrin-coated pits (CCPs) that pinch off as clathrin-coated vesicles (CCVs). Throughout the last decade $PI4,5P_2$ has been defined as a crucial regulator of CCV formation, scission and uncoating (Cremona and De Camilli, 2001; De Matteis and Godi, 2004).

Formation of the clathrin triskelia on the plasma membrane is important for formation of CCVs and adaptor proteins play an important role in the recruitment and polymerization of the outer clathrin layer via simultaneous binding to clathrin, membrane lipids and in many cases, transmembrane cargo proteins (Ehrlich *et al.*, 2004; Owen *et al.*, 2004). Numerous trafficking proteins have been shown to specifically bind $PI4,5P_2$ including epsin via its ENTH (epsin NH2-terminal homology) domain, CALM/AP180 via the closely related ENTH-like domain (Ford *et al.*, 2001; Itoh *et al.*, 2001), the heterotetrameric adaptor complex 2 (AP-2) via the surface-exposed basic patches (Gaidarov and Keen, 1999), Dab2 (Mishra *et al.*, 2002), and dynamin via its PH domain (Schmid *et al.*, 1998). In addition, $PI4,5P_2$ is an important regulatory factor for modulation of the actin cytoskeleton and subsequently vesicular transport (Qualmann and Kelly, 2000; Qualmann *et al.*, 2000).

Clathrin-Mediated Trafficking

The majority of clathrin mediated internalization is coordinated by the AP2 complex comprised of two large subunits (α and $\beta 2$), a medium subunit ($\mu 2$) and a small subunit ($\sigma 2$). Through its association with clathrin, accessory endocytic proteins, membrane lipids and cargo receptors, AP2 may serve as a central protein recruitment hub during CCV assembly (Praefcke *et al.*, 2004). The $\mu 2$-subunit is critical for the function of AP2 by mediating its binding to membrane cargo proteins. In the AP2 crystal structure, the $\mu 2$-subunit C-terminal domain was found to be buried within a pocket formed by the remaining complex subunits and unable to interact with membrane bound cargo proteins (Collins *et al.*, 2002). The current model for AP2 cargo binding suggests that when AP2 docks at a membrane in the presence of cargo it shifts to an 'open' conformation (Collins *et al.*, 2002; Kirchhausen, 2002) and assembly of the AP2 complex onto membranes is mediated by $PI4,5P_2$ (Gaidarov and Keen, 1999). Two $PI4,5P_2$ binding sites have been identified within the AP2 complex; the α- subunit contains a $PI4,5P_2$ binding site that is positioned to dock the AP2 complex at membranes (Collins *et al.*, 2002; Kirchhausen, 1999), while the $\mu 2$-subunit contains a binding site formed by a cluster of conserved lysine residues (Rohde *et al.*, 2002). Recently it has been demonstrated that the $\mu 2$-subunit binds $PI4,5P_2$ only when the AP complex is docked to the membrane and the $\mu 2$-subunit is bound to cargo (Collins *et al.*, 2002; Honing *et al.*, 2005). This evidence emphasizes the critical role for $PI4,5P_2$ in both the recruitment of AP2 to endocytic sites and the stabilization of the $\mu 2$-subunit/cargo interaction at the plasma membrane (Collins *et al.*, 2002; Honing *et al.*, 2005).

While it is clear that $PI4,5P_2$ is critical for clathrin mediated endocytosis, the spatial and temporal regulation of $PI4,5P_2$ for ongoing endocytic events is not understood. In mice, inactivation of synaptojanin 1 leads to elevated levels of $PI4,5P_2$ and decreased rates of presynaptic vesicle cycling resulting in accumulation of CCVs at nerve endings and perinatal lethality (Cremona *et al.*, 1999). Conversely, depletion of $PI4,5P_2$ by overexpression of the membrane-targeted 5-phosphatase domain of synaptojanin 1 impairs receptor-mediated uptake of transferrin, and knockdown of PIPKIγ, the dominant PIPKI isoform in brain, inhibits the uptake of epidermal growth factor (EGF) as a result of mislocalization of AP2 and clathrin (Krauss *et al.*, 2003). When PIPKIγ was genetically depleted from mice, animals displayed defects in both the exocytic and endocytic limbs of the synaptic vesicle cycle and exhibited a postnatal-lethal phenotype (Di Paolo *et al.*, 2004). Previously, it was demonstrated that expression of wild type PIPKIα enhanced endocytosis of EGFR, while expression of a kinase dead mutant had an inhibitory effect in NR6 cells (Barbieri *et al.*, 2001). Alternatively, it was recently reported that either expression or RNAi based knockdown of PIPKIβ had a significant impact on transferrin endocytosis, while expression or knockdown of PIPKIα or PIPKIγ had no effect in CV-1 and HeLa cells (Padron *et al.*, 2003). The

authors concluded, however, that these observed effects were the result of large perturbations of cellular PI4,5P_2 levels upon modulation of PIPKIβ expression (Padron *et al.*, 2003).

