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INVITED REVIEW

Regulation of the actin cytoskeleton by phosphatidylinositol 4-phosphate 5 kinases

Yuntao S. Mao · Helen L. Yin

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Abstract Phosphatidylinositol (4,5)-bisphosphate (PIP_2) is an important lipid mediator that has multiple regulatory functions. There is now increasing evidence that the phosphatidylinositol 4-phosphate 5 kinases (PIP5Ks), which synthesize PIP_2, are regulated spatially and temporally and that they have isoform-specific functions and regulations. This review will summarize the highlights of recent developments in understanding how the three major PIP5K isoforms regulate the actin cytoskeleton and other important cellular processes.

Keywords PIP_2 · PIP5K · Actin cytoskeleton · Arf6 · Endocytosis · Phagocytosis

Phosphatidylinositol (4,5)-bisphosphate (PIP_2) is particularly abundant at the plasma membrane (PM) and is proposed to be a PM organelle marker, which distinguishes the PM from the internal organelles that are more highly enriched in other types of phosphoinositides [35, 111, 140]. As such, PIP_2 is the hub for the docking of multicomponent signaling complexes [56] and the maintenance of cytoskeletal-PM adhesion [122]. PIP_2 is also a regulator of ion channels [58, 130], endocytic and exocytic membrane trafficking [72, 89], integrin signaling [32], cytokinesis [84], epithelial cell morphogenesis [88], and apoptosis [53, 92]. Some of these roles are mediated through PIP_2-dependent modulation of the actin cytoskeleton [59, 120, 149], while others are not [111]. In addition, PIP_2 is the immediate precursor to three pivotal second messengers, diacylglycerol (DAG), inositol (1,4,5)-triphosphate ($InsP_3$), and phosphatidylinositol (3,4,5)-triphosphate (PIP_3) [38].

Y. S. Mao · H. L. Yin (✉)
Department of Physiology,
University of Texas Southwestern Medical Center,
5323 Harry Hines Blvd,
Dallas, TX 75390-9040, USA
e-mail: Helen.Yin@UTSouthwestern.edu

PIP_2 dynamics

PIP_2 is found primarily on the cytoplasmic leaflet of the PM where it accounts for approximately 1% of membrane phospholipids [91]. PIP_2 is generated from phosphatidylinositol monophosphates (PIPs) through two distinct pathways: first, by the type I phosphatidylinositol 4-phosphate 5 kinases (PIP5Ks) which phosphorylate phosphatidylinositol 4-phosphate [PI(4)P] on the D-5 position of the inositol ring, and second, by the type II PIP4Ks which phosphorylate PI(5)P at the D-4 position. These two types of PIP kinases are non-redundant and have distinct functions [60]. PIP4Ks exist as dimers, which form a flattened surface that docks on the lipid bilayer [107]. PIP5Ks are likely to have a similar overall organization. Domain-swapping experiments show that the recognition of PI(4)P vs PI(5)P is dictated primarily by the specificity loop within the kinase core; PIP4Ks have a conserved ala that recognizes PI(5)P, while PIP5Ks have a conserved glu that recognizes PI(4)P [75]. As PI4P is much more abundant than PI(5)P [132], PIP5Ks are likely to be the major source of PIP_2. This is confirmed by pulse-labeling studies [128]. We will focus on PIP5Ks exclusively in this review.

PIP_2 level is determined by a balance between synthesis and dissipation. PIP_2 can be decreased in many ways. First, PIP_2 is hydrolyzed by phospholipase C (PLC) to generate $InsP_3$ and DAG. This provides an effective mechanism for downshifting the PIP_2 signal [109]. Second, PIP_2 is converted by the class I phosphoinositide 3 kinases (PI3Ks) to generate PIP_3, which is important for signaling, growth regulation, and cell migration [44, 144]. Third, the D-5 phosphate on the PIP_2 inositol ring is dephosphorylated by phosphoinositide 5 phosphatases [6], such as synaptojanin [104] and Ocrl [42], which have been implicated in the maintenance of PIP_2 homeostasis at the PM and *trans* Golgi network (TGN), respectively. Fourth, PIP_2 that is generated

locally may be dissipated by diffusion, but a gradient can be maintained by continuous generation locally or by PIP_2 binding to scaffolding molecules at a site of synthesis to immobilize the lipid [27].

There is now overwhelming evidence suggesting that some pools of PIP_2 are generated in a spatially and temporally regulated manner and that downregulation of the PIP_2 signal is critically important for the cycling of almost all PIP_2-dependent processes [18, 28, 119].

New tools for studying PIP_2 dynamics

PH-PLCδ-GFP has been widely used to monitor PIP_2 dynamics in cells by high-resolution live cell imaging [13]. It strongly labels the PM [12], and its ability to accurately report PIP_2 levels at the PM, when overexpressed at low level, has been corroborated in fixed cells using anti-PIP_2 antibody [78, 140].

There have been many attempts to change the PIP_2 level in cells. A constitutively membrane-targeted yeast inositol polyphosphate 5 phosphatase (Inp54p) is used to assess the role of PIP_2 in membrane–cytoskeleton interactions [108]. A cell permeant PIP_2-binding peptide derived from gelsolin's PIP_2-binding domain depletes PM PIP_2 in 3T3-L1 adipocytes and inhibits glucose transport in an actin-dependent manner [45]. PIP_2 is shuttled into intact cells through histone carriers to increase PIP_2 globally [140] or selectively on the basal vs apical side of polarized epithelial cells [88].

