

## One lipid, multiple functions: how various pools of PI(4,5)P<sub>2</sub> are created in the plasma membrane

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Received: 13 April 2010/Revised: 1 June 2010/Accepted: 2 June 2010/Published online: 18 June 2010  
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**Abstract** Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P<sub>2</sub>] is a minor lipid of the inner leaflet of the plasma membrane that controls the activity of numerous proteins and serves as a source of second messengers. This multifunctionality of PI(4,5)P<sub>2</sub> relies on mechanisms ensuring transient appearance of PI(4,5)P<sub>2</sub> clusters in the plasma membrane. One such mechanism involves phosphorylation of PI(4)P to PI(4,5)P<sub>2</sub> by the type I phosphatidylinositol-4-phosphate 5-kinases (PIP5KI) at discrete membrane locations coupled with PI(4)P delivery/synthesis at the plasma membrane. Simultaneously, both PI(4)P and PI(4,5)P<sub>2</sub> participate in anchoring PIP5KI at the plasma membrane via electrostatic bonds. PIP5KI isoforms are also selectively recruited and activated at the plasma membrane by Rac1, talin, or AP-2 to generate PI(4,5)P<sub>2</sub> in ruffles and lamellipodia, focal contacts, and clathrin-coated pits. In addition, PI(4,5)P<sub>2</sub> can accumulate at sphingolipid/cholesterol-based rafts following activation of distinct membrane receptors or be sequestered in a reversible manner due to electrostatic constraints posed by proteins like MARCKS.

**Keywords** Plasma membrane · Phosphatidylinositol 4,5-bisphosphate · Type I phosphatidylinositol-4-phosphate 5-kinases · Rac1 · Talin · AP-2 · Lipid rafts

### Abbreviations

FERM Band 4.1-ezrin-radixin-moesin homology  
BTK Bruton's tyrosine kinase

DAG	Diacylglycerol
DAGK	Diacylglycerol kinase
EGF	Epidermal growth factor
IP <sub>3</sub>	Inositol trisphosphate
MDCK	Madin-Darby kidney cells
MARCKS	Myristoylated alanine-rich C kinase substrate
GAP43	Growth-associated protein 43
OCRL1	Oculocerebrorenal syndrome of Lowe 1 phosphatase
OSBP	Oxysterol-binding protein
PTEN	Phosphatase and tensin homologue on chromosome 10
PA	Phosphatidic acid
PI	Phosphatidylinositol
PIP	Phosphatidylinositol phosphate
PIP3	Phosphatidylinositol 3-phosphate
PIP4	Phosphatidylinositol 4-phosphate
PIP3P	Phosphatidylinositol 3-monophosphate
PIP4P	Phosphatidylinositol 4-monophosphate
PIP5P	Phosphatidylinositol 5-monophosphate
PI(4,5)P <sub>2</sub>	Phosphatidylinositol 4,5-bisphosphate
PI(3,4,5)P <sub>3</sub>	Phosphatidylinositol 3,4,5-trisphosphate
Fapp	Phosphatidylinositol-4-phosphate adaptor protein
IPP 5-Ptase	Inositol polyphosphate 5-phosphatase
PLC	Phospholipase C
PLD	Phospholipase D
PTB	Phosphotyrosine-binding
PH	Pleckstrin homology
PIP5KI	Type I phosphatidylinositol-4-phosphate 5-kinase
PIP4KII	Type II phosphatidylinositol-5-phosphate 4-kinase
OSH2	Yeast oxysterol-binding protein homologue

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## Introduction

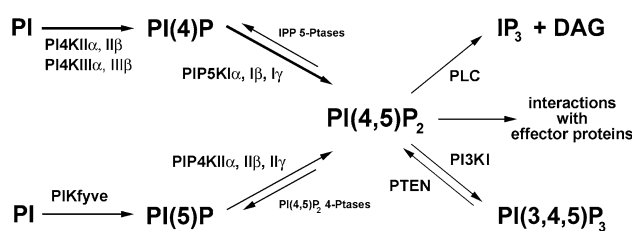
During the five decades that have passed since the discovery of phosphoinositide turnover in the cell [1], it has been established that phosphatidylinositol 4,5-bisphosphate [PI(4,5)P<sub>2</sub>] is located mainly in the plasma membrane and serves as a source of second messengers: inositol trisphosphate (IP<sub>3</sub>), diacylglycerol (DAG), and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P<sub>3</sub>]. IP<sub>3</sub> and DAG are generated by phospholipase C (PLC)-catalyzed hydrolysis of PI(4,5)P<sub>2</sub>, while PI(3,4,5)P<sub>3</sub> arises by phosphorylation of PI(4,5)P<sub>2</sub> by the class I phosphatidylinositol 3-kinases (PI3KI). Aside from the fundamental signaling role of its derivatives, PI(4,5)P<sub>2</sub> itself controls the activity of several integral membrane proteins, like ion channels and transporters, and affects a myriad of proteins associating with the membrane due to PI(4,5)P<sub>2</sub> binding. The latter group includes proteins of the actin cytoskeleton that recognize PI(4,5)P<sub>2</sub> using either specific domains, like the pleckstrin homology (PH) domain or the band 4.1-ezrin-radixin-moesin homology (FERM) domain, or unfolded stretches of basic amino acids. Despite PI(4,5)P<sub>2</sub> comprising only 1% of all plasma membrane phospholipids, its extraordinary versatility puts PI(4,5)P<sub>2</sub> in the center of plasma membrane dynamics governing motility, cell adhesion, endo- and exocytosis. Some of these subjects have been discussed extensively in recent excellent reviews [2, 3].

The diversity of PI(4,5)P<sub>2</sub> functions poses the question of the nature of the mechanisms governing the local and discrete character of PI(4,5)P<sub>2</sub>-dependent processes. This review covers work dealing with this puzzle and pointing to the existence of various pools of PI(4,5)P<sub>2</sub> in the plasma membrane. The ideas proposed as a solution to the above problem include local control of PI(4,5)P<sub>2</sub> synthesis and confinement of PI(4,5)P<sub>2</sub> in the plasma membrane due to an involvement of membrane rafts and positively charged membrane proteins.

## PI(4,5)P<sub>2</sub> synthesis in the plasma membrane

Major role of type I phosphatidylinositol-4-phosphate 5-kinases

Phosphatidylinositol 4-monophosphate [PI(4)P] and PI(4,5)P<sub>2</sub> are two major derivatives of phosphatidylinositol (PI), their amounts reaching about 5% each of the total cellular PI [4]. Due to the relative abundance of PI(4)P, its phosphorylation at the D-5 position of the inositol ring is the prevailing route of PI(4,5)P<sub>2</sub> synthesis in the cell (Fig. 1). This reaction is catalyzed by kinases named the type I phosphatidylinositol-4-phosphate 5-kinases (PIP5KI) belonging to the family of phosphatidylinositol phosphate



**Fig. 1** PI(4,5)P<sub>2</sub> metabolism and activity. PI(4,5)P<sub>2</sub> is generated mainly by phosphorylation of PI(4)P by PIP5KI (the pathway indicated by *thicker arrows*) and, to lower extent, by PIP4KII-catalyzed phosphorylation of PI(5)P. PI(4,5)P<sub>2</sub> directly affects activity of numerous proteins, serves as a source of second messengers: IP<sub>3</sub>, DAG, and PI(3,4,5)P<sub>3</sub>. The lipid can be also dephosphorylated at the D-4 and D-5 positions of the inositol ring

kinases (PIPK). Alternatively, minor amounts of PI(4,5)P<sub>2</sub> arise from phosphorylation of phosphatidylinositol 5-monophosphate [PI(5)P] due to action of the type II phosphatidylinositol-5-phosphate 4-kinases (PIP4KII), a second PIPK subfamily (Fig. 1). Finally, PI(4,5)P<sub>2</sub> can also be generated by PTEN- (phosphatase and tensin homologue on chromosome 10)-driven dephosphorylation of PI(3,4,5)P<sub>3</sub> [5]. Although crucial for termination of PI(3,4,5)P<sub>3</sub> signaling, the importance of this process for PI(4,5)P<sub>2</sub> generation in the plasma membrane remains questionable. Of note, PI(4,5)P<sub>2</sub> can also undergo dephosphorylation catalyzed either by inositol polyphosphate 5-phosphatases (IPP 5-Phases) such as oculocerebrorenal syndrome of Lowe 1 phosphatase (OCRL1) and synaptojanins [6–8] or by PI(4,5)P<sub>2</sub> 4-phosphatases type I and II [9]. These enzymes dephosphorylate the D-5 and D-4 phosphoester linkage of PI(4,5)P<sub>2</sub> yielding PI(4)P and PI(5)P, respectively. Activity of IPP 5-Phases is important for controlling the PI(4,5)P<sub>2</sub>/PI(4)P balance at defined cellular locations and its disturbances lead to homeostatic PI(4,5)P<sub>2</sub> defects bearing serious consequences to health. These are exemplified by the devastating human developmental disorder named Lowe syndrome caused by loss-of function for the OCRL1 [10, 11], mortality of synaptojanin 1-deficient mice [12] and possibly, Down's syndrome connected with trisomy of the synaptojanin 1 gene [13]. On the other hand, PI(4,5)P<sub>2</sub> 4-phosphatases seem to play an essential role in the synthesis of PI(5)P since PI(4,5)P<sub>2</sub> 4-phosphatase I was shown to control nuclear levels of PI(5)P and thereby p53-dependent apoptosis [14].

The third member of the PIPK family, the type III PIPK named PIKfyve in mammals, is a phosphatidylinositol-3-phosphate 5-kinase that phosphorylates phosphatidylinositol 3-monophosphate [PI(3)P] to PI(3,5)P<sub>2</sub>, a lipid that controls membrane trafficking in the endosomal pathway (see [15] for review). Of note, PIKfyve is also implicated in synthesis of PI(5)P either by direct phosphorylation of PI

(see Fig. 1) or by production of PI(3,5)P<sub>2</sub> which can be then converted into PI(5)P by PI(3)P phosphatases of the myotubularin family [16–18]. Both PIP5KI and PIP4PII also utilize PI(3)P as a substrate *in vitro* and are indicated to produce PI(3,4)P<sub>2</sub>, PI(3,5)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub> in the cell [19, 20]. However, the physiological consequences of this substrate promiscuity of PIPKs are beyond the subject of this review.