The small GTPase Arf6 (ADP-ribosylation factor 6) is a stimulator of PIPKIγ (Honda *et al.*, 1999). Arf6 directly binds to and activates PIPKIγ, resulting in increased PI(4,5)P_2 levels within presynaptic membranes and increased recruitment of clathrin and endocytic adaptors (Krauss *et al.*, 2003). The direct activation of PIPKIγ isoenzymes by ARF6 may synergize with other ARF-dependent effector pathways that affect clathrin-mediated endocytosis, such as phospholipase D, another potent activator of PIPKI isoforms. Moreover, ARF6-dependent PIPKIγ activation has been implicated in the fusion of secretory vesicles in neuroendocrine PC12 cells (Aikawa and Martin, 2003).

Phagocytosis and Macropinocytosis

Phagocytosis and macropinocytosis are important endocytic pathways for uptake of exogenous antigens, bacteria and viruses. Phagosomes mediate the specific uptake of opsonized particles while macropinosomes engulf fluid-phase material non-selectively taken up by membrane lamellipodia and ruffles (Simonsen *et al.*, 2001). Studies using the PI4,5P_2-binding pleckstrin homology (PH) domain of PLCδ1 as a probe demonstrated a local rapid accumulation of PI4,5P_2 at the phagosomal cup, which disappears upon recruitment of PLC and local formation of DAG (Botelho *et al.*, 2000). ARF6 is capable of recruiting a PIP 5-kinase to sites of bacterial entry and stimulating local PI4,5P_2 production (Balana *et al.*, 2005) and in addition, activation of ARF6 has been observed to stimulate macropinocytosis (Donaldson, 2003a, 2003b).

Endoplasmic Reticulum (ER)-Golgi Transport

Although some evidence indicates that PI4,5P_2 is generated at the Golgi complex, none of the known PI4P5K isoforms has been visualized at the golgi complex. PI4P5K activity that can be stimulated by ARF6 (Jones *et al.*, 2000) which has been shown to be associated with Golgi membranes (Godi *et al.*, 1999a, 1999b; Jones *et al.*, 2000; Siddhanta *et al.*, 2000) as well as three 5-phosphatases (the 72 kDa 5-phosphatase pharbin, hSac2, and a type-II 5-phosphatase). Defects in these phosphatases cause oculocerebrorenal or Lowe (OCRL) syndrome (Attree *et al.*, 1992), characterized by congenital cataracts, mental retardation and renal ion-transport defects. These evidence strongly suggest that PI4,5P_2 must have a specific function at the Golgi complex.

PI4,5P_2 has been proposed to play a role in ER-to-Golgi transport (Godi *et al.*, 1998), regulation of dynamin (Jones *et al.*, 1998), PLD1 (Freyberg *et al.*, 2001) and profilin (Dong *et al.*, 2000), and in maintaining the structural integrity and function of the golgi complex (Siddhanta *et al.*, 2000; Sweeney *et al.*, 2002). In addition, PI4,5P_2 may generate these effects by controlling the spectrin and actin machineries. PI4,5P_2-sequestering agents, such as neomycin and several PH domains, inhibit ARF-stimulated assembly of spectrin and actin on Golgi membranes *in vitro* (Godi *et al.*, 1998). Recently, OCRL1, a PI4,5P_2-5′-phosphatase localized at the Golgi apparatus, was shown to directly interact with the clathrin heavy chain and play a role in clathrin-mediated trafficking of proteins from endosomes to the TGN (Choudhury *et al.*, 2005), indicating that PI4,5P_2 needs to be removed or PI4P is required for this process to occur.