Recently, the arsenal for manipulating PIP_2 has become even more sophisticated. 5 phosphatase and PIP5K are targeted to the PM using the rapamycin-inducible FKBP and FRB dimerization [56, 131, 137]. In one study, the cytosolic form of Inp54p is fused to CFP-tagged FKBP (CFP-FKBP-Inp54p) and cotransfected with plasmids encoding FRB attached to the membrane targeting domain of Lyn11 (Lyn11-FRB) and YFP-PH-PLCδ [56]. Rapamycin induces translocation of the normally cytosolic CFP-FKBP-Inp54p to the PM and a reciprocal dissociation of YFP-PH-PLCδ from the PM. PIP_2 depletion by PM targeted Inp54p blocks the KCNQ K^+ channels, while rapamycin targeting of PIP5Kγ increases K^+ current. Significantly, targeting of an activator of PI3K, which increases PIP_3 but not PIP_2, has no effect on the K^+ channels. As PIP_2 level is changed acutely in the absence of the cascade of Ca^{2+}, DAG, or $InsP_3$ signals that normally accompany PIP_2 signaling, these results show conclusively that PIP_2, and not the second messengers generated from PIP_2, is the direct regulator of the K^+ channels.

PIP_2 microdomains

It has been reported that approximately half of the cell's PIP_2 is synthesized preferentially in cholesterol/sphingolipid enriched caveolar light membrane fractions (rafts) [48, 96, 105] and that these PIP_2-enriched microdomains exhibit locally regulated PIP_2 turnover and restricted diffusion-mediated exchanges with their environment [48]. There are also reports that PIP_2 is enriched in noncaveolar microdomains that are the staging platforms for choreographing signaling and cytoskeletal dynamics. The existence of PIP_2 microdomains is confirmed by immunofluorescence staining of PM sheets prepared from PC12 cells [3, 94]. The PM PIP_2 microdomains are heterogenous; some contain conventional raft markers, while others are enriched for syntaxin, which is involved in Ca^{2+}-mediated exocytosis and mostly excluded from the low-density raft fraction [3]. Other PIP_2 clustering proteins have also been identified. These include MARCKS, which sequesters PIP_2 under basal conditions and is induced by agonist signaling to release PIP_2 for interaction with other PIP_2 targets [91]. In the case of phagocytosis, PIP_2 accumulates and remains in the phagocytic cup for minutes without diffusing away [27]. This accumulation is not dependent on rafts or the actin cytoskeleton, raising the possibilities that lipids are held in place by the restriction caused by extreme membrane curvature and by binding to proteins within the phagocytic cup.

Raft isolation by floatation on density gradients shows that PIP5K is not enriched in rafts in PC12 cells or platelets [3, 148], while the same PIP5K is recruited to lipid rafts during B cell activation [114]. The yeast PIP5K, Mss4p, is also not present in rafts, although its association with membrane is sphingolipid-dependent [70]. Recently, the existence of rafts per se has been intensely debated [121] because there are suggestions that detergent extraction per se induces artifactual clustering, and optical measurements give mixed results, with only some data supporting the existence of a less mobile lipid population [23].

The questions of whether PIP_2 exists in heterogeneous microdomains, and whether these domains are formed by PIP_2 in cholesterol-rich rafts by interaction with proteins, or by a combination of both, need to be answered. They hold the key to understanding how PIP_2 is regulated spatially and temporally and how the PIP_2 pools generated by the PIP5K isoforms are functionally, and possibly physically, segregated.

PIP_2 regulates multiple actin-binding proteins

Cytoskeletal proteins were among the first shown to be regulated by PIP_2 [59, 149], and many of these proteins regulate actin dynamics at the cell cortex [25, 57, 129]. Some bind PIP_2 through well-defined PIP_2 recognition modules [80]. For example, ezrin, a member of the ERM family, which links actin filaments to the PM, has a FERM domain which binds PIP_2, inducing the relief of the

autoinhibited state [103]. However, the majority of actin regulatory proteins bind PIP_2 using less obviously structured motifs that contain clusters of basic/aromatic amino acids [149]. Some examples are: WASP family proteins, which promote actin assembly by activating the nucleating Arp2/3 complex; gelsolin family proteins, which sever and cap actin filaments to promote dynamic actin reorganization; vinculin, which regulates focal adhesion (FA) turnover; capping protein, which caps the (+) end of actin filaments; and cofilin, which severs actin filaments to accelerate their in vivo treadmilling. In most cases, PIP_2's charged inositol headgroup and hydrophobic acyl chain are both required for binding [50, 67, 77]. The length of the acyl chain is also critical; di-C_{16} and di-C_8 PIP_2 bind cofilin, while di-C_4 PIP_2 does not [50].

Recently, the three-dimensional structure of cofilin bound to di-C_8 PIP_2 has been solved by nuclear magnetic resonance [50]. It reveals rich mechanistic details about how cofilin interacts with PIP_2 and suggests a model in which the interplay between cofilin inactivation by PIP_2 and activation by dephosphorylation can specify the spatial and temporal regulation of cofilin at the PM.

Cloning of PIP5Ks

Yeast has a single PIP5K gene, while mammals have three. In 1996, two mammalian PIP5K isoforms were cloned simultaneously from human and mice [64, 85]. These isoforms were independently named α and β, but unfortunately, the human and mouse isoform designations were reversed. That is, human PIP5Kα is equivalent to mouse β, and human PIP5Kβ is equivalent to mouse α. This disparate nomenclature has generated much confusion in the literature. In this review, we will use the human isoform designation *exclusively* (Table 1). In 1998, a third isoform, named PIP5Kγ, was cloned [65], and it has splice variants that are functionally distinct [47, 65]. The EST database suggests that the α and β isoforms may also have alternative splice variants [85, 138], but they have not been characterized functionally.