The type I PIP5Ks, central to PI(4,5)P<sub>2</sub> synthesis in the plasma membrane, are of three isoforms,  $\alpha$ ,  $\beta$ , and  $\gamma$ , encoded by distinct genes. PIP5KI $\alpha$  and I $\beta$  of human and mouse origin were cloned simultaneously by two groups and given reciprocal names [21–23]. In this work, names adopted for the human enzymes by Anderson's group are used. PIP5KI $\alpha$ 1 consists of 549 amino acids while PIP5KI $\beta$ 1 of 540 amino acids, and they represent one of three (PIP5KI $\alpha$ ) and two (PIP5KI $\beta$ ) splicing variants, all of molecular weight of about 62 kDa [22]. PIP5KI $\gamma$  has three well-characterized splicing variants: PIP5KI $\gamma$ a of 668 amino acids (661 amino acids in murine PIP5KI $\gamma$ a), PIP5KI $\gamma$ b of 635 and PIP5KI $\gamma$ c of 688 amino acids (present in rodents), also called PIP5KI $\gamma$ 90, I $\gamma$ 87 and I $\gamma$ 93 based on their migration in gel electrophoresis [23, 24]. Although all these PIP5KI isoforms coexist in most tissues, PIP5KI $\alpha$  is highly expressed in skeletal muscles and PIP5KI $\beta$  in the heart, whereas PIP5KI $\gamma$  is abundant in the brain, which suggests specific functions for these isoenzymes in distinct cells. The physiological importance of PIP5KI $\gamma$  is indicated by early mortality of *PIP5KI $\gamma$*  knockout mice [25]. The three PIP5KI isoforms display about 80% identity in the amino acid sequence of their catalytic domain consisting of 330–380 amino acids and located in the center of the molecule [22, 23]. Another unique feature of the PIP5Ks common to all the isoforms is the up-regulation (up to tenfold) of their enzymatic activity by phosphatidic acid (PA) [21, 23, 26]. Consequently, phospholipase D (PLD) and diacylglycerol kinase (DAGK), enzymes producing PA, can regulate PIP5KI activity, as discussed below. All PIP5KI isoforms undergo autophosphorylation and phosphorylation catalyzed by protein kinase A within the catalytic domain, which strongly suppresses their lipid kinase activity [27, 28]. The variable amino- and carboxy-terminal tails of PIP5KI $\alpha$ , I $\beta$ , and I $\gamma$  are involved in the regulation of their activity [23] and are likely to participate in the targeting to distinct cellular locations. For example, the C-terminal 28 amino acids (26 in murine kinase) were found to govern interaction of PIP5KI $\gamma$ a with talin at focal contacts organized during adhesion of cells to the matrix [29, 30]. The C-terminus (amino acids 440–562) of PIP5KI $\alpha$ 2 targets the enzyme to the nucleus [31]. A search of the human and mouse genome sequence databases for an amino acid motif conserved in all PIPK has revealed a PIPK homolog composed of 395 amino acids (about

40 kDa). This protein is abundant in brain and testis, has no intrinsic kinase activity but homodimerizes with PIP5KI $\alpha$  and I $\beta$ , thus affecting their activity [32].

Studies on the functions of individual isoenzymes have yet to produce a comprehensive picture. However, it is becoming increasingly clear that the different PIP5KI isoforms play specific roles in individual cell types and one kinase isoform can not compensate for the loss of another [33–37]. PIP5KI $\alpha$  is implicated in actin remodeling that governs ruffling, expansion of the leading edge of migrating cells, and phagosome formation [38–40]. Nevertheless, participation of PIP5KI $\beta$  (murine PIP5KI $\alpha$ ) in the uncapping of actin filaments leading to actin polymerization in thrombin-activated platelets [33], actin polymerization accompanying cell volume changes [34], and actin reorganization during oxidative stress should also be noted [41]. A growing body of evidence suggests that PIP5KI $\alpha$  and I $\beta$  can shuttle between the plasma membrane and cytoplasm being recruited to the membrane upon activation of cell motility [39, 40, 42, 43]. Overexpression of PIP5KI $\alpha$  or I $\beta$  induces various changes of the actin cytoskeleton including the appearance of unusual structures like actin-rich needles and comets [23, 44, 45]. Therefore, both these kinase isoforms have been postulated to generate PI(4,5)P<sub>2</sub> affecting the actin cytoskeleton organization in the cell. However, since the cellular localization of the overexpressed kinases is often different than that of the endogenous ones (see below), and the final outcome of a kinase's activity can be different depending on the cellular context including the activity of the Rho family members [45, 46], it is hard to ascribe a particular aspect of actin remodeling to one of the kinase isoforms based solely on those data. Besides the role in actin cytoskeleton reorganization, PIP5KI $\alpha$  is also found in the nucleus at sites of pre-mRNA processing where it interacts with non-canonical poly(A) polymerase to control the formation of selected mRNAs [31, 47]. On the other hand, PIP5KI $\beta$  is engaged in formation of clathrin-coated pits during receptor endocytosis [48]. Participation of PIP5KI $\gamma$ a in the formation of clathrin-coated pits has also been documented in great detail [49–51]. Additionally, the activity of PIP5KI $\gamma$ a-c emerges as crucial for assembly of focal contacts and cell-cell contacts and for association of the actin cytoskeleton with the plasma membrane mediated by proteins of these structures [29, 30, 35, 52–54].

Mammalian PIP5KI $\alpha$  and I $\beta$ , but not PIP4KII $\beta$ , were able to restore PI(4,5)P<sub>2</sub> synthesis and rescue lethality of yeast mutants lacking Mss4p, the only yeast PIP5KI [55, 56]. The cells do not have PIP4KII but express the type III PIPK called Fab1p, which does not participate in PI(4,5)P<sub>2</sub> synthesis and, hence, is not able to substitute for Mss4p [55]. The Mss4p kinase is localized to the plasma membrane and is required for yeast viability, actin organization,

and cell wall integrity [57]. These data suggest that an involvement of PIP5KI-derived PI(4,5)P<sub>2</sub> in actin skeleton remodeling is evolutionarily ancient.

PIP4KII provides a clue on PIP5KI–membrane interaction: role of PI(4)P

The second pathway for the production of PI(4,5)P<sub>2</sub> relies on phosphorylation of PI(5)P on the D-4 position of the inositol ring catalyzed by type II PIP4K (Fig. 1). Three isoforms of PIP4KII ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) have been identified in mammalian cells, with PIP4KII $\alpha$  being the first PIPK cloned [58–60]. The distinct substrate specificity of PIP5KI and PIP4KII was established by Rameh et al. [61], who also found evidence for the presence of PI(5)P, a PIP4KII substrate, in fibroblasts. Kinases of the PIP4KII subfamily share about 35% amino acid identity with the enzymes of the PIP5KI subfamily within the kinase catalytic domain. A structure-based alignment of the catalytic domain of human PIP4KII $\alpha$  and II $\beta$ , PIP5KI $\alpha$  and I $\beta$ , Mss4p (yeast PIP5KI), and Fab1p (yeast type III PIPK) as well as protein kinase A allowed three residues conserved in all these enzymes to be identified: Lys150, Asp278, and Asp369 (numbering as in PIP4KII $\beta$ ) engaged in ATP and Mg<sup>2+</sup> binding [62]. Accordingly, mutation of the corresponding Lys138 in PIP5KI $\beta$  abolished the kinase activity [23]. Outside the core kinase domain, as well as in the so-called insert of the domain, very little amino acid identity is found between type I and type II PIPKs [22].

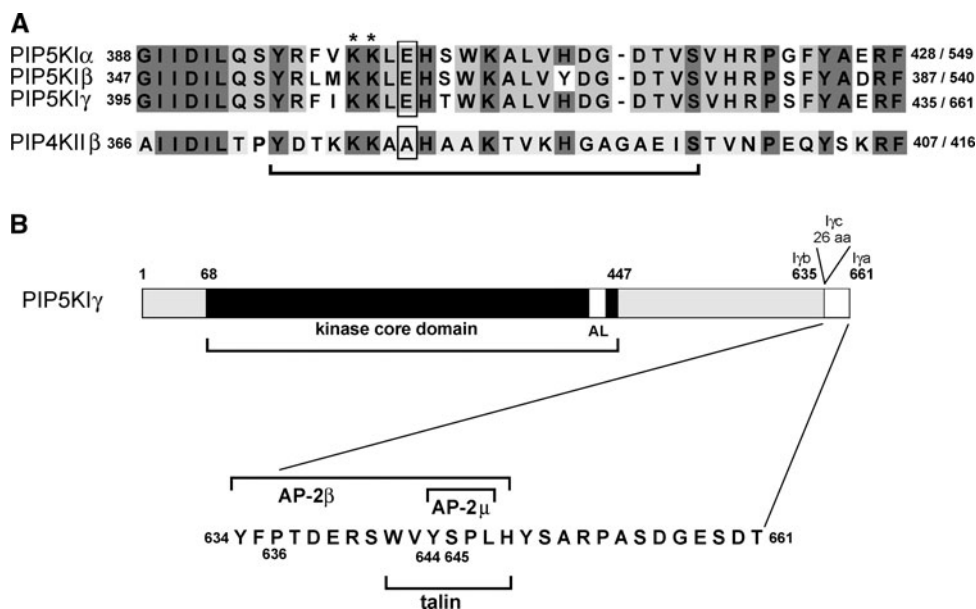
Type II PIP4Ks are found in the cytoplasm, nucleus, and endoplasmic reticulum and their cellular functions remain unclear (see [63] for review). In contrast to PIP5KI, both endogenous and overexpressed PIP4KII are only weakly associated with the plasma membrane and are insensitive to PA [56, 60, 64]. When first purified from erythrocytes, PIP4KII $\alpha$  was found unable to phosphorylate the intrinsic PIP in isolated erythrocyte membrane [65]. This is in agreement with the finding that very small quantities of PI(5)P are present in the cell, reaching only about 2% of the PI(4)P level [61]. Accordingly, in cells subjected to radioactive labeling of phosphoinositides, phosphorylation at the D-5 position of the inositol ring was found to exceed that of the D-4 hydroxyl group two to threefold [66]. It seems, therefore, that PIP4KII activity does not contribute substantially to PI(4,5)P<sub>2</sub> production in the plasma membrane.

Yet, it was the crystallization of PIP4KII $\beta$  that shed light on the PIPK-membrane interaction [62]. The enzyme is a homo-dimer made up of subunits linked head-to-head at their amino-termini. In the dimer, the N-terminal fragments of the catalytic core of both subunits jointly form a highly basic flat surface proposed to provide a site for the

kinase-membrane attachment. When modeled on a membrane bilayer, the dimer appeared as a peripheral protein not penetrating the membrane. Its membrane binding was thought to be directed by electrostatic interactions between the dimer's flat positively charged surface and the anionic lipid head groups.