Using the PH domain of PLCδ in immunoelectron microscopy, the presence of PI4,5P_2 has only very recently been visualized at the Golgi stacks in less than 5% of the total labeled cell (Watt *et al.*, 2002). This could be because PI4,5P_2 is present only transiently, in very low levels in Golgi membranes, or may be bound by PI4,5P_2 binding proteins which reduce its accessibility to probes. While it is not clear which PIPK is responsible for PI4,5P_2 generation in the Golgi complex, activation of ARF increases the levels of PI4,5P_2 in Golgi membranes up to 10-fold (Godi *et al.*, 1999 a). ARF exerts this control through the recruitment and activation of a certain unknown PIP5K. This may be mediated by the stimulation of PLD by ARF, which increases the levels of phosphatidic acid, a powerful stimulator of PIP5K. In addition, ARF as well as some ARF guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) can interact with PI4,5P_2 or with its further phosphorylated derivative PI3,4,5P_3 (Donaldson, 2003a; Donaldson and Honda, 2005; Donaldson and Jackson, 2000). These interactions between PI4,5P_2 and the proteins regulating its synthesis might serve as an explosive yet tightly regulated spatial and temporal generation of PI4,5P_2 in select membrane domains.

PI4,5P_2 has clearly been shown to function in exocytosis, however the underlying mechanism remains unclear. In addition to vesicle budding from the Golgi complex, PI4,5P_2 is essential for vesicle fusion with the plasma membrane. In yeast, PI4,5P_2 has been shown regulate the ATP-dependent priming step, during which NSF disassembles SNARE protein complexes prior to vacuole docking, and a later step that follows vacuole docking but precedes vesicle fusion (Mayer *et al.*, 2000). In addition, PI4,5P_2 may have a role in establishing secretory granule docking sites on the plasma membrane (Holz *et al.*, 2000; Martin, 2001). As previously mentioned, genetic depletion of PIPKIγ in mice resulted in a postnatal lethal phenotype where the knockout mouse demonstrated deficiency in both exocytic and endocytic trafficking in the neuronal system (Di Paolo *et al.*, 2004). Moreover, RNAi mediated knockdown of PIPKIγ in INS-1E cells caused inhibition of calcium stimulated secretion of insulin (Waselle *et al.*, 2005).

PIPKIγ Generates PI4,5P_2 for Endocytosis and Exocytosis

Most recently it was discovered that PIPKIγ directly binds to the μ2-subunit of AP2 (Bairstow *et al.*, 2006; Krauss *et al.*, 2006). This interaction occurs at the Yxxϕ (YSPL) motif in the last 26 amino acids of PIPKIγ661 and can be impaired by tyrosine phosphorylation of this motif. While overexpression or knockdown of PIPKIγ has no effect on the global cellular levels of PI4,5P_2, changes in PIPKIγ expression enhance or inhibit transferrin uptake. Additional evidence demonstrates that the interaction between PIPKIγ and the μ2-subunit is critical for PIPKIγ to facilitate transferrin uptake. This work indicates a striking model resolving the spatial and temporal generation of PI4,5P_2 at sites where clathrin-AP2 mediated endocytosis occurs (Figure 10).

This model was futher reinforced by the recent discover that PIPKIγ661 directly binds to the μ-subunit of AP1B as well (Ling *et al.*, 2006). While the AP2 complex is important for regulation of receptor-mediated endocytosis from the plasma membrane, AP1B has been shown to mediate the basolateral targeting and/or recycling of many basolateral membrane proteins in epithelial cells (Folsch, 2005; Folsch *et al.*, 1999). Further investigation showed that, similar to μ2 adaptin, PIPKIγ661 binds to μ1B adaptin via its YXXϕ motif, the cargo sorting motif recognized by the μ-subunits. Since PIPKIγ661 binds the μ-subunits through a cargo motif it was important to determine if PIPKIγ661 was participating in AP complex mediated trafficking as cargo or as a regulator of other cargoes.

PIPKIγ661 also directly binds the cytoplasmic domain of E-cadherin (Ling *et al.*, 2006). E-cadherin is a major mediator of adhesion between neighboring cells, is critical for the establishment and maintenance of epithelial morphology, and is an important suppressor of tumor metastasis (Perez-Moreno *et al.*, 2003). It has been demonstrated that loss of PIPKIγ from epithelial cells results in loss of E-cadherin from the basolateral membrane and its accumulation in a cytosolic compartment. While global PI4,5P_2 levels remained unchanged, overexpression of wild-type PIPKIγ661 enhanced the internalization and recycling of E-cadherin, whereas kinase dead PIPKIγ661 had the reverse effect, indicating that PIPKIγ661 may be involved in the regulation of E-cadherin trafficking to the basolateral membrane, which is important for E-cadherin availability as well as the stability of epithelial adherens junctions. Based on PIPKIγ661 interactions with AP1B and E-cadherin, these observations indicate an intriguing possibility that PIPKIγ661 might regulate E-cadherin trafficking via AP1B and since then it has been demonstrated that the interaction between PIPKIγ661 and AP1B is essential for the indirect association between AP1B and E-cadherin (Ling *et al.*, 2006).