The three PIP5K isoforms have a highly conserved central kinase homology domain which has approximately 80% sequence identity (Fig. 1). The PIP5Ks are functionally similar in many respects. In vitro, they have similar enzyme kinetics and they are activated by phosphatidic acid (PA) [65], which is generated by phospholipase D (PLD) [126] or DAG kinase [86]. In addition, all PIP5K isoforms are activated by ser/thr dephosphorylation [102, 145] and by small GTPases such as RhoA [24], Rac1 [141], and Arf6 [61]. When overexpressed, all can potentially cause trapping of membrane in recycling endosomes [18, 124], form endosomal tubules [124], inhibit phagosome closure [119], generate actin comet tails [112], and prime secretory granules for exocytosis [138].

Table 1 Mammalian PIP5K isoforms and major splice variants

Human isoforms and splice variants	Mouse counterparts	Molecular weight (kDa)	Number of residues in human (mouse)
hPIP5Kα	MPIP5Kβ	68	549 (546)
hPIP5Kβ	mPIP5Kα	68	540 (539)
hPIP5Kγ87	mPIP5Kγ635	87	640 (635)
hPIP5Kγ90	mPIP5Kγ661	90	668 (661)
hPIP5Kγ93[a]	mPIP5Kγ688	93	NA (688)

[a] Not yet cloned or identified in humans

In addition, the PIP5K isoforms have divergent amino and carboxyl terminal extensions (Fig. 1). These regions are likely to be important for generating isoform-specific function and regulation [36, 37, 82, 92, 99, 116, 139, 145]. In this review, we will summarize what is known about the role of each PIP5K isoform and highlight their isoform-specific roles.

PIP5K localization

PIP5Ks are cytosolic proteins that associate with the membrane as peripheral proteins. PIP5Kα, as well as the yeast and drosophila orthologs, is also present in the nucleus [16]. The mammalian PIP5K isoforms associate with the PM to a different extent [138], and membrane association is regulated by multiple stimuli [21, 40], especially Arf6, RhoA, and Rac [115, 149]. In addition, isoform-specific binding partners that promote site-specific targeting have also been identified. Some examples are talin for PIP5Kγ90 [37, 82], Ajuba [69], and Bruton's tyr kinase [114] for PIP5Kα.

The PIP5K kinase homology domain is necessary for PM association [4], and the minimal targeting motif has been identified. These include two invariant lys residues in the specificity loop, the conserved glu that specifies PI(4)P recognition [76], and two tandem basic residues at the C-terminus of the kinase domain [4]. These five residues are conserved in all known PIP5Ks from yeast to human, suggesting that they may be the universal PIP5K PM targeting code.

Non-mammalian PIP5Ks

PIP5Ks are found in yeast, arabidopsis, drosophila, and *Caenorhabditis elegans*. Mss4p, the *Saccharomyces cerevisiae* PIP5K, is particularly enriched at the PM [8, 60]. It clusters at sites of dynamic cortical assembly that are distinct from cortical actin patches, and the formation