The importance of electrostatic interactions for plasma membrane binding was also shown for PIP5KI $\beta$ . The data presented by Anderson's group indicated that amino acids involved in this linkage were located in the so-called activation loop of about 20 amino acids localized at the C-terminal part of the kinase catalytic core (Fig. 2a). After substitution of two adjacent lysine residues Lys359Lys360 of the activation loop with two asparagine residues, PIP5KI $\beta$  expressed in osteosarcoma cells no longer bound to the plasma membrane. On the other hand, substitution of the dilysine motif with positively charged arginine residues did not interfere with the membrane association of the protein [56]. The dilysine motif is conserved among all PIPK subfamilies [62], suggesting its key role in the membrane binding of the enzymes. However, the membrane association based solely on this dilysine motif cannot explain the targeting of PIP4KII and PIP5KI to diverse cellular membranes, with some PIP5KI isoforms recruited preferably to defined locations at the plasma membrane.

A partial answer to this question lies in the amino acid sequence of the aforementioned activation loop. Aside from the amino acids common to all PIPKs, some amino acids are highly conserved only within the PIP5KI, PIP4KII, and type III PIPK subfamilies, but different between them. Further mutational studies confirmed the participation of the activation loop in the selective recognition of either PI(4)P or PI(5)P as well as proved its involvement in the association of PIP5KI with the plasma membrane. Swapping of the activation loop between PIP4KII $\beta$  and PIP5KI $\beta$  resulted in a reversed substrate specificity of the obtained protein chimeras. Introduction of the PIP4KII $\beta$  activation loop into PIP5KI $\beta$  converted the PIP5KI $\beta$  chimera into a kinase phosphorylating PI(5)P and simultaneously abolished its binding to the plasma membrane. Reciprocally, PIP4KII $\beta$  with the activation loop of PIP5KI $\beta$  phosphorylated PI(4)P and gained the ability to localize to the plasma membrane [56]. A similar reversion of specificity was achieved after substitution of a single amino acid in the activation loop, A381E in PIP4KII $\beta$ , and E362A in PIP5KI $\beta$ . Moreover, the E362A PIP5KI $\beta$  mutant, using PI(5)P as the preferred substrate, no longer associated with the plasma membrane [67]. Thus, the single amino acids determining the substrate specificity of PIP5KI and PIP4KII were identified (Fig. 2a). The data also suggested that the plasma membrane binding by PIP5KI $\beta$  is correlated with its ability to recognize and phosphorylate PI(4)P but not PI(5)P. However, a line of



**Fig. 2** Organization of PIP5KI molecule. **a** Amino acid sequence of activation loop regions of human PIP5KI $\alpha$ , human I $\beta$ , and murine I $\gamma$  (upper panel) and human PIP4KII $\beta$  (lower panel). The first and the last amino acid of the regions as well as the total number of amino acids composing the proteins are indicated. Dark gray background marks amino acids conserved between PIP5KI and PIP4KII subfamilies; amino acids conserved only within the PIP5KI and PIP4KII subfamilies, but different between them are shown by the light gray background [22, 23, 59]. Bar below the alignment indicates regions exchanged between PIP5KI $\beta$  and PIP4KII $\beta$  by Kunz et al. [56]. Asterisks indicate two lysine residues participating in membrane

data argue against PI(4)P as the only factor recruiting PIP5KI $\beta$  to the plasma membrane. Firstly, analysis of the kinetic properties of the E362A PIP5KI $\beta$  mutant revealed that the protein still displayed a relatively high affinity towards PI(4)P. The significant reduction of the mutant kinase activity toward PI(4)P, below that toward PI(5)P, resulted from a low  $V_{\max}$  of PI(4)P phosphorylation by the mutated kinase. Secondly, other fragments of the PIP5KI $\beta$  activation loop were also shown to participate in an optimal plasma membrane binding by the kinase. Thirdly, the A381E PIP4KII $\beta$  mutant, despite its efficient PI(4)P binding and phosphorylation, did not associate with the plasma membrane [67]. Therefore, the binding of PI(4)P is required but not sufficient to target PIP5KI $\beta$ , and presumably other PIP5KIs, to the plasma membrane.

PI(4,5)P<sub>2</sub> also acts as an anchor for PIP5KI in the plasma membrane

Recent data obtained in several laboratories has underscored the idea about electrostatic interactions of PIP5KI with the plasma membrane pointing simultaneously to an involvement of PI(4,5)P<sub>2</sub>, the product of the kinases, in PIP5KI binding. Recombinant PIP5KI $\alpha$  fused with the GST

binding of PIPKs while glutamic acid residue and alanine residue determining substrate specificity of PIP5KI and PIP4KII, respectively, are marked by boxes. **b** A schematic representation of murine PIP5KI $\gamma$ a (661 amino acids) and PIP5KI $\gamma$ b (635 amino acids). PIP5KI $\gamma$ c of rodents is composed of 687 amino acids and contains a 26-amino-acid insert at Ph635 [23, 24]. AL, activation loop. In the lower panel, the amino acid composition of 26-amino-acid tail of murine PIP5KI $\gamma$ a is shown. The motifs engaged in the binding of talin, AP-2 $\beta$  and AP-2 $\mu$ , are marked by bars. In human PIP5KI $\gamma$ a, two amino acids are inserted between Arg652 and Pro653 with the Arg652 exchanged for Glu652 [132]

tag interacted, apart from PI(4)P and PA, also with PI(4,5)P<sub>2</sub>, as shown by ELISA, protein-lipid overlay assay, and binding to PI(4,5)P<sub>2</sub>-containing liposomes [68, 69]. A fragment of PIP5KI $\alpha$  encompassing amino acids 374–440 and containing the activation loop of the kinase was found to interact preferably with PI(4,5)P<sub>2</sub> over PI(4)P. Conversely, the affinity of the whole kinase for PI(4)P exceeded that for PI(4,5)P<sub>2</sub> about 1.8-fold, indicating that multiple electrostatic interactions govern the membrane-kinase interaction [43, 69]. When expressed in cells, the aforementioned fragment of the kinase evoked a significant reduction of the PI(4,5)P<sub>2</sub> level, affected the organization of actin filaments, and inhibited phagocytosis. Similar results were obtained after expression in cells of a recombinant protein containing the PH domain of PLC $\delta$ <sub>1</sub>, a probe known to bind PI(4,5)P<sub>2</sub> with high affinity ( $K_D \sim 1.2 \mu\text{M}$ , [70]). When looking for the mechanism of the probe action, it was found that both its expression and treatment of cells with the Ca<sup>2+</sup> ionophore ionomycin, which activates PLC leading to PI(4,5)P<sub>2</sub> hydrolysis, reduced the amount of endogenous PIP5KI $\alpha$  associated with the plasma membrane [43, 69]. Altogether, the data indicated that PI(4,5)P<sub>2</sub> can serve as an anchor for PIP5KI $\alpha$  in the plasma membrane. Sequestration of PI(4,5)P<sub>2</sub> by the



lipid-binding probes or PI(4,5)P<sub>2</sub> hydrolysis displaced the kinase from the plasma membrane, thus inhibiting PI(4,5)P<sub>2</sub> synthesis and affecting the PI(4,5)P<sub>2</sub>-dependent actin reorganization. Taking into account the accumulation of PI(4,5)P<sub>2</sub> in the plasma membrane [71], an involvement of the lipid in the binding of PIP5KI to the membrane can explain why various kinase isoforms, when overexpressed in the cell, all display submembraneous location [67, 69, 72]. The endogenous enzymes are located in different cellular compartments presumably because their distribution is under more subtle regulation by several other factors.

Results obtained for GFP-tagged PIP5KI $\alpha$ , I $\beta$  and I $\gamma$ , I $\gamma$ b expressed in RAW macrophages were in full agreement with the suggestion about the PI(4,5)P<sub>2</sub> engagement in the binding of the kinases to the plasma membrane [72]. In addition to ionomycin treatment, also depletion of cellular ATP or termination of polyphosphoinositide synthesis released the PIP5KI isoforms from the plasma membrane. Partial dissociation of GFP-PIP5KI $\beta$  from the plasma membrane was also observed in cells co-expressing the kinase and synaptojanin 2, PI(4,5)P<sub>2</sub>-dephosphorylating enzyme [72]. A PIP5KI $\alpha$  mutant in which the positively charged arginine and leucine residues in the activation loop were substituted with the negatively charged aspartic acid residues failed to associate with the plasma membrane, while analogous mutations of amino acids located C-terminally from the activation loop were also effective but to a lower extent. Those data supported the suggestion that multiple electrostatic interactions of PIP5KI are crucial for its binding to anionic lipids of the plasma membrane. The authors took advantage of the crystal structure of PIP4II $\beta$  described earlier [62] and proposed that PIP5KI isoforms form homo-dimers with a flat, positively charged surface of the enzyme oriented towards the plasma membrane. The amino acids contributing to the positive surface charge of PIP5KI are presumably present both outside and within the activation loop of the kinase and are crucial for the proper positioning of the loop for substrate recognition and of the catalytic center for substrate phosphorylation. In the plasma membrane, the negatively charged lipids including PI(4)P, PI(4,5)P<sub>2</sub>, PA, and phosphatidylserine can participate in PIP5KI binding. PA also strongly activates PIP5KI, possibly overcoming the substrate inhibition of PIP5KI [68] to create conditions for efficient local generation of PI(4,5)P<sub>2</sub>. Subsequent hydrolysis of the lipid by PLC can facilitate displacement of the kinase from the membrane, providing an “electrostatic switch”, which terminates local PI(4,5)P<sub>2</sub> synthesis [72]. Such a local reduction of PI(4,5)P<sub>2</sub> level, which follows its rapid generation, was observed during Fc $\gamma$  receptor-mediated phagocytosis [73] and budding of clathrin-coated vesicles [12]. The existence of the “electrostatic switch” does not answer, however, the

question about factors recruiting PIP5KI to distinct sites at the plasma membrane during cell activation. It is conceivable that an interaction of PIP5KIs with specific membrane proteins provides a key for the plasma membrane recruitment of the individual kinase isoforms.

