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Topical Review

Phosphatidylinositol Phosphate Kinases Put $PI4,5P_2$ in Its Place

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Abstract. Phosphatidylinositol 4,5 bisphosphate ($PI4,5P_2$) is a critical second messenger that regulates a myriad of diverse cellular activities including modulation of the actin cytoskeleton, vesicle trafficking, focal adhesion formation, and nuclear events. In order to effectively regulate these disparate cellular events, synthesis of $PI4,5P_2$ by phosphatidylinositol phosphate kinases (PIP kinases) must be both spatially and temporally regulated. Two subfamilies of PIP kinases, types I and II, allow the generation of $PI4,5P_2$ from independent pools of substrate, PI(4)P and PI(5)P respectively. In turn, type I and II PIP kinases show different subcellular localization and thus are involved in distinct signaling pathways. Additionally, several type I isoforms, and their splice variants, have now been shown to be differentially localized throughout the cell and to be involved in the synthesis of $PI4,5P_2$ at distinct sites. These findings implicate PIP kinases as the major regulators of $PI4,5P_2$-mediated events, making them key signaling enzymes in a variety of processes. Understanding the mechanisms regulating spatial and temporal synthesis of $PI4,5P_2$ by PIP kinases is vital for understanding these processes as a whole. This review examines both structural and regulatory features that modulate activity, localization, and substrate usage of PIPKs.

Key words: Phosphatidylinositol bisphosphate — Phosphatidylinositol phosphate kinases — Substrate specificity — Rac — Rho — Arf

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Introduction

The lipid second messenger phosphatidylinositol 4,5 bisphosphate ($PI4,5P_2$) has been shown to be maintained at relatively constant levels in cells [47]. However, some agonists such as growth factor stimulation or cell adhesion to the extracellular matrix cause rapid but modest changes in cellular $PI4,5P_2$ content [3, 76]. Nevertheless, numerous cellular processes are regulated by $PI4,5P_2$ (Fig. 1) [67]. The suggestion is that these processes are regulated by local changes in $PI4,5P_2$ synthesis. Although difficult to quantify, localized $PI4,5P_2$ production has been determined biochemically or by single-cell imaging with $PI4,5P_2$-specific antibodies or with the PH domain of PLCδ (PH-PLCδ) fused to GFP [5, 48]. However, the PH-PLCδ antibody only detects $PI4,5P_2$ changes on the plasma membrane, suggesting a bias towards $PI4,5P_2$ detection using this probe. Other PH domains have also been used to visualize changes in 3′phosphorylated phosphoinositides spatially within cells [5]. Even with these tools, little is known as to how site-specific changes in $PI4,5P_2$ production are coordinated. Thus, the question currently being asked is what are the mechanisms for spatial and temporal $PI4,5P_2$ synthesis by the phosphatidylinositol phosphate kinases.

The enzymes that produce $PI4,5P_2$, are the phosphatidylinositol phosphate kinases (PIP kinases), a family of lipid kinases that is sequence-distinct from all other lipid kinases, but contains a kinase core domain with conserved catalytic residues that bind ATP and Mg^{2+}. These conserved residues are spatially organized similar to the catalytic residues of other lipid kinases and protein kinases, implying that there is a common phosphotransfer mechanism [55].

The PIP kinases are divided into three subfamilies, type I, II, and III according to their signaling specificity (*see* Fig. 2). The type I and II PIP kinase subfamilies include three isoforms α, β, and γ

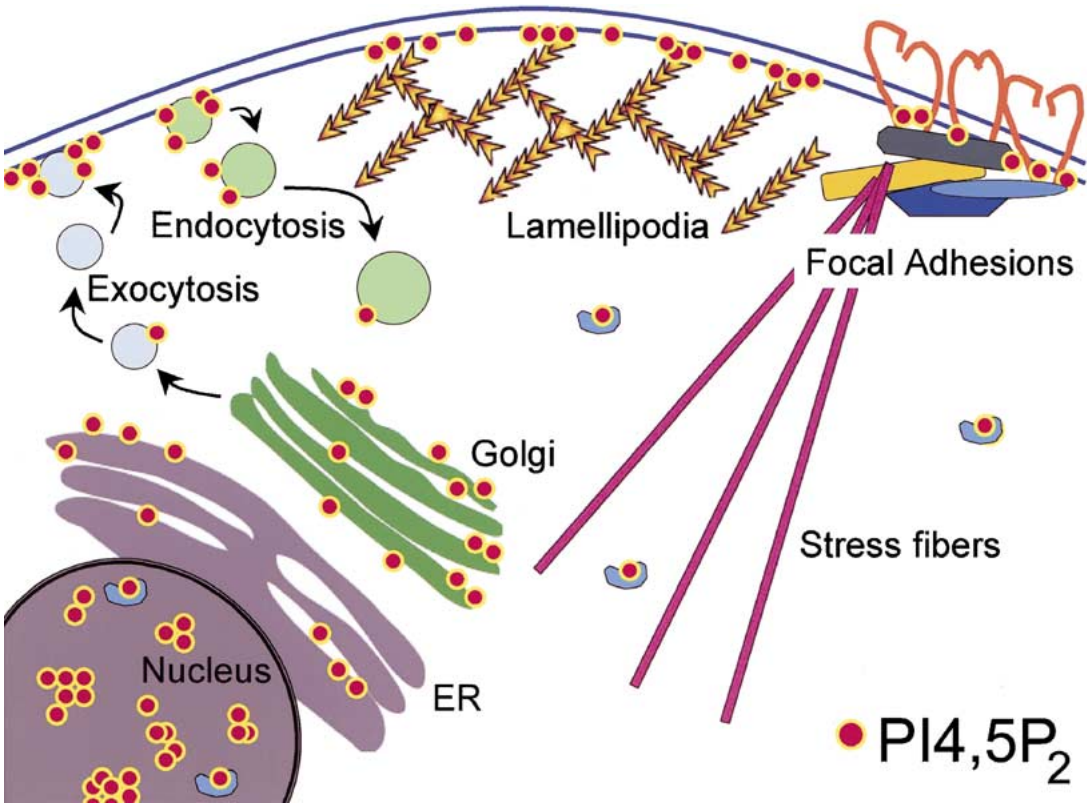

Fig. 1. $PI4,5P_2$ is produced at several cellular sites. Cellular processes such as exocytosis, endocytosis, mRNA processing, focal adhesion formation, and actin remodeling require the localized synthesis of $PI4,5P_2$ (*red*). $PI4,5P_2$ may also associate with soluble protein complexes, but the functional relevance of these complexes has not been defined. The mechanism by which $PI4,5P_2$ is maintained at each site is potentially due to direct regulation of PIP kinase isoforms. For example, PIPKIγ661 produces $PI4,5P_2$ specifically at focal adhesions and is targeted to focal adhesions by talin and regulated by FAK signaling. This is the first direct evidence of PIP kinase isoform-specific functions.