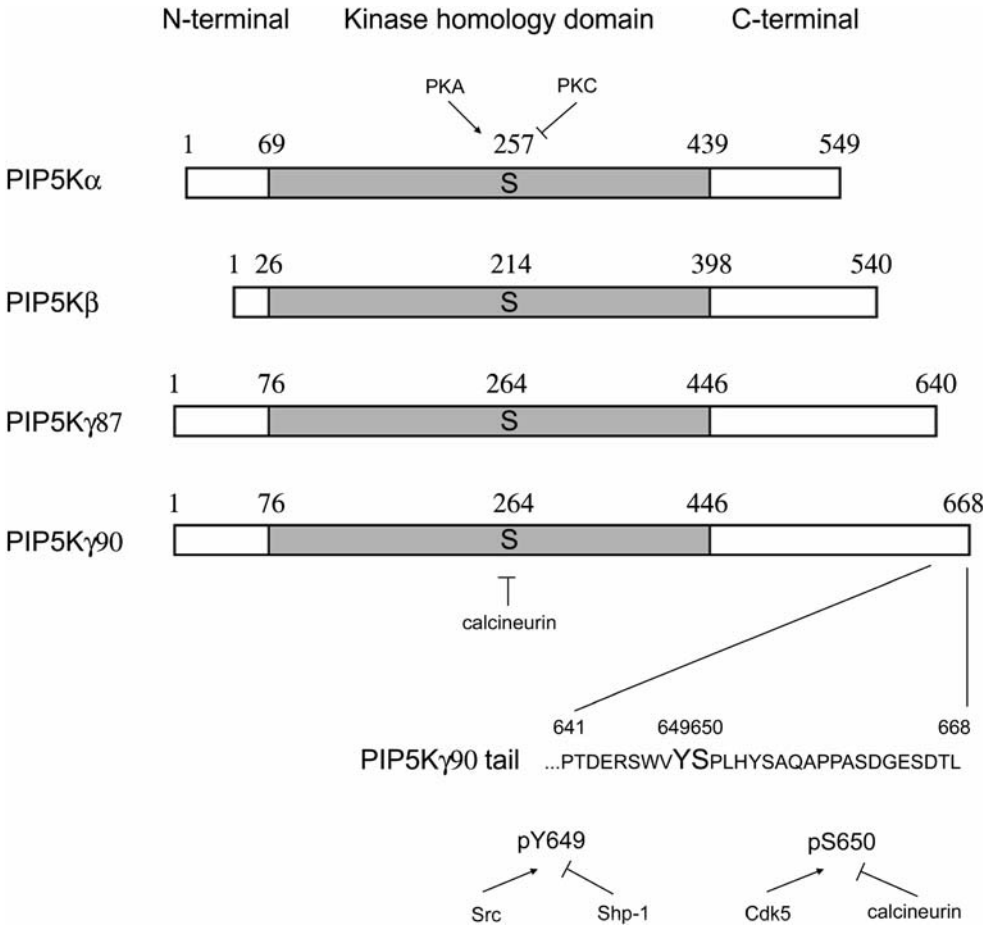

Fig. 1 The domain structure of human PIP5K isoforms and their phosphorylation sites. PIP5Kγ has an 87-kDa isoform and a 90-kDa isoform which has a 28 amino acid extension at its C-terminus (tail). Ser257 in PIP5Kα (equivalent to ser214 in β and ser264 in γ) is constitutively phosphorylated by PKA or autophosphorylation, and it is dephosphorylated by a PKC-dependent pathway [66, 102]. Dephosphorylation activates the lipid kinases. In addition, the PIP5Kγ90 tail has two tandem phosphorylation sites (tyr649 and ser650) which are mutually exclusive. Tyr649 phosphorylation activates PIP5Kγ90, while ser650 phosphorylation inhibits activity and blocks tyr649 phosphorylation. Tyr649 is phosphorylated by Src [83] and dephosphorylated by Shp-1 [10]. In neurons and neuroendocrine cells, PIP5Kγ90's ser650 is phosphorylated by Cdk5 and dephosphorylated by calcineurin in response to K^+-induced depolarization [79, 99]

of these clusters is independent of actin filaments [127]. Mss4p recruitment to the PM is dependent on normal sphingolipid biosynthesis, although it is not located in raft microdomains per se [70]. *Mss4* mutants are unable to form actin cables, have abnormal distribution of actin patches, irregular cell shape, aberrant deposition of cell wall material, and decreased viability [34, 60]. These results establish that PIP_2 has important roles in *S. cerevisiae*, including the regulation of the actin cytoskeleton. The *Schizosaccharomyces pombe* PIP5K, Its3p, is also enriched at the PM. It regulates cell wall integrity of the fission yeast through a PLC-mediated pathway [33], and it is concentrated at the septum during cytokinesis [150].

Drosophila and *C. elegans* each has three putative PIP5K genes, but most of them have not been characterized. The partially characterized drosophila *Sktl* is required for cell viability, germline development, and bristle morphology [54]. It has a nuclear localization signal, which shuttles Sktl between the nucleus and cytoplasm [22]. Its nuclear localization suggests that it might be an ortholog of PIP5Kα, which is also found in the nucleus [16]. Furthermore, as *Sktl* is not required for neurotransmitter release [54], it is not likely to be equivalent to mammalian PIP5Kγs, which have been strongly implicated in synaptic functions [37] (see below).

Mammalian PIP5Kβ

PIP5Kβ is ubiquitously expressed [64, 85] and exists as a soluble protein in the cytoplasm, in association with punctate cytoplasmic structures and the PM [101, 123]. Initially, much of the information about PIP5Kβ was obtained using transient transfection of WT and KD PIP5Kβ plasmids in cultured cells. There was no systemic attempt to distinguish between the function of individual PIP5K isoform. In some cases, the level of overexpression was unphysiologically high, overwhelming the normal mechanisms for specifying unique isoform functions. In addition, some of the putative KD mutants are not actually kinase dead, and most are not consistently dominant-negative (Table 2). Nevertheless, in spite of these caveats, there is convincing evidence that PIP5Kβ has a major role in actin regulation. Recently, RNA interference (RNAi) and gene knockout by homologous recombination have provided definitive evidence for its isoform-specific function.

Table 2 Kinase dead mutants[a] used to probe PIP5K functions

amino acid WT→KD	Residue number in human (mouse equivalent)	Function	References
K→A/M[b]	α181(179), β138(138), γ188(188)	Corresponds to the lys in protein kinases that binds ATP's α-phosphate	[3, 14, 46, 65, 66, 90, 106]
D→A/N/V	α246(244), β203(203), γ253(253)	A putative substrate binding site	[10, 46, 81, 82]
D→A	α270(268), β227(227), γ277(277)	A putative substrate binding site	[9, 18, 135, 143, 146]
D→K/N/A	α309(307), β266(266), γ316(316)	Corresponds to the catalytic asp in protein kinases	[1, 26, 47, 106, 147]
R→Q	α427(425), β386(386), γ434(434)	?	[1, 26, 41]
Truncation	αΔ1-240 (Δ1-238)	Dimerizes with WT PIP5K	[14, 30, 125]

[a] Most KD mutants do not consistently behave as dominant negatives.
[b] This mutant has partial kinase activity when expressed in mammalian cells and is therefore not a true KD mutant [26, 146].

Regulation of actin polymerization

PIP5Kβ overexpression induces actin polymerization, but the type of actin filaments formed is dependent on the cells studied and the extent of overexpression [9, 90, 123, 135, 146]. For example, PIP5Kβ overexpression induces the formation of pine needle-like actin structures in COS-7 cells [123] and robust stress fibers in CV1 cells [146]. The stress fibers are formed in a RhoA-dependent manner by changing the activity of several PIP_2-sensitive actin regulatory proteins including gelsolin, profilin, cofilin, and erzin [146]. PIP5Kβ is also implicated in RhoA-dependent ezrin recruitment to microvilli in HeLa cells [90] and Rac1-dependent recruitment to cell junctions [9].

Frequently, overexpression of PIP5Kβ, or the other PIP5K isoforms, induces the formation of actin comet tails that propel vesicles enriched with PIP5K and PIP_2 [112]. Comet formation is WASP- and Arp2/3-dependent. These comets may be used for vesicle trafficking of "raft"-associated cargoes from the TGN to the PM of nonpolarized cells [112] and selectively to the apical PM of polarized MDCK cells [52].