#### Coupling between PI(4)P and PI(4,5)P<sub>2</sub> synthesis at the plasma membrane

The considerations on PI(4)P and PI(4,5)P<sub>2</sub> as plasma membrane anchors for PIP5KI need to be reconciled with the data on the subcellular location of these lipids. Thus, visualization of PI(4)P with specific antibodies indicated that about 47% of the lipid is accumulated in the Golgi apparatus. Very little PI(4)P was found to reside in the plasma membrane at steady state [74], as confirmed by studies with the PH domains of phosphatidylinositol-4-phosphate adaptor protein 1 (Fapp1) and oxysterol-binding protein (OSBP) as PI(4)P markers [75–77]. This PI(4)P distribution is opposite to the prevailing plasma membrane location of PI(4,5)P<sub>2</sub> that is well documented by cell fractionation studies and image analysis [71, 78]. When combined with reports on PI(3)P enrichment in endosomes [79], these findings spark an idea that the organelle-specific composition of phosphoinositides can define the identity and functions of various cellular organelles by recruiting specific effector proteins and directing membrane traffic [3, 74]. In particular, PI(4)P at Golgi, besides serving as a source for a small local pool of PI(4,5)P<sub>2</sub>, controls the vesicular traffic towards distinct compartments like endo/lysosomes and plasma membrane by recruiting adaptor proteins including AP-1 complex or GGAs or FAPPs [74, 76, 80, 81], and participates in sphingolipid metabolism (see [82] for review). However, the analysis of PI(4)P distribution in the cell based solely on imaging of over-expressed PH domains of Fapp1 or OSBP can be biased to the Golgi at the expense of the plasma membrane staining since the both probes bind not only PI(4)P but also ARF1, a Golgi marker protein. Recently, a pool of PI(4)P has been revealed in the plasma membrane in experiments employing another probe, the PH domain of yeast oxysterol-binding protein homologue (OSH2), or an improved protocol of plasma membrane preservation for PI(4)P staining with antibodies [83, 84].

While the content of PI(4)P in the cellular membranes awaits direct estimation, it is well established that the activity of phosphatidylinositol 4-kinases (PI4K), which generate PI(4)P by PI phosphorylation, is present in the Golgi, the plasma membrane, and lysosomes [85]. Four PI4Ks have been identified in mammalian cells: PI4KII $\alpha$  and II $\beta$ , and PI4KIII $\alpha$  and III $\beta$ , with these two classes being distinguished by the wortmannin-sensitivity of PI4KIIIs. PI4KIII $\alpha$  is localized primarily to the

endoplasmic reticulum. The Golgi apparatus contains PI4KIII $\beta$ , PI4KII $\alpha$ , and PI4KII $\beta$ . Among the kinases, PI4KIII $\beta$  contributes to production of PI(4)P in *cis/medial* Golgi, PI4KII $\alpha$  in *trans*-Golgi network and PI4KII $\beta$  displays more complex pattern of distribution as described below [86–90]. Silencing of PI4KII $\alpha$  expression by RNA interference led to an about 50% reduction of PI(4)P synthesis and a 60% reduction of PI(4,5)P<sub>2</sub> synthesis in HeLa cells, indicating that the activity of this Golgi-associated kinase significantly affects the overall level of PI(4,5)P<sub>2</sub> [74]. Taken together, the data suggest that PI(4,5)P<sub>2</sub> is generated by phosphorylation of PI(4)P “en route” from the Golgi to the plasma membrane. Alternatively, the Golgi-derived PI(4)P can be converted to PI(4,5)P<sub>2</sub> immediately upon reaching the plasma membrane. In either case, the Golgi-plasma membrane trafficking emerges from these studies as an important factor controlling the plasma membrane level of PI(4,5)P<sub>2</sub>. This subject got a new input with the discovery of Balla’s group that substantial amounts of PI(4)P appear in the plasma membrane and in submembrane vesicles during the acute resynthesis of phosphoinositides following Ca<sup>2+</sup>-induced PLC activation and PI(4,5)P<sub>2</sub> depletion [77]. Subsequent work of the group has indicated that also in the course of stimulation of cells with angiotensin II, an agonist of G protein-coupled receptor, PLC utilizes PI(4,5)P<sub>2</sub> pool whose maintenance is limited by local PI(4)P synthesis [83]. Based on the wortmannin sensitivity and the results of siRNA application, the delivery of this plasma membrane pool of PI(4)P was attributed to PI4KIII $\alpha$ , although the kinase itself was not detected at the plasma membrane [77, 83, 86]. Therefore, PI(4)P can reach the plasma membrane not only by exchange of vesicles between the plasma membrane and the Golgi but possibly also as a result of PI4KIII $\alpha$  activity at sites of close apposition between the endoplasmic reticulum and the plasma membrane.

On the other hand, early biochemical studies detected significant activity of PI4Ks, particularly PI4KII, in the plasma membrane [85, 86]. This activity can likely be ascribed to PI4KII $\beta$ , an enzyme cloned simultaneously by two groups [88, 89]. In a series of elegant studies combining immunofluorescence observations and biochemical fractionation it has been determined that PI4KII $\beta$  is recruited to the plasma membrane from the cytosol. This redistribution is controlled by the monomeric small GTPase Rac1 in a GTP-dependent manner. Accordingly, the kinase was found in membrane ruffles evoked by stimulation of PDGF receptor which employs Rac1 as a downstream effector. It was established that the responsiveness of PI4KII $\beta$  to Rac1 was mediated by the catalytic domain of the kinase. The recruitment of PI4KII $\beta$  to the membrane stimulates its enzymatic activity 16-fold. The membrane-bound PI4KII $\beta$  is palmitoylated and this

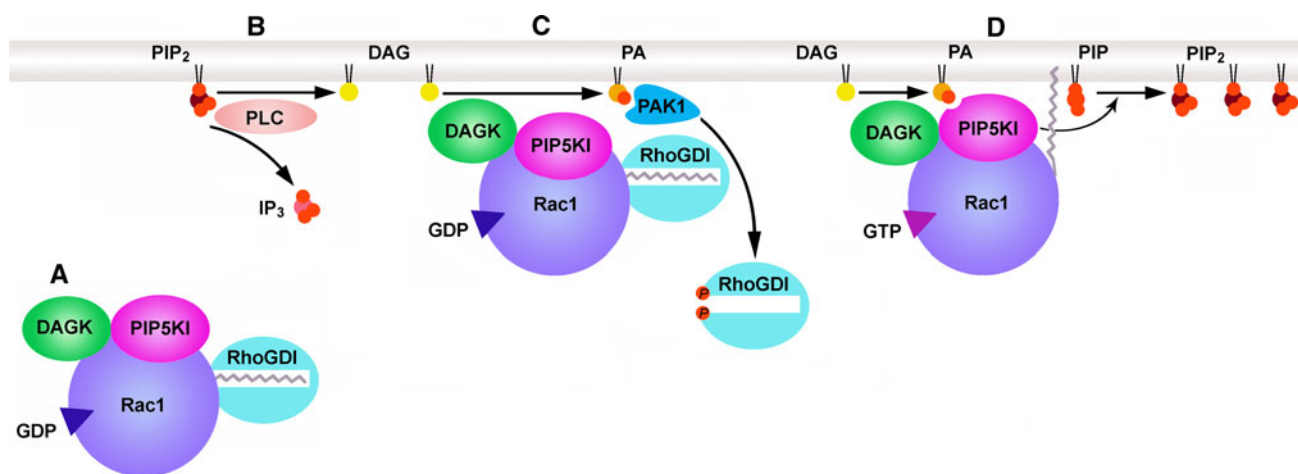
acylation is a prerequisite for the high enzymatic activity of the kinase [88, 91]. Those data indicate that Rac1 controls the recruitment of PI4KII $\beta$  to the plasma membrane and places it in a position allowing activation by other enzymes (palmitoyl acyltransferase?) or lipids present in the membrane.

Taken together, the studies on PI4KII $\beta$  and PI4KIII $\alpha$  support the suggestion that the synthesis of PI(4)P and PI(4,5)P<sub>2</sub> in the plasma membrane can be coupled spatially and temporally providing a way for transient accumulation of PI(4,5)P<sub>2</sub> at discrete plasma membrane locations. One can hypothesize that upon cell stimulation by agonists of G protein-coupled receptors PI(4)P is generated by PI4KIII $\alpha$  residing in the endoplasmic reticulum adjacent to the plasma membrane. At the plasma membrane the lipid is rapidly converted into PI(4,5)P<sub>2</sub> to be then hydrolyzed by PLC and trigger IP<sub>3</sub>/Ca<sup>2+</sup> signaling. Thus far, PIP5KI $\alpha$  (murine I $\beta$ ) and PIP5KI $\gamma$ b have been shown to generate PI(4,5)P<sub>2</sub> subsequently utilized for the IP<sub>3</sub>/Ca<sup>2+</sup> signaling by G protein-coupled receptors [36, 92]. On the other hand, PI4KII $\beta$  catalyzes the synthesis of PI(4)P converted into a pool of PI(4,5)P<sub>2</sub> acting on cytoskeletal proteins. In the latter case, the coupling of PI(4)P and PI(4,5)P<sub>2</sub> production can be achieved by Rac1, which recruits to the plasma membrane and thus facilitates activation of both PI4KII $\beta$  [88] and PIP5KI, as discussed below.

### Regulators of PIP5KI activity and PI(4,5)P<sub>2</sub> synthesis at the plasma membrane

Rac1 recruits PIP5KI to the plasma membrane and facilitates its activation

Rac1 has been reported to associate with PIP5KI $\alpha$  and, to a lower extent, with PIP5KI $\beta$  and I $\gamma$  [33, 42, 93–95]. An association of RhoA, but not Cdc42, with all three isoforms of PIP5KI was also reported [94–96]. The association of Rac1 and RhoA with PIP5KI was independent of the GDP/GTP loading of the GTPases. In the cited reports, the binding of the two Rho family GTPases to PIP5KI was examined by the pull-down assays relying on incubation of immobilized Rho GTPases with homogenates prepared from rat brain or liver, or with cell lysates, and subsequent measurements of PIP5KI activity and/or kinase isoform identification. The data were supported by co-immunoprecipitation of Rac1 and RhoA with PIP5KI. Moreover, a direct binding of GDP/GTP-loaded Rac1 and RhoA to PIP5KI $\alpha$  and I $\beta$  was also shown using recombinant proteins [33]. However, neither Rac1 nor RhoA activated PIP5KI $\beta$  in another *in vitro* assay [97], while activation of PIP5KI $\alpha$ , I $\beta$  and I $\gamma$  by Rac1, RhoA, and Cdc42 was demonstrated *in vivo*. Only GTP-bound



**Fig. 3** Rac1 forms multimolecular complex engaging PIP5KI. **a** In resting cells, PIP5KI $\alpha/\beta$  and DAGK $\zeta$  are bound to the C-terminus of GDP-loaded Rac1. The interaction of Rac1 with the plasma membrane is blocked by RhoGDI, which masks the prenyl group attached to the C-terminal CAAX motif of Rac1. **b** Upon cell activation, PLC produces DAG. **c** DAG is subsequently phosphorylated by DAGK $\zeta$  to PA. The PA activates PAK1, which

phosphorylates RhoGDI at two sites. Phosphorylated RhoGDI dissociates from Rac1 exposing its prenyl group. **d** Rac1 binds to the membrane via the prenyl group and adjacent polybasic region and this facilitates replacement of GDP with GTP by membrane-bound exchange factors. PIP5KI $\alpha/\beta$  recruited to the plasma membrane together with Rac1 is activated by PA and phosphorylates PI(4)P to PI(4,5)P<sub>2</sub>

proteins activated PIP5KIs in cells since no increase in PIP5KI activity and no PI(4,5)P<sub>2</sub> production was observed in cells expressing dominant negative mutants of Rac1, RhoA, and Cdc42 [46, 95, 98, 99]. Taken together, the data implicate that Rho family GTPases can interact with and control the activity of PIP5KI and also suggest that the activation can be indirect. Since the association of Rac1 and RhoA with PIP5KI does not depend on GDP/GTP binding to the GTPases, the complex of Rac1 or RhoA with PIP5KI can be maintained in both resting and activated cells. Therefore, the GTPases should be able to shift PIP5KI to the plasma membrane upon cell activation. The data on the participation of Rac1 and RhoA in PIP5KI regulation vary and point to either RhoA [46, 97, 100, 101] or Rac1 [33, 39, 42] or both [95, 99] as dominant factors affecting PIP5KI activity, probably depending on the cell type and stimulus used in those studies.