Before reaching the basolateral membrane, newly synthesized E-cadherin is first delivered from the trans-Golgi network to the recycling endosome, where AP1B specifically localizes and may mediate cargo sorting and assembly. It has been shown that PIPKIγ661 colocalizes with both E-cadherin and recycling endosomes strongly suggesting that PIPKIγ661 may mediate the recruitment of E-cadherin to the recycling endosome via its dual interaction with E-cadherin and AP1B. As shown in Figure 10, these observations reveal a novel mechanism where PIPKIγ661 functions as both scaffolding and signaling molecule during E-cadherin trafficking and represents a novel paradigm in which PIPKIγ661 serves as a cargo adaptor for AP complexes (Ling *et al.*, 2006). In this model, the AP complex indirectly interacts with cargo via the scaffolding molecule PIPKIγ661, which directly binds to AP complexes via a Yxxϕ sorting motif in its C-terminus.

PIPKIγ661 may also have additional lower affinity binding contacts with AP1B, as Krauss and colleagues

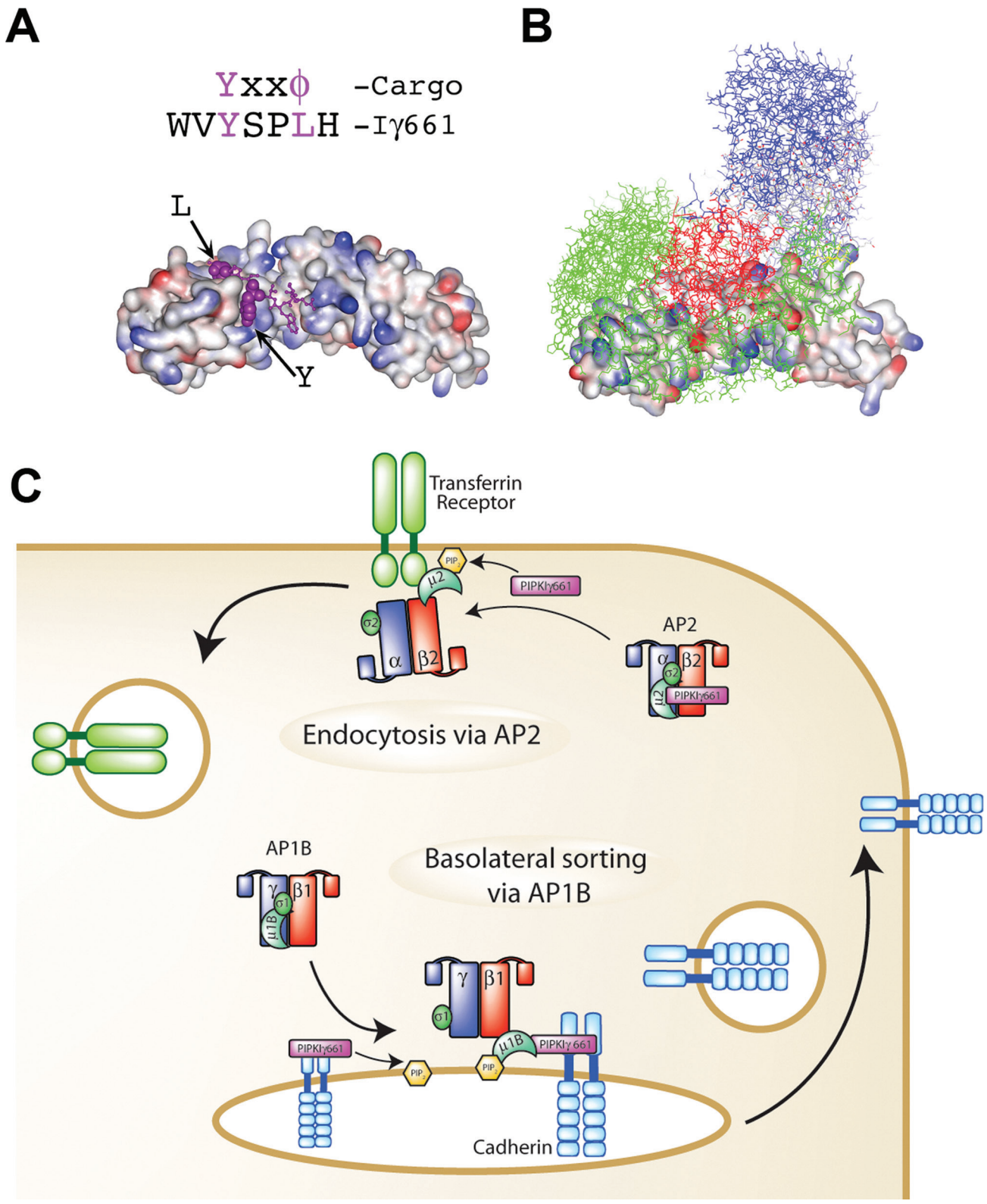

Figure 10 **PIPKIγ661 directly binds the μ-subunit of AP2 and AP1B *via* a cargo binding motif in its C-terminal extension. The interaction between PIPKIγ661 and AP2 regulates AP2 function and endocytosis of the transferrin receptor and possibly others. PIPKIγ661 has also been shown to function as both a