as well as a number of splice variants [7, 30, 31, 32, 42]. It is important to note that the nomenclature used throughout this review is designated for the human isoforms (hPIPKIα is mPIPKIβ). The most striking differences between these subfamilies is their substrate usage and subcellular targeting [38]. Biochemical and molecular analysis has revealed that each subfamily is responsible for the production of different lipid second messengers. Interestingly, both the type I and type II PIP kinases produce $PI4,5P_2$, but by unique mechanisms. The classical mechanism involves the phosphorylation of PI(4)P to form $PI4,5P_2$ [1]. Alternatively, $PI4,5P_2$ is also synthesized by the type II PIP kinases via the recently discovered lipid PI(5)P [53]. Hence, type I PIP kinases are PI(4)P 5-kinases since they utilize PI(4)P as substrate [53, 82], whereas the type II PIP kinases are consequently PI(5)P 4-kinases due to usage of PI(5)P to generate $PI4,5P_2$ [53]. The third family of PIP kinases, the type III PIP kinases, which are homologous to yeast Fab1p, have also been identified in mammalian cells. These kinases produce $PI3,5P_2$ in vivo, using PI(3)P as substrate, hence are PI(3) P5-kinases [20, 59]. Overall, the PIP kinase subfamilies are able to generate a variety of other lipid second messengers. The type I PIP kinases are the most versatile enzymes, as they are able to phosphorylate PI(3)P in vitro on both the 4- and 5-hydroxyls forming $PI3,4P_2$ and $PI3,4,5P_2$ [70]. The synthesis of $PI3,4,5P_2$ occurs by a concerted double phosphorylation of PI(3)P, as $PI3,4P_2$ is a very poor substrate; thus, the type I PIP kinases can be both 4- and 5-kinases [23, 70, 82]. In fact, PIPKIβ, has recently been shown to generate $PI3,4,5P_3$ in response to oxidative stress in platelets [23]. Type II PIP kinases also use PI(3)P as a substrate to produce $PI3,4P_2$, although this reaction is less efficient in vitro [38]. The type III PIP kinase family, in addition to the type I PIP kinases, can generate PI(5)P from PI and may be responsible for the synthesis of this novel lipid [59, 70]. Importantly, these findings suggest that the PIP kinases have the potential to generate phosphoinositide second messengers, which were previously attributed to the action of PI 3-kinases and phosphoinositide phosphatases.

The PIP kinases share significant sequence identity throughout the kinase domain, but are functionally unrelated and localize to distinct subcellular compartments. Type I PIP kinases are found at the

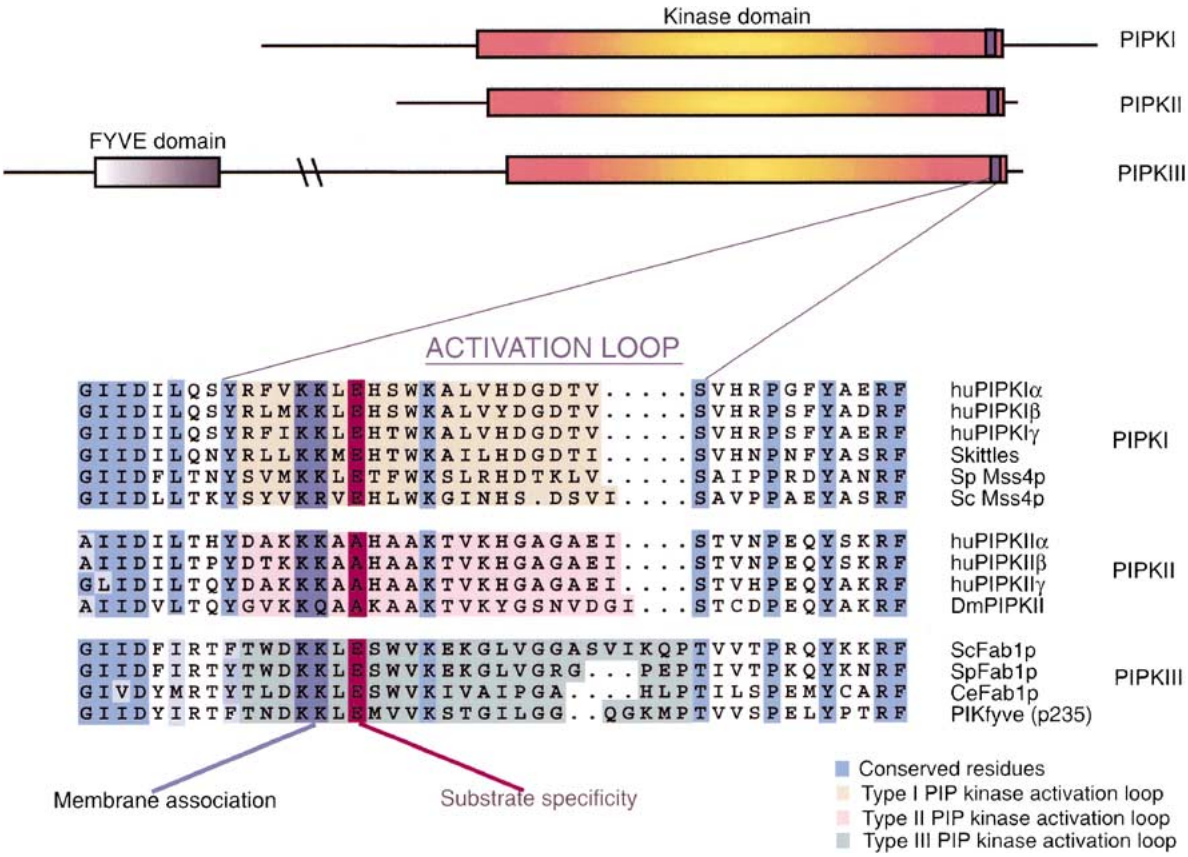

Fig. 2. The activation loop of the PIP kinase determines substrate specificity and plasma membrane targeting. The type I, II, and III PIP kinase subfamilies contain a conserved kinase domain (*yellow*). The activation loop is positioned at the C-terminus of the kinase domain and is evolutionarily conserved within each subfamily. Residues conserved between the subfamilies are highlighted in blue. Type I, II, and III PIP kinase activation loops are labeled yellow, pink, and green respectively. The KK motif is important for membrane targeting (*blue*), while the glutamate (*red*) of type I and type III PIP kinases or alanine (*red*) of type II PIP kinases is necessary for substrate recognition. The activation loop is a means by which the PIP kinases selectively generate lipid second messengers.