Among the GTPases of the Rho family, Rac1 is of special interest because of its ability to recruit to the plasma membrane and activate PI4KII $\beta$  as well [88]. Hence, this GTPase could in principle coordinate the synthesis of PI(4)P and PI(4,5)P<sub>2</sub> at distinct regions of the membrane. Rac1 affects actin organization and focal complex assembly contributing to ruffling and lamellipodia formation [102], which is in line with the crucial role of PI(4,5)P<sub>2</sub> in those events. PIP5KI was shown to associate with the C-terminus of Rac1 in a nucleotide-independent fashion based on the ability of a recombinant peptide encompassing amino acids 166–188 of Rac1 to compete with full-length Rac1 for pull-down of PIP5KI activity from rat brain homogenate [42]. In further studies, the <sup>185</sup>ArgLysArg<sup>187</sup> sequence in the C-terminus of

Rac1 was found crucial for the PIP5KI pull-down from fibroblast lysates [94]. The C-terminal fragment of Rac1 also associated with DAGK, catalyzing PA production. Similarly to PIP5KI, DAGK associated with both GDP- and GTP-bound Rac1. However, the association of the two lipid kinases with Rac1-GTP was enhanced in the presence of PA, phosphatidylserine, and PI(4)P. Combined with the detected association of PIP5KI and DAGK with RhoGDI the data suggested the existence of a multimolecular complex able to control PIP5KI recruitment to the plasma membrane [42]. In resting cells, RhoGDI is able to sequester the lipid moiety of Rac1 and prevent the association of the whole multimolecular complex containing Rac1, DAGK, and PIP5KI with the plasma membrane. Upon cell activation, RhoGDI releases Rac1, which can then anchor the protein complex to the plasma membrane leading to the synthesis of PA and, in turn, PI(4,5)P<sub>2</sub> (Fig. 3a, d).

The above idea was developed further by Abramovici et al. [103], who identified ubiquitously expressed DAGK $\zeta$  as a component of a multimolecular complex containing Rac1 and RhoGDI. Moreover, the activity DAGK $\zeta$  was found to be a crucial factor controlling Rac1 activation: the PA produced by DAGK $\zeta$  activates p21-activated kinase 1 (PAK1), which in turn phosphorylates RhoGDI leading to its dissociation from Rac1. In fibroblasts derived from DAGK $\zeta^{-/-}$  mice and stimulated with PDGF, a decreased Rac1 activation and inhibition of ruffling, spreading, and migration were found [103]. On the other hand, association of DAGK $\zeta$  with PIP5KI $\alpha$  was also documented, and expression of DAGK $\zeta$  significantly enhanced PIP5KI $\alpha$  activity in thrombin-stimulated cells [104]. Those data put



DAGK $\zeta$  and its product, PA, in the center of events controlling Rac1-mediated PIP5KI recruitment and activation at the plasma membrane. The PA generated by DAGK $\zeta$  activates PAK1 enabling RhoGDI dissociation from Rac1, subsequent anchoring of Rac1 at the plasma membrane and activation of the Rac1-associated PIP5KI, leading eventually to local PI(4,5)P<sub>2</sub> synthesis (Fig. 3c, d). The initial step of the cascade requires an appearance of DAG in the plasma membrane. The lipid arises most likely from hydrolysis of PI(4,5)P<sub>2</sub> catalyzed by PLC at the onset of cell stimulation (Fig. 3b). Of note, Rac proteins were found to associate with and stimulate the activity of PLC $\beta_2$  and PLC $\gamma_2$  isoforms [105–107].

The physiological importance of the Rac1-PIP5KI interaction for PI(4,5)P<sub>2</sub> synthesis is well documented. Studies performed on permeabilized yet living platelets allowing introduction of recombinant proteins revealed that interaction of Rac1 with PIP5KI $\beta$ , but not PIP5KI $\alpha$ , determined the uncapping of actin filaments as a result of PI(4,5)P<sub>2</sub> synthesis. A point mutation introduced in the C-terminus of Rac1, which abolished binding of PIP5KI $\beta$ , also inhibited actin assembly in thrombin-stimulated cells, while exogenous PIP5KI $\beta$  introduced to permeabilized platelets restored the process [33]. Those studies put the I $\beta$  isoform of PIP5K downstream of Rac1 in the signaling pathway leading to PI(4,5)P<sub>2</sub> synthesis and actin polymerization relying on the exposure of the barbed ends of microfilaments. Another line of data pointed to the I $\alpha$  isoform of PIP5KI as the one acting downstream of Rac1 during stimulation of thrombin receptor [99]. In that case, Rac1-GTP was found to mediate recruitment of PIP5KI $\alpha$  to the plasma membrane, but this processes required Rho-GTP involvement as well. Both Rac-GTP and Rho-GTP increased the activity of PIP5KI $\alpha$  five to sevenfold when co-expressed with it [99]. Finally, PIP5KI $\alpha$  rather than PIP5KI $\beta$  was reported to act downstream of Rac1 during actin remodeling, which underlies ruffling in PDGF-stimulated MG-63 fibroblasts [39]. Taken together, the data indicate that local activation of Rac1 can result in the recruitment of PIP5KI and its activation at the plasma membrane, however, the kinase isoform involved and the mode of Rac1 action (direct or indirect) varies from cell to cell.

Another link between Rac1 and PIP5KI $\alpha$  seems to be provided by the LIM protein Ajuba. Ajuba on the one hand influences the activity of Rac1 at nascent adhesive sites in a migrating cell by promoting the assembly of the p130Cas-Crk complex that interacts with the DOCK180-ELMO complex, a guanine nucleotide exchange factor of Rac1 [108]. On the other hand, Ajuba was also found to associate with PIP4KII $\beta$ , PIP5KI $\alpha$ , and PIP5KI $\beta$ , but not PIP5KI $\gamma$  in vivo. The binding of PIP5KI $\alpha$  was enhanced 20-fold during cell migration. Further in vitro studies indicated that the

interaction of PIP5KI $\alpha$  with Ajuba stimulated the activity of the lipid kinase sixfold [40]. Hence, the Ajuba-Rac1-PIP5KI $\alpha$  interactions can regulate PI(4,5)P<sub>2</sub> synthesis in the course of assembly of adhesive complexes required for cell migration. Taking into account that PIP5KI $\gamma$ , the kinase isoform neglected by Ajuba, is localized to focal contacts and activated at these sites by another protein—talin (see below), the assembly of focal contacts provides an example of complexity of mechanisms fine-tuning the functions of PIP5KI isoforms at the plasma membrane.

Besides the GTPases of the Rho family, an involvement of ARF6 from the ARF-family GTPases, often acting in cooperation with PLD, is also considered in activation of PIP5KI at the plasma membrane. Combined in vitro and in vivo studies have indicated that myristoylated membrane-bound ARF6 activates PIP5KI $\alpha$ , I $\beta$  and I $\gamma$ , and PLD2 [97, 109–111]. Therefore, the ARF6-induced association of PIP5KI with the plasma membrane can increase its activity by bringing the kinase close to PI(4)P and PA and additionally facilitating the reciprocal stimulation of PIP5KI and PLD by the respective products of their activities, PI(4,5)P<sub>2</sub> and PA [111–113]. The ternary ARF6-PLD-PIP5KI complex has been implicated in controlling Ca<sup>2+</sup>-regulated exocytosis, ruffling, and receptor endocytosis [97, 109, 114, 115]. In terms of actin cytoskeleton rearrangements, ARF6 cooperates with Rac1 and the cross-talk between the proteins can be coordinated at the level of guanine nucleotide exchange factors of the two GTPases [116, 117].

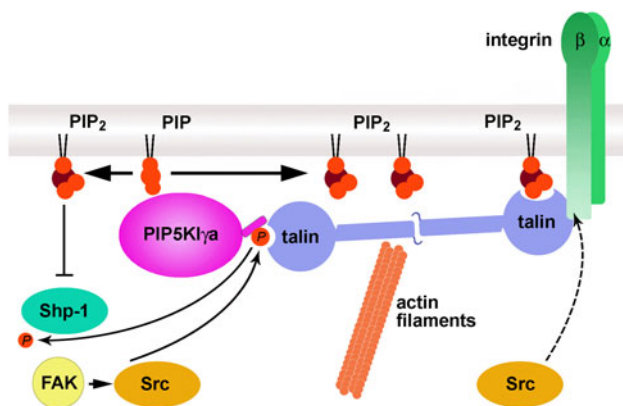
#### PIP5KI $\gamma$ a and I $\gamma$ c bind talin at focal contacts

Talin is a large, rod-shaped 270-kDa protein containing a globular head with the FERM domain, which binds PI(4,5)P<sub>2</sub>, PIP5KI $\gamma$ a/c, F-actin, focal adhesion kinase (FAK),  $\beta$ 1- and  $\beta$ 3-integrin. The flexible rod fragment of talin enables its dimerization and contains additional actin- and  $\beta$ -integrin-binding sites. The assembly of dimers engages the C-terminal domain of talin, which suggests that full-length talin can adopt variable conformations ranging from a parallel dimer to an extended one [118, 119]. The PIP5KI $\gamma$ a-binding site in talin was mapped to so-called subdomain F3 of the FERM domain, which structurally resembles the phosphotyrosine-binding (PTB) domain [29, 30]. The F3 subdomain of talin is also a site of  $\beta$ -integrin association, however, despite such overlapping, distinct amino acid motifs mediate the interactions of PIP5KI $\gamma$ a and  $\beta$ 1-integrin with talin [29, 52, 120, 121]. In the kinase, the <sup>642</sup>WVYSPLH<sup>648</sup> motif located within the last 26 amino acids of the C-terminus (28 amino acids of human PIP5KI $\gamma$ a) was found to participate in talin binding [29, 52, 121] as shown in Fig. 2b. This amino acid sequence is also present in the rodent PIP5KI $\gamma$ c allowing

its binding to talin as well, while PIP5KI $\gamma$ b, devoid of the C-terminal 26/28-amino acid extension does not interact with talin.