PIP5Kβ has also been implicated in neurite retraction downstream of RhoA and its effector, ROCK. PIP5Kβ overexpression in neuroblastoma N1E-115 cells induces neurite retraction even when ROCK is inactive; while overexpression of the PIP5Kβ KD mutant induces spontaneous neurite extension [136, 147]. PIP5Kβ may also be important for the phagocytosis of *Yersinia* [143], which binds to the host integrin β1 receptor to activate Rac1. Overexpression of either PIP5Kβ or Arf6 bypasses the Rac1 requirement, suggesting that they act downstream of Rac1 during *Yersinia* phagocytosis. As FcγR-mediated phagocytosis is also regulated by Rac and Arf6 [51], the possibility that PIP5Kβ is also involved merits investigation.

The importance of PIP5Kβ in actin regulation is supported by gene knockout. Mast cells from the *PIP5Kβ−/−* (mouse *PIP5Kα−/−*) mice have 35% less PIP_2 and less polymerized actin at the cell cortex [116]. They have abnormally robust responses to Fcε receptor I cross-linking, and hyperresponsiveness is supported by the finding that the *PIP5Kβ−/−* mice exhibit enhanced passive cutaneous and systemic anaphylaxis. Latrunculin, which depolymerizes actin, increases degranulation and cytokine generation in WT mast cells, establishing that *PIP5Kβ−/−* phenotype can be directly attributed to decreased actin polymerization. Taken together, these results indicate that PIP5Kβ negatively regulates mast cell functions by maintaining a cortical actin network that dampens the dynamics of Fcε receptor I signaling and downstream responses. Paradoxically, although in vitro and in vivo evidence suggest that PIP5Kβ has an important role in actin regulation, the *PIP5Kβ−/−* mice have no other reported phenotype. They are viable and develop normally [116]. Furthermore, no compensatory change in the expression of the other PIP5Ks is detected.

Receptor-mediated endocytosis

PIP5Kβ also has roles that are not related to actin regulation. For example, PIP5Kβ overexpression promotes receptor-mediated endocytosis in HeLa cells. These are manifested by an increase in transferrin uptake, the number of nascent clathrin-coated pits at the PM, and the amount of membrane-associated clathrin adaptor protein AP-2 complexes [101]. Cytochalasin D or latrunculin A, agents that depolymerize actin by different mechanisms, does not block the PIP5Kβ effects. Significantly, PIP5Kβ depletion by RNAi inhibits transferrin uptake in HeLa cells, while depletion of the other PIP5Ks has little effect [101]. Taken together, these results establish that PIP5Kβ is the primary regulator of receptor-mediated endocytosis in HeLa cells. Neurons use another PIP5K isoform to regulate endocytosis, although the mechanism for increased AP-2 recruitment may be similar [11, 99].

Regulation by Rho and Arf family small GTPases

There is now extensive evidence to suggest that PIP5Ks are regulated by RhoA, Rac1, and Arf6. RhoA and Rac1 have potent, and sometimes opposite, effects on the actin cytoskeleton, and their ability to recruit and activate PIP5Ks provides a very attractive model for how they may regulate the actin cytoskeleton. This relation has been reviewed extensively [100, 115, 149] and will not be discussed further here.

Arf6, which regulates membrane trafficking between endosomes and the PM, has also been implicated in the regulation of cell motility and the actin cytoskeleton through PIP5Ks. Overexpression of the constitutively active Arf6 mutant or PIP5K induces trapping of PM-derived PIP_2-rich vesicles in the recycling endosome compartment by polymerized actin [18]. Honda et al. [61] reported that PIP5Kβ (as well as PIP5Kα) is recruited to membrane ruffles by Arf6 and that recombinant Arf6-GTP binds PIP5Kβ and stimulates its lipid kinase activity. Surprisingly, Rac1 and RhoA do not stimulate PIP5Kβ activity under similar conditions, although they are reported to do so in other studies [24, 133, 134, 141]. The relation between Arf6 and PIP5K is further consolidated by the finding that Arf6 also recruits PLD2 to membrane ruffles [61, 126]. PLD has been intimately linked to PIP5Ks because it generates the PIP5K activator, PA [61, 126] and, in addition, it is itself activated by PIP_2 [39, 118]. Thus, Arf6 recruitment of both PIP5Ks and PLD to membrane ruffles establishes a positive feedback loop between PIP5K and PLD to synergistically amplify the initial Arf6 signal. Now there is also evidence that Arf6 regulates PIP5Kγ in neurons and chromaffin cells to promote membrane trafficking and vesicle priming for exocytosis [1, 73] (see below). In addition, Arf6 induces formation of endosomal tubules that contain PIP5Ks [124].

Although Arf6 is likely to be a primary regulator of PIP5K at the PM, some of the Arf6 responses may be coordinated with the activity of Rho GTPases because Arf6 may act upstream or downstream of RhoA and Rac, depending on the cellular context. This web of interactions could involve elaborate positive and negative feedback loops that are only beginning to be understood.

Regulation by phosphorylation/dephosphorylation

a. Ser/thr phosphorylation

Unexpectedly, the PIP5Ks kinase core, which has no identifiable homology to any known protein kinase [15], can phosphorylate itself on ser/thr residues in vitro, and phosphorylation inhibits lipid kinase activity [66]. Although there is no evidence that PIP5K autophosphorylates in the context of the cell, this could explain why PIP5Ks are constitutively phosphorylated and provide a mechanism to dampen basal PIP_2 generation under resting conditions. PIP5Ks' phosphorylation is also regulated by conventional kinases and phosphatases. The cAMP-dependent protein kinase A (PKA) phosphorylates PIP5Kβ in vitro [102], and the PKA phosphorylation consensus is located in the kinase homology domain. Mutation of ser214 within the consensus (Fig. 1) to ala decreases basal PIP5Kβ phosphorylation by 60% [102].