plasma membrane, at focal adhesions, in the nucleus, and on Golgi [7, 19, 22, 31, 41]. Type I PIP kinase activity has been shown to be necessary in actin rearrangements adhesion, secretion, endocytosis, and ion channel regulation [2, 16, 19, 25, 31, 41, 62, 63]. The yeast type I PIP kinase, Mss4p, localizes to the plasma membrane and is required for cell cycle-dependent actin reorganization and yeast cell wall integrity, suggesting that type I functions are evolutionarily conserved [17, 27]. Type II PIP kinases, in contrast, are not present in yeast but are found in multicellular organisms from *Drosophila* to mammals. Unlike type I PIP kinases, the type II enzymes do not regulate actin cytoskeleton reorganization in mammalian cells and are unable to complement the lethality of *mss4* mutants in yeast [27, 31]. In mammalian cells, type II PIP kinases are found in the cytosol, nucleus, and associated with the endoplasmic reticulum, but are not strongly associated with the plasma membrane [1, 7, 32]. The physiological functions of type II PIP kinases are not yet well defined, although a specific type II isoform, PIPKIIβ, interacts with the p55 TNF receptor in mammalian cells and thus may play a role in TNFα-mediated signaling [10]. In addition, nuclear functions of type I and type II PIP kinase isoforms have been implicated in regulation of gene expression, pre-mRNA processing, and mRNA export [1, 7, 81]. In summary, the targeting of type I and II PIP kinase to different cellular compartments and their use of distinct substrates make these enzymes prime candidates for the differential regulation of synthesis of PI4,5P$_2$ and other phosphoinositide second messenger pools in response to stimuli, as they are involved in multiple regulatory pathways.

The type III PIP kinases, although strikingly unique compared to the type I and II PIP kinase subfamilies, localize to internal membranes and maintain vesicle membrane integrity [29, 59]. Interestingly, the mammalian type III PIP kinase, PIKfyve (p235), contains a FYVE domain shown to be responsible for subcellular targeting to endomembranes [60]. The yeast homologue Fab1p is also important in regulating vacuole homeostasis and protein sorting [18, 78]. Defects of Fab1p mutants in yeast can be rescued with mammalian PIKfyve, but not with a mammalian type

I PIP kinase. This is consistent with the signaling of the type III PIP kinases, which create $PI3,5P_2$ in vivo, using the product of PI 3-kinase [46].

The arrays of phosphoinositide second messengers produced by the PIP kinases are central to the regulation of distinct cellular functions. Therefore, this review will emphasize the known and possible mechanisms that regulate localized $PI4,5P_2$ production and substrate specificity. In addition, the function of specific PIP kinase isoforms, signaling pathways, and the effectors that modulate PIP kinase activities will be discussed.

The Activation Loop Determines Substrate Specificity

The molecular mechanisms by which PIP kinases recognize and bind closely related substrates were identified as being regulated by an activation loop that mediates substrate specificity and subcellular targeting (Fig. 2). Since the PIP kinase subfamilies appear to have preferential substrate usage, Kunz et al. [38] addressed the mechanisms of substrate specificity in a series of studies that identified the activation loop as the determinant for substrate specificity. The activation loop consists of 20–25 amino acids in the C-terminus of all PIP kinases and corresponds topologically in the PIP kinase structure to the activation loop in protein kinases [55]. Although the activation loop is disordered in the crystal structure of PIPKIIβ, it spans the active site of the PIP kinases, suggesting that it plays an important role in second messenger generation [55]. This positioning of the activation loop over the PIPKIIβ active site implied a role in determining substrate recognition. Additionally, the amino-acid sequence of the activation loop is highly conserved within members of a given PIP kinase subfamily, but is divergent between them, suggesting that specific residues within the activation loop are important for coordination of phosphates on the inositol ring. Finally, the loop is adjacent to the membrane-interaction interface and it therefore is critically positioned for roles in substrate binding in membrane association and potentially in subcellular targeting.

The approach taken to determine the role of the activation loop was to engineer chimeric kinases containing reciprocal swaps of the PIP kinase activation loops. By this strategy, Kunz et al. [38] showed that the type Iβ PIP kinase (PIPKIβ) containing the activation loop from PIPKIIβ (PIPKIβ(IIβloop)) became a PI(5)P 4-kinase and no longer localized to the plasma membrane. The converse swap with PIPKIIβ containing the activation loop from PIPKIβ (PIPKIIβ(Iβloop)), also changed specificity and became a PI(4)P 5-kinase that localized to the plasma membrane.

The activation loop sequence was further characterized and the specific amino acids required for substrate specificity were identified. An array of mutations generated within the type I and type II activation loops demonstrated that substrate preference was determined predominantly by a single amino-acid residue corresponding to E362 of PIPKIβ [37]. The type II PIP kinases contain an alanine (A381) at this position (Fig. 2). Changing only this PIPKIβ glutamate to alanine (E362A) swapped substrate recognition from PI(4)P to PI(5)P. The reciprocal mutation in PIPKIIβ (A381E) consequently swapped substrate recognition from PI(5)P to PI(4)P. Moreover, PIPKIβ E362A no longer targeted to the plasma membrane, illustrating that substrate interaction is important for targeting of the type I PIP kinases to the plasma membrane when overexpressed. The glutamate 362 of PIPKIβ may determine specificity by stereo-specific exclusion of phosphoinositides phosphorylated at the D-5 position, preventing PI(5)P binding. In contrast, the alanine of PIPKIIβ enables recognition and binding of the D-5 phosphorylated substrates. From these studies, the activation loop determined substrate specificity of PIP kinases in vitro and in vivo and is both necessary and sufficient for targeting of type I PIP kinase isoforms to the plasma membrane upon overexpression [37, 38].

Signaling specificity can be achieved in part by recruitment of PIP kinases to specific membrane sites. The results show that the activation loop is both necessary and sufficient for targeting the type I PIP kinase to the plasma membrane. The type I PIP kinase isoforms contain a dilysine motif (PIPKIβ(KK)) and another invariant lysine residue within the activation loop (Fig. 2). Mutagenesis of PIPKIβ(KK) to PIPKIβ(NN) resulted in a 3-fold decrease in affinity for substrate and a loss of plasma membrane targeting, illustrating the importance of the positively charged residues [38]. Thus, several features of the PIP kinase activation loop may be critical in determining subcellular localization in addition to substrate specificity. However, current data indicate that subcellular targeting of the endogenous type I PIP kinases clearly requires interaction beyond the activation loop and this will be addressed below.