Phosphorylation of PIP5KI $\gamma$ a on Tyr644 within the <sup>642</sup>WVYSPLH<sup>648</sup> motif strengthens the interaction of the kinase with talin 15-fold and activates the kinase as well [29, 52]. Accordingly, the NMR structure of talin PTB in complex with the <sup>641</sup>SWVpYSPLH<sup>648</sup> peptide revealed an unusual electrostatic cluster involving the phospho-Tyr644 and basic amino acids of the PTB [122]. As shown in Fig. 4, the phosphorylation of PIP5KI $\gamma$ a is catalyzed by Src kinase, which associates with PIP5KI $\gamma$ a, and both these events are additionally upregulated by FAK kinase [30, 52]. It is assumed that the tyrosine phosphorylation releases autoinhibitory restraints within the PIP5KI $\gamma$ a molecule [121]. Of note, Src phosphorylates  $\beta$ -integrin as well, however, in contrast to phosphorylated PIP5KI $\gamma$ a, affinity of phosphorylated  $\beta$ 1-integrin for talin is diminished. Eventually, this Src kinase activity weakens the interaction of  $\beta$ -integrin with talin [52]. The reciprocal effect exerted by tyrosine phosphorylation of PIP5KI $\gamma$  and  $\beta$ 1-integrin on their interactions with talin ensue from different amino acid composition of motifs involved in the binding [121].

The enzymatic activity of PIP5KI $\gamma$ a is a prerequisite for the formation of talin-containing focal adhesions [30]. This is because PI(4,5)P<sub>2</sub> produced locally by the kinase can



**Fig. 4** Talin and PIP5KI $\gamma$ a/c interact during focal contact assembly. Upon cell activation, PIP5KI $\gamma$ a/c undergoes phosphorylation on Tyr644 by Src kinase, which also associates with it. Both events are positively regulated by FAK kinase. When phosphorylated, PIP5KI $\gamma$ a/c binds to the FERM domain located in the globular N-terminal part of talin. The kinase produces PI(4,5)P<sub>2</sub>, which binds to the FERM domain of talin and relieves autoinhibitory interaction of the talin head with rod. At these conditions, talin is able to bind  $\beta$ -integrins, inducing additionally their aggregation and activation. Intracellularly, talin binds actin filaments, which enables the formation of stress fibers. The Tyr644 can be dephosphorylated by Shp-1, which associates with PIP5KI $\gamma$ . The activity of Shp-1 is inhibited by PI(4,5)P<sub>2</sub> favoring the lipid accumulation. On the other hand, the binding of  $\beta$ -integrin to talin can be inhibited by phosphorylation of  $\beta$ -integrin by Src kinase

bind to the FERM domain and relieve the autoinhibitory head-tail association within the talin molecule, thus exposing the integrin-binding site [123, 124]. As a result, PI(4,5)P<sub>2</sub> provides a positive signal for the interaction of talin with  $\beta$ 1- and  $\beta$ 3-integrin, also promoting integrin clustering [125] and enabling their interaction with the extracellular matrix (Fig. 4). Simultaneously, talin interacts with actin filaments, thereby initiating the assembly of focal complexes. In addition to talin, PI(4,5)P<sub>2</sub> also binds to other proteins of focal complexes, like vinculin,  $\alpha$ -actinin, and syndecan-4, promoting their association with the complexes. PI(4,5)P<sub>2</sub> can also support its own generation in a positive-feedback manner (as shown in Fig. 4) by inhibition of Shp-1 tyrosine phosphatase, which associates with and dephosphorylates PIP5KI $\gamma$ a [126]. Besides the phosphorylation-based activation of PIP5KI $\gamma$ a/c, stimulation of PIP5KI $\gamma$ b by PLD2 also adds to the synthesis of PI(4,5)P<sub>2</sub>, which facilitates cell adhesion [53]. All of these mechanisms enhance the activity of PIP5KI $\gamma$  and production of PI(4,5)P<sub>2</sub> during formation and maturation of focal contacts. Recently, PIP5KI $\gamma$ a was found to associate with PLC $\gamma$ 1 and the association was inhibited by phosphorylation of Tyr634 in PIP5KI $\gamma$ a. This phosphorylation was catalyzed by the receptor for epidermal growth factor (EGF), which in this way can diminish PI(4,5)P<sub>2</sub> hydrolysis and favor PI(4,5)P<sub>2</sub> accumulation at the focal contacts required for cell migration [127].

On the other hand, a line of data indicates that PIP5KI $\gamma$ a and  $\beta$ -integrin compete for binding to talin [52, 120], which suggests that PI(4,5)P<sub>2</sub>, generated by tyrosine-phosphorylated talin-bound PIP5KI $\gamma$ a can limit its own production. The lipid promotes the interaction of talin with  $\beta$ 1-integrin, therefore facilitating the displacement of PIP5KI $\gamma$ a from talin. This fine-tuned interaction of talin with  $\beta$ 1-integrin, strengthened by PI(4,5)P<sub>2</sub> and weakened by the competitive binding of phosphorylated PIP5KI $\gamma$ a and by  $\beta$ -integrin phosphorylation, has been proposed to confer a dynamic character on focal adhesions, enabling their assembly and disassembly [52]. Taking into account that talin forms dimers and that recently a second  $\beta$ -integrin-binding site in the talin rod has been characterized [117, 128, 129], possible combinations of talin interactions during focal contact assembly/disassembly are complex.

#### Interaction of PIP5KI $\gamma$ a with AP-2 adaptor at clathrin-coated pits

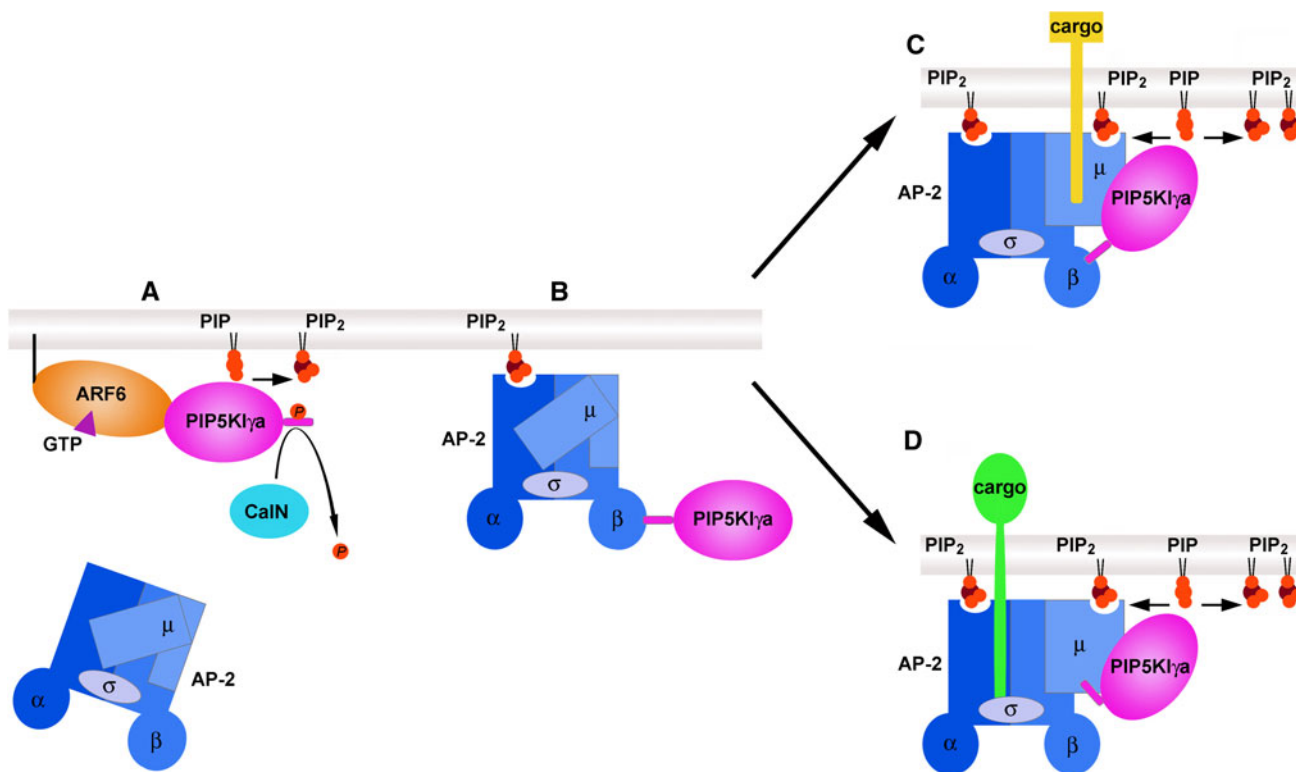
PIP5KI $\gamma$  is the major PIP5KI producing PI(4,5)P<sub>2</sub> in synapses where the lipid controls both exocytosis of synaptical vesicles and subsequent clathrin-mediated endocytosis of membranes [23, 25, 130]. A combination of co-immunoprecipitation, pull-out and yeast two-hybrid studies has indicated that PIP5KI $\gamma$ a binds to the AP-2 adaptor of the

clathrin-based coat [49–51]. The AP-2 adaptor is a heterotetramer composed of large  $\alpha$  and  $\beta$  subunits, a medium-sized  $\mu$  subunit recognizing cargo proteins, and a small  $\sigma$  subunit. The subunits form a core with two appendage domains of  $\alpha$  and  $\beta$  subunits linked to them via flexible linkers (Fig. 5). Besides the cargo recognition, the complex acts a scaffold binding an array of clathrin-accompanying proteins.