scaffolding and signaling molecule in E-cadherin trafficking. (*A*) The PIPKIγ661 cargo-like sequence is aligned with another cargo sequence and the structural model of the PIPKIγ661 cargo-like sequence and immediately adjacent residues bound to the C-terminal domain of the μ-subunit are shown. (*B*) The full structure of the AP2 complex is shown with the μ-subunit illustrating that in the closed conformation the μ-subunit is inaccessible. (*C*) A model depicting the putative roles of PIPKIγ661 associations with the μ-subunits of AP2 and AP1B in the regulation of receptor endocytosis and E-cadherin trafficking to the basolateral membrane, respectively, are shown.**

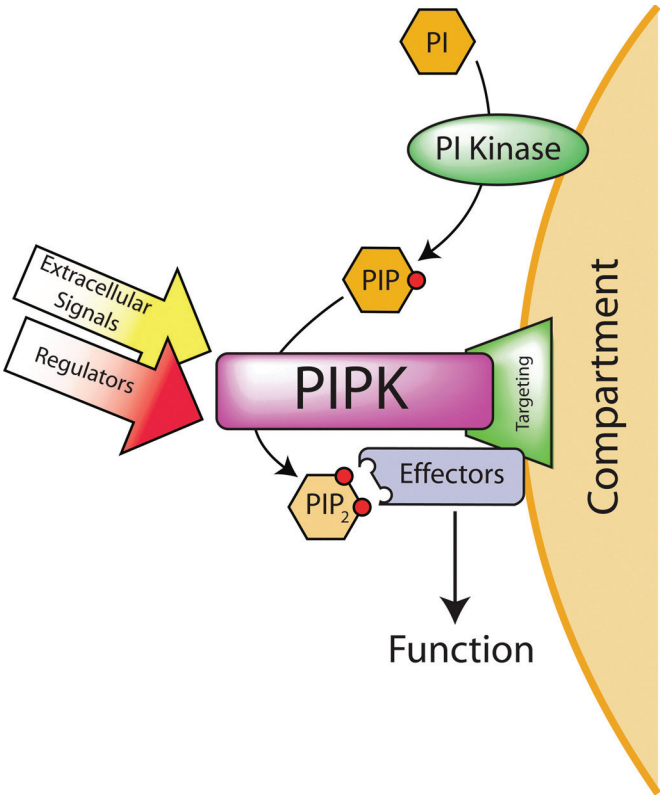

Figure 11 **Model for localized generation of $PI4,5P_2$ by PIP kinases. PI kinases potentially function synergistically with PIP kinases by generating their substrates at specific compartments. PIP kinases are targeted to specific subcellular compartments by targeting factors for the localized generation of $PI4,5P_2$. The spatial and temporal generation of $PI4,5P_2$ near effectors allows $PI4,5P_2$ to act as a second messenger as $PI4,5P_2$ is channeled directly to effectors as it is made. Effectors require $PI4,5P_2$ to carry out functions such as vesicular trafficking, focal adhesion dynamics, cytoskeletal reorganization, and other functions yet to be defined.**