Cellular Functions of the Type I PIP Kinase Isoforms

In addition to substrate recognition and membrane interaction, other structural features of the PIP kinases are required for cellular function. As previously stated, PIPKIIβ can be targeted to the plasma membrane by swapping the activation loop with PIPKIβ, and then it produces $PI4,5P_2$ from PI(4)P. This mutant can rescue many of the Mss4 null phe-

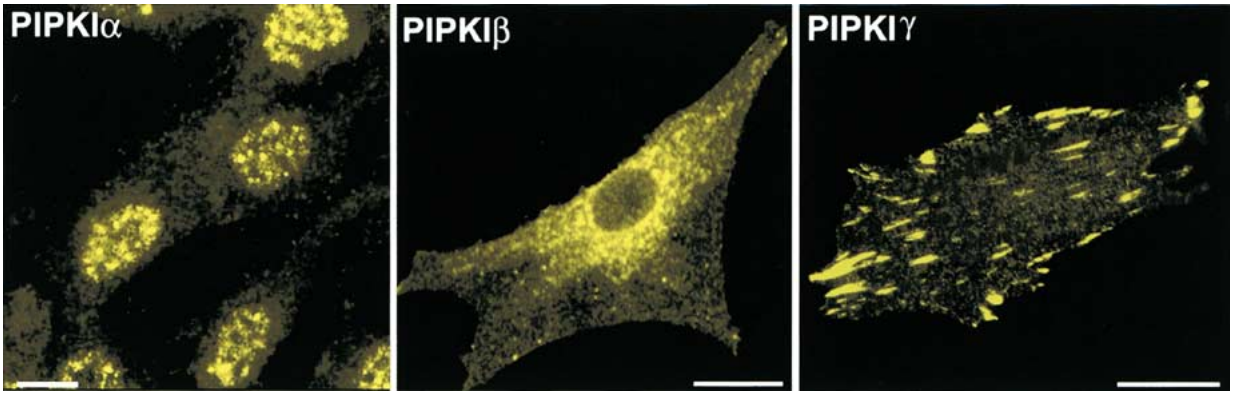

Fig. 3. Type I PIP kinase isoforms have specific and distinct subcellular localization. Endogenous PIPKIα localizes to nuclear speckles where it putatively functions in mRNA processing (*left*). The PIPKIβ localizes to vesicular structures in the perinuclear region (*middle*). Conversely, PIPKIγ661 localizes to focal adhesions (*right*). The selective subcellular localization of the type I PIP kinases provides a means for local generation of $PI4,5P_2$.

notypes when expressed in yeast, including normal actin assembly [38]. However, in mammalian cells this isoform cannot function as a type I PIP kinase and it very poorly induces actin remodeling [38]. These data suggest that additional features outside of the activation loop are necessary for specific type I PIP kinase functions.

The C-termini of the type I PIP kinase isoforms are sequence divergent, indicating that this is a region that may potentially be important for functional divergence. Each type I PIP kinase isoform is alternatively spliced and this generates multiple type I PIP kinases; and each PIP kinase isoform is differentially localized for specific cellular functions [7, 19, 41, 42]. Endogenous PIPKIα localizes to the nucleus and associates with nuclear speckles, which are sites of mRNA processing [7] (Fig. 3). PIPKIα also functions in phagocytosis, which is a process dependent upon local changes in actin dynamics [9, 14]. PIPKIα is also targeted to membrane ruffles and is required for membrane ruffle assembly; ruffle targeting and PIPKIα activity are dependent upon an association with Rac [83]. Conversely endogenous PIPKIβ is present in a vesicular perinuclear region [83]. Finally, PIPKIγ confirms both an isoform-and a splice variant-specific function. PIPKIγ is alternatively messenger RNA-spliced to form PIPKIγ635 and PIPKIγ661, which differ by a 26-amino-acid C-terminal extension [31]. Recent results showed that the 26-amino-acid C-terminus of PIPKIγ661 was sufficient for targeting PIPKIγ to focal adhesions (Fig. 3). The focal adhesion targeting was dependent upon an interaction between the PIPKIγ661 26-amino-acid C-terminus and the talin FERM domain [19, 41]. This suggests that the type I PIP kinase isoforms and splice variants have functional diversity, and at least in this case, the ability to target to specific subcellular sites, regulating spatial production of $PI4,5P_2$ and function.

Actin Remodeling Induced by the Type I PIP Kinases

The movement of cells in response to chemoattractants or growth factors is mediated by coordinated changes in the actin cytoskeleton, involving polymerization and depolymerization of actin filaments [39]. The nucleation of polarized actin filaments leads to formation of protrusions and membrane ruffles, which are critical for migration and phagocytosis [9, 14, 65]. Several actin-binding proteins, such as the WASP Arp2/3 complex, α-actinin, talin, vinculin, and gelsolin are needed for actin remodeling and membrane structure dynamics [21, 24, 26, 35, 57]. Many of these proteins are themselves modulated by $PI4,5P_2$ [4, 61].

Type I PIP kinase and actin remodeling have been examined in both yeast and mammalian systems. Mss4 is the yeast type I PIP kinase and when the *mss4* gene is knocked out, there is a dramatic defect in actin cytoskeleton integrity. In yeast, proper orientation and regulation of actin filaments is required for polarized cell growth. The knockout of Mss4 caused yeast to become enlarged and to exhibit disorganized cortical actin patches. Mss4-deficient yeast also has retarded cell growth due to the loss of actin filaments required for cell division and likely has other defects due to a loss of $PI4,5P_2$ synthesis [17]. The Mss4 knockout phenotype can be rescued however, by either reintroduction of a type I PIP kinase or of the small G protein, Rho2. The studies in yeast identified a type I PIP kinase as an essential mediator of actin dynamics [17]. In mammalian cells, all type I PIP kinase isoforms have the ability to induce actin remodeling, forming actin foci and actin comets when overexpressed [31, 58, 62]. Remodeling of the actin cytoskeleton by the type I PIP kinases is dependent upon kinase activity as well as membrane targeting [31, 38, 58, 62]. However, cell type-specific effects on actin remodeling have also been observed