Stimuli that activate PIP5Kβ by ser/thr dephosphorylation have also been identified. Lysophosphatidic acid, which activates RhoA, induces PIP5K dephosphorylation in a PKC-dependent manner in NIH 3T3 cells [102]. Hypertonicity, which increases PIP_2 level and induces actin assembly in many types of cells, activates PIP5Kβ by dephosphorylation [145]. In addition, hypertonicity also promotes PIP5Kβ association with the PM. As neither actin disruption nor stabilization by pharmacological agents blocks PIP5Kβ dephosphorylation, PIP5Kβ is dephosphorylated upstream of actin remodeling. The RhoA effector, ROCK, is not involved because the PIP5Kβ response is not blocked by a ROCK inhibitor. Significantly, PIP5Kα and γ, which are also constitutively ser/thr phosphorylated under isotonic conditions, are not dephosphorylated by hypertonicity. These results clearly establish that PIP5Kβ is regulated by a balance between protein kinase and phosphatase activity in response to hypertonic stress and that the PIP5Ks have isoform-specific function and distinct modes of regulation.

b. Tyr phosphorylation

It has been known for some time that tyr phosphorylation is involved in the control of PIP_2 homeostasis. Pervanadate, a potent tyr phosphatase inhibitor, increases PIP_2 in HEK293 and REF52 cells [112, 113], while oxidative stress, which activates multiple tyr kinases, decreases PIP_2 generation by isolated cardiac PM [93] and by HeLa cells [53]. The paradox of how stimuli that promote tyr phosphorylation have opposite effects on cellular PIP_2 can be explained. First, PIP5K isoforms are expressed at different levels in different types of cells, and second, tyr phosphorylation appears to have different effects on the PIP5Ks. PIP5Kγ90 is activated by Src kinases [82] (see below), while preliminary evidence suggests that PIP5Kβ is inhibited [53]. PIP5Kβ inhibition is inferred from the finding that the PIP5Ks immunoprecipitated from H_2O_2-treated HeLa cells are less active in vitro than those from control cells [53]. Although this antibody recognizes all PIP5K isoforms, PIP5Kβ should dominate in the immunoprecipitate because it accounts for most of the PIP_2 in HeLa cells [138]. Immunofluorescence studies also show that oxidative stress dissociates PIP5Kβ from the PM [53]. Thus, the large decrease in PM PIP_2 in oxidant-stressed cells may be due to PIP5Kβ inactivation and dissociation from the PM. This decrease may be an early signal for apoptosis because

PIP5Kβ overexpression, which prevents oxidant-induced PIP_2 decrease, protects cells from apoptosis [53].

Mammalian PIP5Kγ

Unlike PIP5Kβ knockout, PIP5Kγ knockout mice die within a day of birth [36]. The primary cause of death has not been determined, but may be due to generalized neuronal defects (see below) which are manifested in the inability to suckle and move normally. Humans have at least two major PIP5Kγ splice variants: a short 87-kDa protein (PIP5Kγ87) and a slightly longer one that has 28 additional amino acids at its C-terminus (PIP5Kγ90) [65] (Fig. 1). PIP5Kγ87 is more abundant than PIP5Kγ90 in most cells [10, 49, 139], while PIP5Kγ90 dominates in the brain [37, 142]. Mice have an additional brain-specific 93-kDa splice variant which has not yet been described in humans [47]. As all three splice variants are knocked out in the currently available mouse model [36], it is difficult to attribute a defect to the knockout of a particular splice variant.

Focal adhesion dynamics

PIP_2 has long been implicated in the regulation of FAs, which are sites of actin filament attachment to the extracellular matrix through integrin receptors and mediators of bidirectional integrin signaling [63]. The PIP_2 level increases transiently during cell attachment to extracellular matrix, and PIP_2 activates several key FA components including vinculin, α-actinin, and talin. Although vinculin mutants that do not bind PIP_2 are recruited to FA normally, their FAs are static and turnover slowly [20, 117]. These results suggest that vinculin is a sensor of PIP_2 in the FA and that it promotes dynamic FA assembly and disassembly. Talin, which binds vinculin, actin, and integrin, has a key role in coupling the cytoskeleton to integrins [29]. An early study suggests that PIP_2 promotes talin/integrin interaction [87]. Now, there is strong evidence that talin has a direct role in increasing PIP_2 at FA because it binds to the carboxyl-terminal tail of PIP5Kγ90, and binding recruits PIP5Kγ90 to FA [37, 82].

Talin/PIP5Kγ90 interaction is reciprocally regulated by phosphorylation of the tandem tyr649 and ser650 residues in PIP5Kγ90's talin binding tail (WVYSPL) [79, 82] (Fig. 1). Under basal conditions, ser650 is constitutively phosphorylated, and as a result, lipid kinase activity and talin binding are suppressed. Integrin signaling via FAK and Src promotes tyr649 phosphorylation, either directly [82] or indirectly, by suppressing phosphorylation of the adjacent ser650 [79]. Tyr649 phosphorylation promotes interaction with talin and stimulates lipid kinase activity [82]. The model that emerges is that integrin signaling increases PIP_2 synthesis at FA by recruiting and activating PIP5Kγ90, and the localized increase in PIP_2 activates talin, which then binds and further activates integrins. The signal is turned off by PIP5Kγ90 dephosphorylation, and Shp-1 tyr phosphatase, which has been previously implicated in the regulation of FA dynamics, dephosphorylates PIP5Kγ90 [10]. FA turnover is therefore dynamically regulated via the reciprocal actions of multiple FA components by Src and Shp-1.

Unlike PIP5Kγ90, PIP5Kγ87 is not found in FA and does not bind talin [37, 82]. Nevertheless, it has also been implicated in integrin adhesion via a PLD2-mediated, but actin-independent, mechanism [106]. PIP5Kβ overexpression has no effect on spreading. These results raise the possibility that PIP5Kγ90 and 87 may be involved in different stages of FA formation or turnover, while PIP5Kβ is not.