The interaction between PIP5KI $\gamma$  and AP-2 involves multiple sites (see Fig. 5): the  $\mu$ 2 subunit binds to the catalytic core of the kinase [50] and to the <sup>644</sup>YSPL<sup>647</sup> motif of the 26-amino-acid C-terminal extension of the kinase [49, 131]. In addition, the C-terminus of PIP5KI $\gamma$  interacts with the  $\beta$ 2 chain of AP-2 [51]. PIP5KI $\gamma$  is the only PIP5KI $\gamma$  isoform interacting with AP-2 [132]. Further site-directed mutagenesis studies and structural analysis indicated that the amino acid motifs of the kinase C-terminus involved in the binding of the  $\mu$ 2 and  $\beta$ 2 chains partially overlap (Fig. 2b). Therefore, the two interactions of the kinase C-terminus are mutually exclusive [131, 132]. Important contacts formed between the PIP5KI $\gamma$

C-terminal tail and the  $\beta$ 2 chain involve aromatic residues (Tyr635, Phe636, Trp642, and Tyr644) and Ser645 of the kinase [51, 131, 132]. Phosphorylation of Ser645 by cdk5 kinase inhibits the interaction between PIP5KI $\gamma$  and AP-2  $\beta$ , while dephosphorylation of Ser645, catalyzed presumably by calcineurin, allows the binding. It is striking that the amino acid sequences involved in AP-2  $\beta$  and talin binding overlap (Fig. 2b). Notably, while Ser645 phosphorylation inhibits the association of PIP5KI $\gamma$  with both talin and AP-2, phosphorylation of Tyr644 facilitates association of PIP5KI $\gamma$  with talin but inhibits the association of the kinase with the AP-2 complex. These data suggest that depending on the phosphorylation state of Tyr644, PIP5KI $\gamma$  can shuttle between sites of cell adhesion and endocytosis [131].

The complex nature of the interactions between PIP5KI $\gamma$  and the AP-2 adaptor creates an elaborate molecular mechanism controlling rapid PI(4,5)P<sub>2</sub> synthesis during assembly of clathrin-based coat. In resting neurons, PIP5KI $\gamma$  is located on the presynaptic membrane but, being phosphorylated on Ser645, it does not interact with AP-2.



**Fig. 5** PIP5KI $\gamma$  interacts with AP-2 at the onset of receptor endocytosis. **a, b** Initially, GTP-loaded ARF6 activates PIP5KI $\gamma$  leading to synthesis of PI(4,5)P<sub>2</sub>. The lipid provides an anchor for the  $\mu$ 2 chain of the AP-2 complex. **d** The C-terminus of the kinase can also shift from  $\beta$ 2 to the  $\mu$ 2 and binds to the  $\mu$ 2 chain via the <sup>644</sup>YSPL<sup>647</sup> motif. This interaction promotes the recognition of cargo receptors devoid of the canonical YXX $\emptyset$  motif; it also activates PIP5KI $\gamma$  facilitating local PI(4,5)P<sub>2</sub> accumulation

also interacts with PIP5KI $\gamma$  catalytic core, activating the kinase and leading to PI(4,5)P<sub>2</sub> production. The lipid serves as an anchor for the  $\mu$ 2 chain of the AP-2 complex. **d** The C-terminus of the kinase can also shift from  $\beta$ 2 to the  $\mu$ 2 and binds to the  $\mu$ 2 chain via the <sup>644</sup>YSPL<sup>647</sup> motif. This interaction promotes the recognition of cargo receptors devoid of the canonical YXX $\emptyset$  motif; it also activates PIP5KI $\gamma$  facilitating local PI(4,5)P<sub>2</sub> accumulation

Excitation of cells and depolarization of the presynaptic membrane leads to dephosphorylation of the kinase allowing AP-2 binding, as shown in hippocampal neurons [51]. There are data suggesting that initially PIP5KI $\gamma$  is activated by ARF6 [109, 133], producing a pool of PI(4,5)P<sub>2</sub> that anchors AP-2 at the plasma membrane by binding to the  $\alpha$  subunit of the complex (Fig. 5a, b). This pool of AP-2 can, in turn, interact with dephosphorylated PIP5KI $\gamma$ , most likely by engaging the  $\beta$ 2 appendage and the kinase C-terminus via relatively weak interactions (Fig. 5b). Subsequent conformational changes of the  $\mu$ 2 chain induced by its phosphorylation allow binding of cargo proteins containing the conventional YXX $\emptyset$  sorting motif. They can also facilitate the interaction of the  $\mu$ 2 chain with the PIP5KI $\gamma$  catalytic core leading to activation of the kinase and thus to local production and accumulation of PI(4,5)P<sub>2</sub> (Fig. 5c). A shift of the C-terminus of the kinase from the  $\beta$ 2 to the  $\mu$ 2 chain (Fig. 5d) also stimulates the PIP5KI $\gamma$  activity [130]. This displacement of the kinase tail from the  $\beta$ 2 appendage can be facilitated by clathrin in the course of coat assembly [132]. Eventually, local production of PI(4,5)P<sub>2</sub> enables recruitment of the components of the clathrin coat, including AP-2 itself, epsin, AP180, Dab2 and dynamin 2. As the <sup>644</sup>YSPL<sup>647</sup> sequence of the PIP5KI $\gamma$  C-terminus corresponds to the conventional YXX $\emptyset$  sorting motif of cargo receptors, the occupation of  $\mu$ 2 by the kinase tail may also favor sorting of cargo lacking the YXX $\emptyset$  motif. This mechanism can be characteristic to internalization of proteins of presynaptic vesicles helping to sort them away from constitutively internalized cargo receptors [134].

It is noteworthy that PIP5KI $\beta$  rather than PIP5KI $\gamma$  has been implicated in the production of PI(4,5)P<sub>2</sub> required for AP-2 recruitment and transferrin receptor uptake in HeLa and CV-1 cells [48], while PIP5KI $\alpha$  (murine PIP5KI $\beta$ ) was indicated to participate in endocytosis of EGF receptor in NR6 fibroblasts [135]. These data led to a suggestion that clathrin-mediated endocytosis in nonneuronal cells, especially endocytosis of nutrient receptors like transferrin receptor, can be regulated differently from endocytosis taking place in synapses [48]. However, thus far no specific structural basis for the binding of PIP5KI $\alpha$  and I $\beta$  isoforms to AP-2 or other components of the clathrin-containing coat has been proposed. In addition, the above-mentioned studies were based on overexpression of the kinases or their knockdown by RNA interference and the results discussed above may reflect the effects of perturbations of cellular PI(4,5)P<sub>2</sub> level on clathrin coat assembly. The PIP5KI $\gamma$  participation in AP-2 recruitment at synapses is well documented and this may be the major function of this kinase isoform in the brain, judging from the high expression of PIP5KI $\gamma$  there. However, an engagement of PIP5KI $\gamma$  in receptor endocytosis in other cell types is conceivable [49, 50].

The termination of PI(4,5)P<sub>2</sub> signals at clathrin-coated vesicles in synapses comes from cessation of its synthesis due to phosphorylation of Ser645 of PIP5KI $\gamma$  [51]. Equally important is the dephosphorylation of PI(4,5)P<sub>2</sub> by synaptojanin 1. The reduction of PI(4,5)P<sub>2</sub> level shortly after scission of clathrin-coated vesicles is required for the coat turnover, judging from an excessive coat association in neurons in synaptojanin 1-null mice [12]. Such rapid and transient production of PI(4,5)P<sub>2</sub> accompanies phagocytosis as well. Similarly to the clathrin-based coat assembly, production of PI(4,5)P<sub>2</sub> by PIP5KI $\alpha$  is required during phagocytosis for actin polymerization and particle internalization. The subsequent actin depolymerization and phagosome maturation requires, in turn, reduction of the PI(4,5)P<sub>2</sub> level in the phagosome membrane by PI(4,5)P<sub>2</sub> hydrolysis and phosphorylation to PI(3,4,5)P<sub>3</sub> [73, 136].

### Are PI(4,5)P<sub>2</sub> clusters formed in the plasma membrane?

Type I PIP5Ks can efficiently increase the local concentration of PI(4,5)P<sub>2</sub> when the lateral mobility of the lipid is restricted. If the diffusion coefficient ( $D$ ) of PI(4,5)P<sub>2</sub> was comparable to that of freely diffusible membrane lipids, PI(4,5)P<sub>2</sub> would rapidly move away from the sites of its synthesis. This subject was thoroughly studied by McLaughlin and colleagues [137] who microinjected micelles of long-chain BODIPY-labeled PI(4,5)P<sub>2</sub> into fibroblasts and epithelial cells and measured the diffusion coefficient of the lipid in the inner leaflet of the plasma membrane using confocal imaging and fluorescence correlation spectroscopy. The averaged  $D$  value of PI(4,5)P<sub>2</sub> in the examined cells was  $0.8 \pm 0.2 \mu\text{m}^2/\text{s}$  in comparison to  $1.5 \pm 0.9 \mu\text{m}^2/\text{s}$  found for rhodamine-phosphatidylethanolamine (25°C). The diffusion coefficient of the fluorescent PI(4,5)P<sub>2</sub> was significantly higher when the lipid was incorporated into the outer leaflet of the plasma membrane of various cells or into giant unilamellar vesicles. The  $D$  values of PI(4,5)P<sub>2</sub> in those membranes reached  $2 \pm 1.3 \mu\text{m}^2/\text{s}$  and  $3.7 \pm 0.8 \mu\text{m}^2/\text{s}$ , respectively, with the latter value characteristic also of other lipids in model membranes [137–139]. The estimated values of the diffusion coefficient of PI(4,5)P<sub>2</sub> in the outer leaflet was in agreement with earlier FRAP (fluorescence recovery after photobleaching) measurements for BODIPY-labeled PI(4,5)P<sub>2</sub> incorporated into the outer leaflet of the plasma membrane of N1E-155 neuroblastoma cells [140]. The over twofold lower  $D$  value of PI(4,5)P<sub>2</sub> in the cytoplasmic than in the extracellular leaflet of the plasma membrane suggests that the lateral diffusion of the lipid in the inner leaflet is indeed restricted.

Notably, the diffusion coefficient of PI(3,4,5)P<sub>3</sub> estimated at  $0.5 \mu\text{m}^2/\text{s}$  by analysis of translocations of the PH



domain of Akt kinase, a molecular sensor of PI(3,4,5)P<sub>3</sub> expressed in NIH 3T3 fibroblasts [141], is in approximate agreement with the  $D$  of PI(4,5)P<sub>2</sub> found by McLaughlin's group [137]. When combined with the short lifetime of PI(3,4,5)P<sub>3</sub> (<1 min) this rate of PI(3,4,5)P<sub>3</sub> mobility allows a polar gradient of the lipid to be formed in moving cells. The gradient is generated by localized synthesis of PI(3,4,5)P<sub>3</sub> at the cell front coupled with its degradation toward the rear and enables efficient cell motility. Therefore, accumulation of PI(4,5)P<sub>2</sub> at particular plasma membrane locations, like forming phagosomes and ruffles (1–10  $\mu\text{m}$  in length) is also conceivable [137, 142], although contradictory opinions should be noted [140].