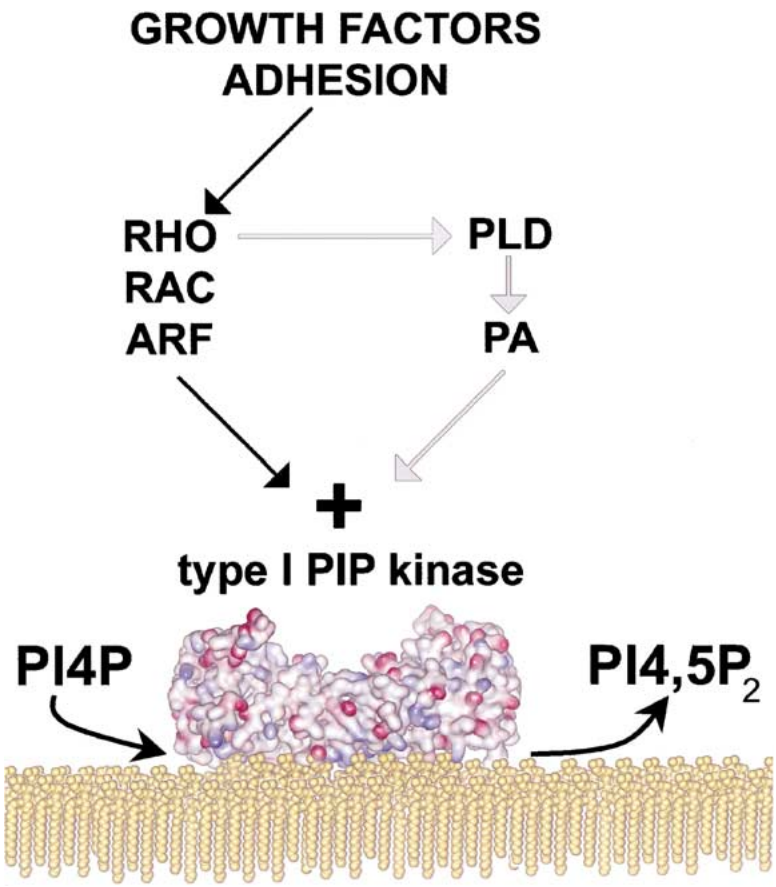

Fig. 4. Potential signaling pathways that regulate type I PIP kinases. Type I PIP kinase activity is regulated by the Rho family of small G proteins. Type I PIP kinase activity can be stimulated either directly by small G protein signaling (*black arrows*) or by activation of PLD. PLD cleaves phosphatidylcholine to produce phosphatidic acid (PA) which is a potent activator of type I PIP kinases. The Rho small G proteins may regulate intracellular targeting of the type I PIP kinases as well.

when type I PIP kinases are overexpressed. In CV1 cells, overexpression of PIPKIβ results in an increased number of stress fibers, which were dependent on Rho and ROCK signaling [79]. In HeLa cells, PIPKIβ induces the formation of membrane ruffles downstream of Arf6 signaling [28]. Upon overexpression, all type I PIP kinases are targeted to the plasma membrane and induce actin remodeling, forming actin foci and actin comets [31, 62]. When compared to the endogenous location of the type I kinases, the overexpressed condition may be an artifact. Thus, conclusions generated from overexpression data have to be interpreted cautiously.

The physiological significance and the associated signaling pathways leading to actin remodeling induced by type I PIP kinases are not well understood. Analysis of the proteins associated with actin comets may help to clarify this pathway. Type I PIP kinase actin comets are similar to those made when *Listeria* infects cells. The formation of actin comets by PIPKIβ was modestly enhanced by growth factor stimulation and tyrosine phosphorylation of comet-associated proteins. Furthermore, PI 3-kinase activity was not required for actin comet formation, providing evidence that $PI4,5P_2$ may directly modulate actin dynamics. The actin tail of comets is comprised of newly formed actin filaments, making them highly dynamic structures. The Wasp/Arp2/3 complex is involved in nucleation of the PIPKIβ-induced actin comets from lipid rafts at the plasma membrane [58]. PIPKIβ is localized at the head of comets, which were also shown to be associated with membrane lipids, endocytic vesicles, and Golgi-derived vesicles [58]. Interestingly, it was hypothesized that actin foci and actin comets were formed by different modes of actin polymerization. Actin comets are asymmetrically formed, while actin foci are formed by symmetric actin polymerization [58]. The cargo and signaling molecules associated with the vesicle may potentially mediate the differential polymerization of actin from the comets. The formation of actin comets is not type I PIP kinase isoform-specific. Recently, actin comets induced by PIPKIγ or PIPKIβ were characterized and it was discovered that dynamin was associated with the comet head. As PDGF and tyrosine phosphorylation were able to modulate the dynamics of type I PIP kinase induced actin comets, dynamin activity also contributed to the dynamics of the actin comets [40, 50]. The localized production of $PI4,5P_2$ is able to mediate the formation of actin comets via regulation of Wasp or dynamin. These studies provide a link between actin dynamics and vesicular trafficking, both processes known to be modulated by the type I PIP kinase isoforms.

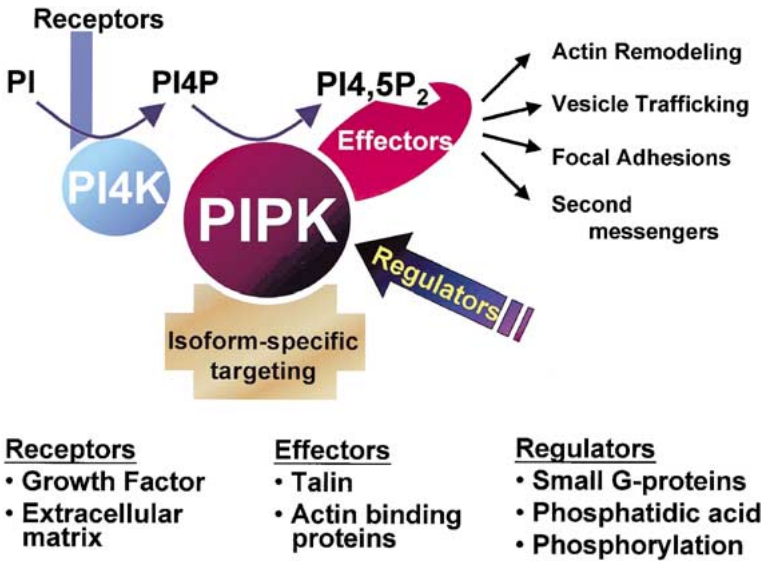

Fig. 5. Model for local synthesis of $PI4,5P_2$ by PIP kinases. PIP kinase associate with a signaling complex where effector molecules directly utilize $PI4,5P_2$. PIP kinases are localized to distinct subcellular sites either by an isoform-specific targeting sequence or by binding to targeting proteins. The signaling complex also contains regulators of PIP kinase activity such as small G proteins or protein kinases. In addition, PI4K would be present so as to supply substrate to the PIP kinases. Extracellular signals are received either via growth factor receptors or extracellular cellular matrix receptors such as integrins. Finally, effector proteins require $PI4,5P_2$ for activity or subsequent protein:protein interactions. Examples of effector proteins are talin or actin-binding proteins.