recently reported that multiple PIPKIs bind to $\mu 2$-subunit of AP2 complex via the kinase domain (Krauss *et al.*, 2006). It is plausible that other type I PIP kinase isoforms could also be involved in E-cadherin trafficking or other AP complex-dependent trafficking events which may be mediated by other protein-protein interactions. The spatial and temporal specificity might be mediated by other specific binding partners. The roles of PIPKIs in AP complex-dependent vesicular trafficking is incompletely understood. Nevertheless, since $PI4,5P_2$ acts as a second messenger to regulate the recruitment and assembly of trafficking machinery (Roth, 2004; Simonsen *et al.*, 2001), the key step is localized generation of $PI4,5P_2$ at sites where E-cadherin and other cargoes are assembled into the trafficking machinery.

Type III PIP Kinases and $PI3,5P_2$

While the functional roles of type I PIP kinase isoforms in vesicular trafficking have been investigated, much less is known about the roles of the type III PIP kinases and the products they generate, including $PI3,5P_2$. $PI3,5P_2$ is the most recently identified phosphoinositide that is synthesized from PI3P by the type III PIP kinases Fab1p in *S. cerevisiae* (Loijens and Anderson, 1996; Yamamoto *et al.*, 1995) and a FYVE-domain-containing PI kinase (PIKfyve) in mammalian cells (Sbrissa *et al.*, 1999).

As the yeast analog of the mammalian lysosome, the *Saccharomyces cerevisiae* vacuole serves as the main storage compartment for essential amino acids, nutrients and ions, functions in the turnover of macromolecules, and is required for sporulation and osmotic homeostasis (Bryant and Stevens, 1998). Rapid changes in size, shape and number of vacuoles are critical to ensure an immediate response to dramatic fluctuations in extracellular osmolarity and nutrient concentrations. The FYVE domain of PIKfyve appears to target the kinase to PI3P-rich membranes, and if the above model is accurate, the specificity loop may play a role as well. A set of evidence indicates that Fab1p mediated $PI3,5P_2$ generation is important for membrane-to-lysosome trafficking, vacuole acidification, packaging and sorting of proteins in multivesicular bodies (MVBs) and growth at elevated temperatures (Efe *et al.*, 2005) and suggests that functionally distinct $PI3,5_2$ binding proteins are most likely involved in these processes. Like $PI4,5P_2$, several effector proteins bind $PI3,5P_2$ and comprise Ent3p, Ent5p, and Vps24p activity, linking Vps7p-mediated ubiquitin sorting into MVBs (Duncan *et al.*, 2003; Eugster *et al.*, 2004), Svp1p-dependent membrane recycling from the vacuole (Dove *et al.*, 2004), and sorting nexins that mediate transport from endosomes to the *trans*-Golgi (Carlton *et al.*, 2004).

It has been reported that overexpression of Fab1p does not result in a significant increase of $PI3,5P_2$. However, *vac7* mutants had a phenotype similar to *fab1* mutants but did not synthesized $PI3,5P_2$, suggesting that there are limiting regulatory factors (Gary *et al.*, 1998). Recently is was discovered that Vac14p (Bonangelino *et al.*, 2002), Vac7p (Dove *et al.*, 2002), and Fig4p (Gary *et al.*, 2002) have important roles in regulating Fab1p-synthesized $PI3,5P_2$. These findings demonstrate that Vac7p, Vac14p, and Fig4p are active in the same pathway as Fab1p and potentially function

as part of a multiprotein complex. Vac7p has been proposed to serve as an upstream activator of Fab1p (Gary *et al.*, 1998), whereas Vac14p and Fig4p were recently demonstrated to form a complex and couple PI3,5P_2 synthesis and turnover independent of Vac7p (Duex *et al.*, 2006). While it has been proposed that Vac14p functions as an osmo-sensor and Fig4p regulates the turnover of PI3,5P_2 as a phosphoinositide phosphatase, the mechanism by which Fab1p is recruited to membranes of organelles to generate PI3,5P_2 in a spatially and temporally regulated manner remains unclear.

REGULATION OF PI4,5P_2 AS A SECOND MESSENGER

In most cell types the cellular content of PI4,5P_2 does not change globally in response to agonists. Since PI4,5P_2 can rapidly diffuse through membranes these observations are not surprising, however this raises questions surrounding the ability of PI4,5P_2 to function as a second messenger if its membrane contents does not vary substantially. In order for PI4,5P_2 to work as a second messenger, localized pools of PI4,5P_2 may be generated and channeled directly to effectors.