Synaptic vesicle physiology

PIP_2 level is increased in neurons in response to K^+-induced depolarization [36], and it regulates synaptic transmission by multiple mechanisms. PIP_2 is the immediate precursor of PLC-generated $InsP_3$ and DAG, which activate Ca^{2+} signaling and PKC, respectively. PIP_2 also directly regulates exocytosis and endocytosis by binding to clathrin adaptors and other endocytic proteins, by priming exocytic vesicles, by promoting membrane fusion and fission, and by regulating the actin cytoskeleton. Synaptojanin, which dephosphorylates PIP_2, is also required for normal synaptic vesicle cycling and actin dynamics at the synapse [28].

PIP5Kγ90 overexpression in chromaffin cells increases the amount of PM PIP_2 as well as the number of vesicles in the docked releasable pool [94]. The importance of PIP5Kγ in vesicle trafficking is confirmed by gene knockout. Synaptosomes prepared from the brains of the *PIP5Kγ−/−* mice have 40% less PIP_2 than WT, and they do not generate PIP_2 in response to K^+ depolarization. Although primary cultures of cortical neurons develop normally in vitro in spite of the lack of PIP5Kγ, they have severe defects in synaptic transmission, which correlate with abnormal exocytosis and clathrin-mediated endocytosis [36]. Likewise, chromaffin cells isolated from these mice have defective vesicle priming and fusion dynamics [49].

Talin, which regulates PIP5Kγ90 in FA, is also present in synapses. Disruption of talin/PIP5Kγ90 interaction induces actin depolymerization and decreases clathrin-mediated synaptic vesicle endocytosis [95]. These results suggest that PIP5Kγ90 is regulated by talin in neurons using mechanisms similar to those in FAs. Likewise, neuronal PIP5Kγ90 is also reciprocally regulated by ser

and tyr phosphorylation. In neurons, PIP5Kγ90 is constitutively phosphorylated on ser650 by p35/Cdk5 and MAPKs, and it is dephosphorylated during K^+-induced depolarization by calcineurfin [1, 142]. Ser650 dephosphorylation activates PIP5Kγ90 and facilitates tyr649 phosphorylation by Src. Significantly, K^+-induced depolarization also promotes PIP5Kγ90 interaction with Arf6, which would further promote PIP_2 synthesis [61, 73]. Thus, PIP5Kγ is activated through a confluence of different and interrelated signals during neuronal stimulation.

Interaction with clathrin adaptor protein complexes

The clathrin adaptor protein complex AP-2 is activated by PIP_2 to bind its transport cargoes at the PM [62]. Now, there is evidence that AP-2 directly participates in increasing PIP_2 synthesis at the nascent endocytic site by binding and activating PIP5Ks. Therefore, it is attractive to hypothesize that the coincidence detection of membrane cargoes and PIP_2 by AP-2, together with AP-2 activation of PIP5Ks, specify the site-specific generation of a local PIP_2 pool that is dedicated to clathrin/AP-2-dependent endocytosis [72].

The details of how this occurs remain to be explored. One study reports that the μ subunit of AP-2 binds all PIP5Ks [74]. Thus, AP-2 binding to PIP5Kβ may explain how PIP5Kβ promotes endocytosis in HeLa cells [101] (see above). However, other studies show that the interaction with AP-2 is mediated primarily through the PIP5Kγ90 tail, which is not present in the other PIP5Ks. Tail binding to AP-2 potently stimulates PIP5Kγ90's lipid kinase activity [11, 99]. Overexpression of the PIP5Kγ90 tail, which competes with endogenous PIP5Kγ90 for AP-2, decreases AP-2 recruitment and synaptic vesicle endocytosis [99]. The trafficking abnormalities are similar to those described in *PIP5Kγ−/−* neurons [36], suggesting that PIP5Kγ90 binding to AP-2 is physiologically relevant and that the neuronal defects in *PIP5Kγ−/−* mice can be attributed at least partly to the lack of PIP5Kγ90.

PIP5Kγ has been implicated in membrane trafficking in non-neuronal cells as well. Overexpression of WT PIP5Kγ90 in MDCK cells increases transferrin uptake, while overexpression of KD PIP5Kγ90 inhibits [11]. These effects are specific for the long splice variant of PIP5Kγ because PIP5Kγ87 overexpression has little effect [11].

Recently, the PIP5Kγ90 tail has been reported to bind to another clathrin adaptor AP-1 [81, 99]. This interaction is proposed to be critical for E-cadherin trafficking and adhesion junction formation [81]. As AP-1 is located primarily at the TGN and it binds PI(4)P instead of PIP_2 [55, 140], this interaction is perplexing. Furthermore, PIP5Kγ87, which does not have the tail, has also been implicated in cadherin and actin-enriched cell/cell adhesion in the epithelial A431 cells [2].

$InsP_3$-mediated Ca^{2+} signaling

PIP_2 is critical to intracellular Ca^{2+} signaling because it is the obligatory precursor of $InsP_3$. RNAi studies show that although depletion of both PIP5Kγ90 and 87 isoforms together (using siRNA directed against a common sequence) decreases total PIP_2 by less than 15% in HeLa cells, it blocks histamine-induced, heterotrimeric G-protein-activated $InsP_3$ generation by more than 70% [139]. Ca^{2+} flux is also inhibited. However, depletion of PIP5Kγ90 with the unique tail-specific siRNA has no effect. Therefore, these results suggest that PIP5Kγ87 is important for G-protein-coupled receptor signaling. Remarkably, depletion of PIP5Kβ or α, which individually accounts for a larger fraction of total PIP_2 than PIP5Kγ in HeLa cells, has almost no effect on $InsP_3$ generation. Single cell immunofluorescence imaging shows that the PIP5Kγ- and PIP5Kβ-depleted HeLa cells have a similar drop in PM PIP_2, but that the latter has more PIP_2 loss in internal membranes as well. The exquisitely selective effect of PIP5Kγ87 depletion on Ca^{2+} signaling suggests that the PM PIP_2 pools generated by PIP5Kγ87 and β are functionally compartmentalized [139].