Regulation of Vesicle Trafficking by Type I PIP Kinases

Phosphoinositide signaling is involved in the process of vesicular trafficking by modulating endocytosis, regulated and constitutive exocytosis, and clathrin coat assembly [13]. As previously discussed, there is growing evidence linking actin dynamics to vesicle trafficking and $PI4,5P_2$ is a key player in both processes [40, 44, 50, 58]. Pools of $PI4,5P_2$ are non-uniformly distributed throughout the plasma membrane and on vesicles to provide sites of vesicle fusion, clathrin coat assembly, or regulated actin dynamics [44]. The type I PIP kinases have defined roles in exocytosis, endocytosis, and clathrin coat assembly [2, 6, 16, 25]. Regulated exocytosis requires the coordinated assembly of many proteins for proper vesicle docking, priming, and fusion. Early studies identified three proteins important for exocytosis, including a PIP kinase, which was identified as a type I PIP kinase. Immunodepletion of type I PIP kinase results in both a decrease in vesicle priming and $PI4,5P_2$ synthesis [25]. Type I PIP kinase activity is also required for endocytosis. Studies have shown that when the N-terminus of PIPKIα is truncated, endocytosis was effectively blocked. This mutant was isolated from a genetic screen to rescue defects in colony-stimulating factor 1 signaling. The truncated PIPKIα lacked kinase activity and was found to rescue the cellular phenotype by inhibiting endocytosis, thus allowing sufficient levels of receptor to remain at the plasma membrane [6, 16]. The mechanism by which the truncated PIPKIα inhibits endocytosis may be by inhibition of clathrin coat assembly. Type I PIP kinase and $PI4,5P_2$ are required for the recruitment of adapter proteins, specifically AP-2, to sites of endocytosis [2], The type I PIP kinases are indeed responsible for producing $PI4,5P_2$ on vesicles and plasma membrane, but where each type I PIP kinase isoform is positioned remains unclear.

Regulation of Type I PIP Kinase Activity by Small G Proteins

$PI4,5P_2$ mediates actin dynamics by modulating signal transduction pathways and the activity of multiple actin-binding proteins [4, 61, 67]. Members of the Rho family of small G proteins, Rho, Rac, and Arf, regulate actin remodeling and vesicular trafficking. First, it has been known for some time that the small G proteins, RhoA, Rac1, and Arf6, coordinate the formation of stress fibers and focal adhesions, lamellipodia, and membrane ruffles, respectively [49]. In addition, each small G protein regulates type I PIP kinase activity and thus $PI4,5P_2$ synthesis in a targeted manner (Fig. 4). Recently, clear links have emerged between small G-protein signaling and $PI4,5P_2$ synthesis. The ability of Rho, Rac and Arf6 to stimulate PIP kinase activity and regulate localization will be discussed.

Rho

Early studies in Swiss 3T3 cells identified Rho as the major small G protein that regulates $PI4,5P_2$ synthesis [12]. Later studies showed that a type I PIP kinase associates with Rho in erythrocytes and Swiss 3T3 cells. Interestingly, the type I PIP kinase association with Rho was independent of Rho activation and was enhanced by ADP ribosylation of Rho by C3 exoenzyme [56]. Rho stimulated PIP kinase activity in a GTP-independent manner and PIP kinase activity was also enhanced by phosphatidic acid [56]. Together these results provided evidence that in vivo

signaling pathways incorporated both PIP kinase and small G-protein signaling.

The mechanism by which Rho activated type I PIP kinase activity was found to be partially dependent upon Rho effectors. The Rho effector, Rho kinase (ROCK), is implicated in the regulation of the actin cytoskeleton as well as the downstream signaling of Rho [43]. Studies have shown ROCK potently stimulates type I PIP kinase activity in vitro. Therefore, Rho activates type I PIP kinase activity through the Rho effector, ROCK [51]. Type I PIP kinases were shown to function downstream of Rho and ROCK signaling in neuronal cells by inducing the reorganization of cortical actin filaments, thus inhibiting neurite formation [73, 80]. Rho and ROCK signaling are required for the retraction of neurites and the type I PIP kinase dead mutant is able to potently block neurite retraction induced by Rho and ROCK. This illustrates the importance of $PI4,5P_2$ synthesis in neurite remodeling. Furthermore, pharmacological inhibition of ROCK by Y-27632, blocking neurite retraction, is rescued by type I PIP kinase overexpression. This data shows PIP kinase to be downstream of Rho and ROCK signaling, supporting a mechanism by which Rho stimulates PIP kinase activity. Interestingly, other studies have shown that type I PIP kinases are direct effectors of Rho signaling, independent of ROCK [45]. Rho signaling has been shown to activate the ERM (ezrin, radixin, moesin) family of proteins, inducing their translocation to the plasma membrane and leading to the subsequent cross-linking of actin filaments. Dominant active Rho is able to induce actin remodeling and also increases the amount of activated ERM proteins at the plasma membrane. Furthermore, expression of a type I PIP kinase results in similar actin remodeling and ERM activation when compared to Rho, and PIP kinase activity is required for ERM activation. [45]. The ability of PIP kinase or Rho to induce actin remodeling and ERM activation was not blocked by ROCK inhibition, suggesting a mechanism by which type I PIP kinase is directly downstream of Rho signaling and is potentially being activated by Rho [45]. Together, this data illustrates that type I PIP kinase signaling is downstream of Rho and that it mediates actin remodeling by both a ROCK-dependent and-independent mechanism [45, 73, 80].

Rac

Type I PIP kinase association with Rac was first demonstrated in vivo by immunoprecipitation of Rac from rat liver. Both PIPKIα and PIPKIβ are able to associate with Rac in vitro, independent of GTP binding to Rac. Interaction with Rac was enhanced by specific phospholipids, such as phosphotidylserine, phosphatidic acid (PA), and PI(4)P, consistent with previous reports showing PA is able to activate type I PIP kinases [34, 69]. The functional significance of Rac and type I PIP kinase association was linked to regulation of Rac-dependent actin assembly. In permeabilized platelets, Rac association with PIPKIβ, but not PIPKIα, regulates actin assembly [71]. Together, Rac and PIPKIβ function by uncapping actin filaments to expose barbed ends, leading to an increase in actin assembly. The increase in actin assembly requires both the association of PIPKIβ with Rac as well as PIP kinase activity [71]. When the association of Rac and PIPKIβ was disrupted by a single point mutation in Rac (RacK186E), there was a decrease in PIP kinase activity and in actin assembly [71]. This data supports a role for isoform-specific PIP kinase function downstream of Rac, leading to actin assembly as well as points to the importance of local $PI4,5P_2$ synthesis. Interestingly, immunoprecipitation of Rac also resulted in an association of PI 3-kinase activity [68]. PI 3-kinase activity is necessary for activation of Rac signaling [68]. We have found that Rac-induced membrane-ruffle formation requires both PI 3-kinase and type I PIP kinase activity. Membrane ruffling is inhibited by overexpression of kinase-dead PIP kinase as well as by inhibiting PI3K [83]. Together these studies now provide insight into the functional significance in the regulation of actin dynamics by Rac and type I PIP kinase signaling.