Spatial and temporal generation of PI4,5P_2 is a key mechanism for the function of PI4,5P_2 as a second messenger. Unique targeting factors facilitate the generation of discrete pools of PI4,5P_2 next to effectors allowing PI4,5P_2 to be synthesized where its utilized. Immediate channeling of PI4,5P_2 to effectors as it is synthesized is an effective mechanism for the localized generation of PI4,5P_2 allowing PI4,5P_2 to function as a second messenger. The interaction between PIPKIγ661 and talin at focal adhesions exemplifies this paradigm. Tyrosine phosphorylation dramatically enhances the association between PIPKIγ661 and talin and targeting to focal adhesions, enabling locally generated PI4,5P_2 at focal adhesions to improve the talin-integrin interaction and modulate the activities of other PI4,5P_2 binding proteins. As PI4,5P_2 strengthens the talin-integrin interaction, this may displace PIPKIγ661 from talin causing a reduction in PI4,5P_2 production. By this mechanism, generation of PI4,5P_2 is highly dynamic and is self limiting (Ling *et al.*, 2003).

Additionally, PI4,5P_2 can be sequestered in a reversible fashion by proteins that can act as a buffer to bind and passively concentrate PI4,5P_2 in lateral membrane domains contributing to local pools of PI4,5P_2 (McLaughlin *et al.*, 2002). Biophysical experiments on model membranes have provided evidence that proteins such as Myristoylated Alanine-rich C Kinase Substrate (MARCKS) are concentrated in membranes and can fulfill the role of sequestering a significant fraction of PI4,5P_2 in membrane domains (Allen and Aderem, 1995; Arbuzova *et al.*, 2000; Myat *et al.*, 1997; Wang *et al.*, 2001). Cell biology data provide indications that MARCKS sequester a significant fraction of PI4,5P_2 *in vivo*. When cells are stained with antibodies specific for PI4,5P_2 and MARCKS, a strong colocalization is observed and overexpression of MARCKS was shown to generate a concomitant rise in cellular PI4,5P_2 which is anticipated given the cell maintains a near constant level of free PI4,5P_2 (Laux *et al.*, 2000). These results would be expected as it logically follows that if MARCKS acts to sequester PI4,5P_2 they should be present in the same cellular domain at comparable levels and should have a high affinity for PI4,5P_2. CAP23 a cytoskeletal protein, and GAP43 a growth associated protein, are neuronal proteins that have garnered attention as PI4,5P_2 binding proteins with parallels to MARCKS. Sequestration of PI4,5P_2 acts as a mechanism to provide PI4,5P_2 'sinks' which the cell can call upon when PI4,5P_2 is needed to activate downstream effectors. Sequestration performed by regulatory proteins such as MARCKS, GAP43 and CAP23 could potentially lay the ground work for generating a model to understand how PI4,5P_2 in nuclei is stored.

How PI4,5P_2 is stored and sequestered in nuclei has not been sufficiently resolved (Irvine, 2006). When nuclei are treated with detergents to remove the nuclear envelope a significant fraction of inositol lipids such as PI4,5P_2 remain, suggesting PI4,5P_2 is not stored in a lipid bilayer. Similar to proteins that sequester PI4,5P_2 in the cytosol, it has been suggested that proteins such as PI carrier proteins (Cunningham *et al.*, 1995; Wirtz, 1997) are present in nuclei which can store PI4,5P_2 and assure its precise localization.

PIP kinase knockout mice raise interesting questions about localized generation of PI4,5P_2 and the function of the PIP kinases. When PIPKIα is genetically deleted in mice the major physiological defect is enhanced anaphylaxis as the result of increased degranulation and cytokine production by mast cells. Evidence suggests that by generating PI4,5P_2 PIPKIα regulates actin reorganization in mast cells in a manner that is functionally important for degranulation suggesting that PIPKIα has a unique physiological role. In contrast, PIPKIα

seems to be dispensable for supplying $PI4,5P_2$ for the generation of second messengers PIP_3 and IP_3 which are required for the development and effector function of mast cells. Since PIPKIα knockout mice are viable and display no overt histological abnormalities despite the wide tissue distribution, this suggests redundancy may exist among the PIP kinases (Sasaki *et al.*, 2005).