This finding again raises the important question of how the PIP_2 pools existing on the same PM can have distinct functions. One possibility is that PIP5Kγ87 is part of a supramolecular PLCβ signaling scaffold that specifies rapid local Ca^{2+} generation and propagation [31], while PIP5Kβ is not. In this model, the PIP_2 generated by PIP5Kγ87 would be immediately available for hydrolysis by PLCβ within the signaling scaffold, thus, behaving like the previously proposed agonist-sensitive, de novo synthesized PIP_2 pool [71, 98]. In contrast, the PIP_2 generated by PIP5Kβ may represent a preexisting agonist-insensitive pool that maintains the PM's status quo. The two PIP_2 pools may physically segregated from the other PIP_2 pool by interaction with scaffolding proteins. Studies using heart sarcolemma support the existence of agonist-sensitive and -insensitive PIP_2 pools [23, 96].

Mammalian PIP5Kα

PIP5Kα is ubiquitously expressed [64, 85] and found in multiple cell compartments. Like other PIP5Ks, it is partly cytosolic and partly PM-associated. However, PIP5Kα is also found in the nucleus [16]. The *PIP5Kα−/−* (mouse *PIP5Kβ−/−*) mouse has not been described in the literature.

Membrane ruffling

The PM ruffles in response to many types of stimuli and requires remodeling of cortical actin networks downstream

of the activation of Rac [110]. PIP_2 has long been implicated in this process because Rac and Arf6 regulate PIP5Ks and induce ruffling. In MG-63 fibroblasts, PIP5Kα, but not β, translocates to the PM after PDGF stimulation [41]. Overexpressed PIP5Kα promotes the formation of actin foci formation when Rac1 is inhibited, but stimulates ruffle formation when Rac1 is activated. These results suggest that PIP5Kα promotes actin assembly and that additional inputs from Rac1 are required to generate active ruffles. The LIM protein ajuba, which is a component of the integrin-mediated adhesive complex and a Rac activator, is a potential effector [69]. It promotes PIP5Kα localization to membrane ruffles and leading lamellipodia.

B cell and platelet activation

Upon the engagement of B cell receptors, PIP5Kα is recruited to the PM by Bruton's tyr kinase which has a PH domain [114]. Membrane fractionation shows that PIP5Kα is translocated to lipid rafts where PIP_2 is converted to second messengers including PIP_3, $InsP_3$, and DAG. Thrombin activation of platelets induces recruitment of PIP5Kα to the PM in a Rho and ROCK-mediated, but Rac1-independent, manner [148]. RhoA also recruits PIP5Kα (and β, but not γ) to the cleavage furrow during cytokinesis [43].

Phagocytosis

Actin remodeling during FcγR-mediated phagocytosis is regulated by a highly orchestrated series of events [17, 26]. One of the initial changes is a localized increase in PIP_2 at the nascent phagocytic cup. PIP5Kα is recruited to the phagocytic cup, and overexpression of a PIP5Kα KD mutant blocks actin remodeling and PIP_2 accumulation there [17, 26]. It is not known at present whether PIP5Kβ or γ is also important for this type of phagocytosis, although PIP5Kβ has been implicated in integrin-mediated *Yersinia* phagocytosis [143]. The PIP_2 increase is critical for actin modeling at the phagocytic cup. PIP_2 promotes actin assembly by recruiting WASP family proteins to induce Arp2/3-dependent actin nucleation, and PIP_2 also inhibits gelsolin to prevent actin severing during the ingestion phase [5].

Receptor-mediated endocytosis

Like PIP5Kβ and γ, PIP5Kα has also been implicated in receptor-mediated endocytosis. Overexpression of a PIP5Kα truncation mutant (Table 2), which has little kinase activity and is not recruited to the PM, inhibits the endocytosis of epidermal growth factor receptor [14] and mutated colony stimulating factor-1 receptor which is endocytosed more rapidly [30].

Apoptosis

PIP5Kα, but not PIP5Kβ or γ, is cleaved by caspase 3 during apoptosis, and overexpression of PIP5Kα protects cells against apoptosis by inhibiting caspase activity [92]. Protection is dependent on PIP_2 generation because KD PIP5Kα shows no protection. The mechanism of protection is, however, different from that ascribed to PIP5Kβ, which is not cleaved by caspase 3 and which prevents upstream of caspase activation [53].

Nuclear PIP_2

There is increasing evidence that the nucleus has its own phosphatidylinositol machinery [19]. PIP_2 is found in nuclear speckles, which contain mRNA-processing components that are used for chromatin remodeling [16]. The PDZ domain-containing protein syntenin-2 is targeted to the nuclear speckles by binding PIP_2, and syntenin-2 depletion by RNAi disrupts PIP_2 nuclear speckles and impairs cell survival [97]. Nuclear PIP_2 has also been implicated in mRNA processing, transcriptional regulation, and stress responses [19]. PIP5Kα and PIP4K, which synthesize PIP2 using different routes, are both found in the nucleus, and type III PI4Kα, which generates the PIP5K substrate PI(4)P, has been identified there as well [68]. PIP5Kα does not have a recognizable nuclear localization signal, and the mechanism for its shuttling between the nucleus and cytoplasm remains to be identified. The *S. cerevisiae* and *Drosophila* PIP5Ks are also partially nuclear localized, and they have a nuclear localizing signal [7, 22].

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