Arf

ADP ribosylation factors (ARF) are a family of small G proteins primarily involved in regulation of vesicular trafficking and the actin cytoskeleton [54]. The subcellular localization of Arf has been limited to the Golgi and plasma membrane for Arf1 and Arf6 respectively. Type I PIP kinase isoforms have been shown to function downstream of Arf6 signaling [8, 28]. Studies with Arf have provided evidence of a potential mechanism by which $PI4,5P_2$ is synthesized on the Golgi and plasma membrane. Arf activation of $PI4,5P_2$ synthesis was first identified on the Golgi, but the specific type I PIP kinase isoform is not known [22]. Arf1 and Arf6 have both been shown to increase $PI4,5P_2$ synthesis via activation of type I PIP kinase and phospholipase D (PLD) [28, 36, 64]. Small G-protein activation of PLD causes production of phosphatidic acid, a potent activator of the type I PIP kinases [34] (Fig. 4). However, Honda et al. demonstrated that Rac and Rho are unable to stimulate PIPKIβ and that its activity was dependent only upon Arf and PA [28]. PA-stimulated type I PIP kinase activity was found to be associated with the assembly of clathrin coats by recruitment of AP-2 to the plasma membrane [2]. Together this data suggests a mechanism for Arf-dependent activation of type I PIP kinase at the Golgi or plasma membrane in the regulation of vesicular trafficking.

Subcellular Distribution

Subcellular distribution of type I PIP kinase has also been addressed in response to the various small G proteins discussed. Rho and Rac also influences the subcellular distribution of type I PIP kinase, specifically PIPKIα. Coexpression of dominant active Rac or dominant active Rho with PIPKIα results in targeting of overexpressed PIPKIα to the plasma membrane. The translocation of PIPKIα to the plasma membrane is dependent upon Rho and Rac activation, and is blocked by the dominant negative forms of Rac or Rho, but not Arf6 [11]. However, other studies have shown that Arf6 induces translocation of PIPKIβ to membrane ruffles, resulting in local accumulation of $PI4,5P_2$, and this occurs by both a Rac-dependent and Rac-independent mechanism [28]. In addition to membrane ruffling, Brown et al. demonstrated that Arf6-dependent activation of type I PIP kinase and localized $PI4,5P_2$ synthesis are required for vesicle trafficking through the Arf6 plasma membrane-endosomal recycling pathway [8]. Together these studies suggest that small G proteins are important in targeting of the type I PIP kinases resulting in local $PI4,5P_2$ synthesis.

In summary, the small G proteins modulate the type I PIP kinases by complex mechanisms. The association of the small G proteins in the case of Rho and Rac with type I PIP kinases are not GTP-dependent. In the case of Rac, interaction with type I PIP kinase occurs outside of the Rac effector domain, suggesting that Rac may not be directly activating type I PIP kinases. However, the data suggests that small G-protein interaction with type I PIP kinases modulates the intracellular targeting of PIP kinases. As spatial production of $PI4,5P_2$ is key for actin dynamics and vesicle trafficking, type I PIP kinase intracellular targeting would be a critical step in regulation of PIP kinase activity.

Posttranslational Modifications

There are now several examples demonstrating that type I PIP kinase activity can be directly regulated by protein phosphorylation. These examples cover both yeast and mammalian enzymes and indicate that, overall, phosphorylation has an inhibitory effect on type I PIP kinase activity [33, 52, 72]. In fission yeast, *Schizosaccharomyces pombe*, a type I-like PIP kinase was shown to be phosphorylated in vitro by the casein kinase I homologue Cki1. The resulting in vitro phosphorylation was then shown to strongly inhibit type I PIP kinase activity [72]. Further experiments demonstrated that the phosphorylation of a single serine residue was responsible for the inhibitory activity. Interestingly, it was also shown that overexpression of Cki1 in *S. pombe* reduces the cellular levels of $PI(4,5)P_2$ [72]. Studies of mammalian type I PIP kinases have also demonstrated an inhibitory effect of phosphorylation on type I PIP kinase activity.

Itoh et al. [33] showed that purified recombinant type I enzymes possess protein kinase activity and are able to autophosphorylate in the presence of ATP and Mg^{2+}. Autophosphorylation was dramatically enhanced by the addition of PI and resulted in a potent inhibition of kinase activity. As with the *S. pombe* type I PIP kinase, inhibitory phosphorylation was shown to involve mainly serine residues. The physiological significance of this autophosphorylation is unknown, but Itoh and colleagues were able to show that immunoprecipitated PIPKIβ was similarly phosphorylated and that subsequent phosphatase treatment of the enzyme was able to increase PIP kinase activity. The recently solved structure of PIPKIIβ demonstrates that key positive residues are required for enzyme interaction with the phosphohead group of substrate [55]. Based on these observations it was suggested that serine phosphorylation in proximity to these key residues may perturb substrate binding, providing a mechanism for the reduced catalytic activity [33]. These assertions, however, are somewhat in conflict with the kinetic data from the *S. pombe* type I PIP kinase, which implicated the decrease in catalytic activity to a reduction in V_{max} and no change in K_m [72].

Further work on the mammalian type I PIP kinases has suggested a physiological role for phosphoregulation of these kinases in signaling pathways [41, 52, 77]. LPA stimulation of type I PIP kinase activity was shown to directly correlate with dephosphorylation of the enzyme and furthermore it was shown that the dephosphorylation event was sensitive to the phosphatase inhibitor okadaic acid, suggesting the involvement of protein phosphates type 1 or 2 (PP1, PP2A) [52]. Park and colleagues [52] went on to show that in vitro treatment of cellular PIPKIβ with PP1 could reduce PIPKIβ phosphorylation and increase its kinase activity. Additionally, the group showed that PKA may be involved with the initial phosphorylation event, as treatment of 3T3 cells with the PKA inhibitor, H89, but not with other protein kinase inhibitors, was able to reduce the resting phosphorylation of PIPKIβ [52]. Similarly, depolarization of synaptosomes is known to induce an increase in phosphoinositide turnover, and it has recently been reported that the major neuronal PIPKIγ isoform undergoes a depolarization-dependent dephosphorylation [77]. A correlating change in activity for this enzyme, however, has not been shown.