However, when PIPKIγ is genetically inactivated in mice the animals suffer severe synaptic defects and die soon after birth. While the levels of PI and PIP in the brain appear to be unchanged, $PI4,5P_2$ is reduced by almost half suggesting $PI4,5P_2$ synthesis in the brain is a major function of PIPKIγ in a whole organism. Multiple steps of the endocytic and exocytic limbs of synaptic vesicle trafficking are $PI4,5P_2$ dependent and as a result, are severely impaired in PIPKIγ knockout mice. While neuronal defects appear to be a major physiological consequence, it seems unexpected these animals do not exhibit major developmental defects, as PIPKIγ is also an important mediator of cell adhesion dynamics required for proper development (Di Paolo *et al.*, 2004).

Highly specific protein-protein interactions are thought to be an important mechanism for the localized generation of $PI4,5P_2$ suggesting PIP kinases work in a non-redundant manner. In this model, alternative PIP kinases would be unable to compensate for the loss of a particular PIP kinase, since its directed function is dependent not only on substrate specificity, but on specific targeting interactions that are unique to each kinase. This idea seems reasonable, since a recognizable phenotype can be detected when a particular isoform or splice variant is deleted, suggesting that each of the PIP kinases has specific functions that cannot be compensate for by other PIP kinases. In contrast however, since it is expected that the PIP kinases serve a number of critical functions throughout life, it seems unexpected that loss of a specific PIP kinase isoform in a whole organism does not result in a broader phenotype prior to birth. If this is accurate, this suggests the PIP kinases may have some overlapping functions. Resolving this paradigm could be a major focus of the field in the future.

FUTURE OF THE PIP KINASE FAMILY

The family of PIPKs are a distinct family of enzymes whose intrinsic specificity loop as well as direct protein interactions with effectors works as a mechanism to target the respective PIPK to discrete cellular locations, where $PI4,5P_2$ is generated in a spatial and temporal manner to modulate cellular events. While a number of protein interactions that direct PIP kinase activity have been identified, it can be anticipated that an array of protein interactions that function in a similar manner to regulate PIP kinase activity have yet to be characterized. A number of these direct interactions appear to be isoform- or even splice-variant-specific; because of the diverse functions of the PIP kinases it seems reasonable that additional splice variants that have yet to be discovered may exist and function in a manner consistent with those previously described in order to facilitate the precise regulation of an assortment of cellular events.

While protein interactions seem to function as an additional layer of targeting, substrate specificity is still at the heart of PIP kinase function. In mammalian cells there are four distinct PI4 K that generate PI4P and there is emerging evidence that these kinases are targeted to distinct cellular compartments to carry out specific cellular functions (Balla and Balla, 2006). Whether these enzymes recruit the PIP kinases by the products they generate has not been explored, it seems likely that specific PIP kinases would function synergistically with PI kinases that generate their preferred substrate.

While the vast majority of evidence surrounding the biology of the PIP kinase family of enzymes relates to cytosolic events, there is emerging evidence of the existence of a nuclear phosphoinositide cycle autonomous from that occurring in the cytosol (Cocco *et al.*, 1987, 2004; Divecha *et al.*, 1993a, 1993b; Irvine, 2003). Phosphoinositide signaling pathways in nuclei of mammals have been shown to be key for numerous events such as the cell cycle, DNA repair and tumor progression (Irvine, 2002; Lo Vasco *et al.*, 2004). There is evidence for a role for inositol-polyphosphates in cell proliferation and DNA synthesis (Asano *et al.*, 1994; Clarke *et al.*, 2001; Kuriki *et al.*, 1992; Liu *et al.*, 1996; York and Majerus, 1994; York *et al.*, 1994), as nuclear, but not total cellular $PI4,5P_2$ levels decrease as cells progress into the S-phase of the cell cycle (York and Majerus, 1994). Additionally, the role $PI4,5P_2$ plays in nuclei may expand into the processing of pre-mRNA. Both PIPKIα and $PI4,5P_2$ co-localize at nuclear speckles with components of the pre-mRNA processing machinery, suggesting they may play a direct role in regulating pre-mRNA processing. Moreover, $PI4,5P_2$ co-immunoprecipitates with snRNPs and the

hyperphosphorylated form of RNA Pol II, strongly suggesting a function for PI4,5P_2 in the processing of nascent pre-mRNA. Whether PI4,5P_2 plays roles in regulating transcription itself, or any of the obligatory post-transcriptional steps such as capping, splicing, and/or polyadenylation could be a future focus of the field.

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