Very recently, there has been a report of a tyrosine-phosphorylation event that affects a type I PIP kinase activity [41]. Our laboratory has shown that PIPKIγ661 is tyrosine phosphorylated downstream

of focal adhesion kinase (FAK) and that this event serves to increase the activity of the kinase and its association with interacting protein talin. Interestingly, it was also recently shown that talin itself may stimulate PIPKIγ661 kinase activity [19]. Thus, it is not known at present whether tyrosine phosphorylation is directly or indirectly involved in the kinase activity increase.

Localized Production of $PI4,5P_2$ by the Type I PIP Kinases

$PI4,5P_2$ is found at a variety of subcellular sites, such as different domains of the plasma membrane, organelles, vesicle membranes, and the nucleus [75]. Functionally this is critical, as many cellular processes and signaling pathways utilize $PI4,5P_2$ to recruit proteins to specific membrane domains and to generate additional second messengers [15, 47, 67]. Early studies of cellular phosphoinositide levels revealed that upon stimulation by growth factors, $PI3,4,5P_3$ and IP_3 levels dramatically increase, while cellular $PI4,5P_2$ levels decrease slightly after stimulation, but are quickly resynthesized [3]. This approach provided little insight into the changes in local $PI4,5P_2$ levels.

A key question that remains to be elucidated is, how local pools of $PI4,5P_2$ accumulate within the cell. Local accumulation of $PI4,5P_2$ is proposed to occur by two mechanisms: lateral sequestration and local synthesis. The lateral sequestration model is discussed by McLaughlin et al., suggesting $PI4,5P_2$ is sequestered by electrostatic interactions [47]. Recent studies have suggested that proteins with a highly basic cluster of amino acids are able to interact with polyphosphoinositides by electrostatic interactions, limiting diffusion of phospholipids from sites of signaling. MARCKS (myristolyated alanine-rich C kinase substrate) is a candidate for such sequestration that leads to local accumulation of $PI4,5P_2$. In vitro studies have shown MARCKS to bind to $PI4,5P_2$ membranes with high affinity and to be present at high cellular concentrations similar to $PI4,5P_2$ [47]. MARCKS is found in phagosomes and membrane ruffles, sites rich in $PI4,5P_2$ as well as actin, placing MARCKS in a cellular context consistent with $PI4,5P_2$-dependent signaling. An alternative model for local accumulation of $PI4,5P_2$ is local synthesis by the type I PIP kinases. Local synthesis is known to be responsible for creating gradients of $PI3,4,5P_3$ at phagosomes and lamellipodia [47]. Recent studies have also contributed transient accumulation of $PI4,5P_2$ at phagosomes to local synthesis by type I PIP kinases [9]. In cells, a combination of sequestration by MARCKS or other $PI4,5P_2$-binding proteins and local synthesis is a potential mechanism for accumulation of $PI4,5P_2$. Accumulation of $PI4,5P_2$ pools may occur at specific subcellular sites enriched in $PI4,5P_2$ effector proteins, forming a signaling complex that leads to generation of second messengers and activation of signaling pathways.

An example showing the regulation of pools of $PI4,5P_2$ is in the control of actin dynamics and focal adhesions. Actin remodeling is important for the formation of filopodia, lamellipodia, membrane ruffles, and phagosomes. The activities of many factors involved in regulating the dynamics of actin filaments are controlled by $PI4,5P_2$. Recent studies have begun to unravel the role of lipid second messengers in phagocytosis. PIPKIα is recruited to the phagosomal cup upon stimulation with opsinized beads in macrophages. As PIPKIα is recruited, a rise in local $PI4,5P_2$ is observed at the phagosomal cup. This pool of $PI4,5P_2$ is transient, as levels of DAG dramatically increase and $PI4,5P_2$ decreases due to the activation of PLC [9]. The local generation of $PI4,5P_2$ is critical in the process of phagocytosis. When local $PI4,5P_2$ synthesis is inhibited with kinase-dead PIPKIα, stimulated phagocytosis is inhibited [14]. These recent studies illustrate that $PI4,5P_2$ is locally synthesized at sites of actin remodeling. An other group has shown that when pools of $PI4,5P_2$ are decreased by the expression of PLC, cortical actin remodeling is also decreased [74]. This again illustrates that spatial and temporal regulation of $PI4,5P_2$ is required for many cellular processes.

Local accumulation of $PI4,5P_2$, may occur at specific subcellular sites enriched in $PI4,5P_2$ effector proteins forming a signaling complex. The signaling complex contains $PI4,5P_2$ binding proteins important for generation of second messengers and activation of signaling pathways [1]. Recent advances made in PIP kinase signaling provides evidence that this model depicts a mechanism for local $PI4,5P_2$ production (Fig. 5). An example is the requirement of $PI4,5P_2$ at focal adhesions. PIPKIγ661 specifically localizes to focal adhesions and interacts directly with talin. Talin-binding to the β-integrin is mediated by $PI4,5P_2$. In addition, a key regulator of PIP kinase activity at focal adhesions is FAK signaling. Activation of FAK results in increased tyrosine phosphorylation of PIPKIγ661, resulting in increased PIP kinase activity [41]. Other studies have identified a PI4K to localize to focal contacts via an interaction with a member of the tetraspanin-4 family, which forms complexes with integrins [66]. PI4K recruitment to focal complexes provides local generation of substrate for the PIP kinases. The formation of this signaling complex provides evidence for regulation of $PI4,5P_2$ synthesis at focal adhesions.

Conclusion

$PI4,5P_2$ is a key lipid second messenger that is involved in the regulation of numerous cellular processes. The spatial and temporal regulation of $PI4,5P_2$ synthesis is critical in cell survival and function.

Currently, work has shifted towards studying the mechanisms by which PIP kinases produce $PI4,5P_2$ in response to signaling pathways, and towards identifying factors that interact with and regulate PIP kinase activity. From regulation of the PIP kinases at the level of substrate specificity to activation by small G proteins and phosphorylation, current studies are providing insight into how $PI4,5P_2$ is synthesized at the Golgi versus the plasma membrane. The significant differences in effects of the various small G proteins on type I PIP kinase activity and cellular distribution due to Rho, Rac, and Arf remains to be clarified. The role of small G-protein activation of specific type I PIP kinase isoforms and the functional impact on vesicular trafficking and actin rearrangements remain highly controversial.

Several type I PIP kinase splice variants have been identified to date, but only the function of the PIPKIγ661 splice isoform has been elucidated. The existence of multiple type I PIP kinase splice isoforms may permit the type I kinase to function individually in distinct cellular functions. Determining the role of each type I PIP kinase isoform and of splice variants will further address the mechanism of $PI4,5P_2$ spatial and temporal synthesis and function. Significant advances have been made in determining type I PIP kinase isoform-specific function, especially in focal adhesion formation [19, 41]. As studies progress, the family of PIP kinases continues to put $PI4,5P_2$ in its place.

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