Some studies indicate even lower lateral mobility of PI(4,5)P<sub>2</sub> [143, 144]. It was shown that long-chain NBD-labeled PI(4,5)P<sub>2</sub> mixed with a cationic shuttle and delivered to the plasma membrane of mouse atrial myocytes through a micropipette attached to the cells failed to leave the patch of the membrane surrounded by the pipette walls. Accompanying FRAP measurements confirmed that PI(4,5)P<sub>2</sub> moved extremely slowly in the plane of the myocyte plasma membrane with the  $D$  calculated at 0.00039  $\mu\text{m}^2/\text{s}$  [143]. This is 10<sup>4</sup> time less than the  $D$  values found in the other studies discussed above. A similar severe restriction of lateral mobility of NBD-labeled PI(4,5)P<sub>2</sub> was found in the plasma membrane of HEK293 cells [144]. The confinement of PI(4,5)P<sub>2</sub> was abrogated by depolymerization of actin pointing to the cytoskeleton as a factor responsible for the low mobility of the lipid. On the other hand, NBD-PI and NBD-PI(4)P rapidly crossed the boundaries of micropipettes attached to the cell surface [143, 144]. Notably, the same approach has revealed that in CHO cells PI(4,5)P<sub>2</sub> is as mobile as PI(4)P [144] leaving open the question as to how common is the extremely slow diffusion of PI(4,5)P<sub>2</sub> in cells.

Several mechanisms can limit the lateral mobility of PI(4,5)P<sub>2</sub> in the plasma membrane [145]. One can envisage the existence of boundaries or "fences" imposed by the submembrane cytoskeleton and strong bending of the plasma membrane, which could hinder PI(4,5)P<sub>2</sub> diffusion, for example, at the neck of the phagosome. Recently, confinement of PI(4,5)P<sub>2</sub> within so-called lipid rafts or its sequestration by proteins due to electrostatic interactions are being considered, as discussed below.

Lipid rafts and raft-related caveolae are plasma membrane microdomains enriched in sphingolipids and cholesterol revealed in the 1990s [146–150]. Since this discovery, several attempts have been undertaken to examine whether PI(4,5)P<sub>2</sub> can be accumulated within these membrane domains. Rafts/caveolae-derived membranes are resistant to detergent solubilization, and during ultracentrifugation of detergent cell lysates over density gradients they float to fractions of low density. After fractionation of

lysates derived from cells depleted of cholesterol, rafts/caveolae-residing lipids and proteins shift to medium- and high-density fractions reflecting disintegration of the microdomains [151–154]. Based on such biochemical analysis, accumulation of PI(4,5)P<sub>2</sub> has been postulated in rafts isolated from epithelial Madin-Darby kidney cells (MDCK), epidermal A431 cells, and neuroblastoma cells [155–159]. Those early biochemical data indicated also that rafts harbored enzymes governing PI(4)P and PI(4,5)P<sub>2</sub> synthesis, hydrolysis, and dephosphorylation [155, 158, 160]. Accumulation of PI(4,5)P<sub>2</sub> and production of PI(3,4,5)P<sub>3</sub> was also implicated to take place in platelet rafts [161]. However, fractionation of detergent cell lysates may lead to an enrichment of *bona-fide* raft-originating membranes in non-raft proteins and lipids [162–164]. Moreover, the dependence of the PI(4,5)P<sub>2</sub> distribution and activity on cholesterol level in the plasma membrane does not necessarily prove an association of PI(4,5)P<sub>2</sub> with rafts [165, 166]. Due to these limitations, in another approach the PH domain of PLC $\delta_1$  was used as a probe to localize PI(4,5)P<sub>2</sub> in frozen ultrasections of fibroblasts and HEK293 cells by immunoelectron microscopy and for *in vivo* analysis of PI(4,5)P<sub>2</sub> clustering by the FRET technique [71, 166]. No indications of PI(4,5)P<sub>2</sub> clusters have been found in those studies. This is in agreement with calculations based on the diffusion coefficient value of PI(4,5)P<sub>2</sub>, suggesting that PI(4,5)P<sub>2</sub> patches of nanometer scale should rapidly dissipate (within milliseconds according to [142]; see also [140, 167]). One should bear in mind, however, that the PI(4,5)P<sub>2</sub>-binding probes can have a limited access to PI(4,5)P<sub>2</sub> already preoccupied by other proteins at steady state, leaving the problem of PI(4,5)P<sub>2</sub> distribution in the plasma membrane unsolved.

Despite the technical difficulties of the raft studies, a line of data indicates that during activation of a distinct class of plasma membrane receptors named immunoreceptors rafts can contribute to PI(4,5)P<sub>2</sub> synthesis and clustering. All these receptors have a tyrosine-based activation motif whose phosphorylation is catalyzed by kinases of the Src family known to reside in rafts. Upon activation, the immunoreceptors merge with the rafts, which enables their phosphorylation and triggers signaling cascades. Concomitantly, the rafts merge into spatially organized signaling platforms accommodating proteins and lipids of the cascades [168–171]. Upon activation of B cell antigen receptor, PIP5KI has been found to become recruited to plasma membrane rafts by Bruton's tyrosine kinase (BTK) catalyzing local synthesis of PI(4,5)P<sub>2</sub>, which could then be used by PI3K and PLC $\gamma$  to produce PI(3,4,5)P<sub>3</sub>, DAG, and IP<sub>3</sub> [172]. During T cell antigen receptor stimulation, rafts assemble into a structure known as the immunological synapse at the T cell/antigen-presenting cell contact site. The integrity of the immunological synapse is maintained



through anchoring to the underlying actin cytoskeleton, possibly via PI(4,5)P<sub>2</sub>-binding proteins. Among these, vinculin and talin were identified by mass spectroscopic analysis in raft fractions isolated from T cells [173]. Accordingly, clusters of PI(4,5)P<sub>2</sub> were visualized in vivo in T cells transfected with the PH domain of PLCδ<sub>1</sub>, following induction of raft coalescence by cross-linking of GM1 ganglioside. PI(3,4,5)P<sub>3</sub> and DAG were also found in the GM1-enriched domains, indicating that rafts can serve as sites of PI(4,5)P<sub>2</sub> phosphorylation and hydrolysis [174]. Activation of another immunoreceptor, Fcγ receptor II involved in phagocytosis, induces an enrichment of raft fractions in PIP5K1α and PI(4,5)P<sub>2</sub>. Further ultrastructural studies performed on sheets of plasma membrane obtained by mechanical cleavage of cells revealed that PI(4,5)P<sub>2</sub> accumulated at the margins of raft conglomerates [43]. Such structures form during Fcγ receptor II activation and accommodate the receptor and proteins of its signaling cascade [175, 176]. At these particular locations, PI(4,5)P<sub>2</sub> may function in tethering the actin cytoskeleton to the plasma membrane (as proposed for T cell receptor) and govern internalization of particles. Taken together, the data suggest that PI(4,5)P<sub>2</sub> can accumulate at margins of raft conglomerates whose formation accompanies activation of immunoreceptors.

It has also been hypothesized that the spatial confinement of PI(4,5)P<sub>2</sub> in the plasma membrane can result from the lateral sequestration of the lipid by proteins. Indeed, a significant fraction of the lipid is bound to proteins since PI(4,5)P<sub>2</sub> controls the adhesion between the plasma membrane and the submembrane cytoskeleton [177]. This finding is in agreement with the results of studies on lipid composition of microvesicles released from human erythrocytes [178]. The microvesicles were depleted by half in PI(4,5)P<sub>2</sub> and PI(4)P despite having a composition of the major phospholipids very similar to that of native erythrocyte membrane. Based on those data it was proposed that about 50% of PI(4,5)P<sub>2</sub> in the erythrocyte membrane was bound to the integral membrane proteins glycophorin and proteins of the spectrin-based membrane skeleton [178]. Such interactions could slow the diffusion of PI(4,5)P<sub>2</sub> into budding microvesicles. Subsequent measurements of the diffusion of PI(4,5)P<sub>2</sub> in the plasma membrane of Rat1 cells indicated that the erythrocyte results were representative of other cells as well [137]. The diffusion coefficient of long-chain BODIPY-labeled PI(4,5)P<sub>2</sub> in the inner leaflet of the plasma membrane ( $0.9 \pm 0.2 \mu\text{m}^2/\text{s}$ ) was 2.8-fold lower than the  $D$  value of PI(4,5)P<sub>2</sub> in blebs formed by the cells ( $2.5 \pm 0.8 \mu\text{m}^2/\text{s}$ ) and presumably lacking most of the cytoskeleton and integral membrane proteins interacting with the cytoskeleton. The authors interpreted those results to mean that two-thirds of PI(4,5)P<sub>2</sub> in Rat1 plasma membrane were bound reversibly to the cytoskeleton or

other proteins which, in turn, hindered the lateral diffusion of the lipid [137]. Further theoretical calculations support this hypothesis. A hypothetical spherical cell 10 μm in diameter contains about 30,000 PI(4,5)P<sub>2</sub>/μm<sup>2</sup> of the plasma membrane, accounting for 30 μM PI(4,5)P<sub>2</sub> concentration (assuming PI(4,5)P<sub>2</sub> is dispersed uniformly in the interior of the cells) [137, 142]. Out of this, 20 μM PI(4,5)P<sub>2</sub> is sequestered while 10 μM diffuses freely, as indicated by the diffusion measurements [137]. Accordingly, a predominant fraction of the PH domain of PLCδ<sub>1</sub> expressed in cells is bound to the plasma membrane ( $K_D \sim 1.2 \mu\text{M}$ ) while the PH domain of pleckstrin ( $K_D \sim 30 \mu\text{M}$ ) remains in the cytoplasm, suggesting that the concentration of free PI(4,5)P<sub>2</sub> is in the range between 2 and 30 μM [137, 142].

Ample data indicate that PI(4,5)P<sub>2</sub> can be sequestered by a group of integral and peripheral membrane proteins that contain stretches of positively charged amino acids. Thus far, the ability to sequester and cluster PI(4,5)P<sub>2</sub> has been ascribed to a ubiquitously expressed protein named myristoylated alanine-rich C kinase substrate (MARCKS). The highly conserved effector domain of MARCKS contains three serine residues whose phosphorylation is catalyzed by protein kinase C; the domain also contains sites of calmodulin and actin binding. MARCKS binds to actin filaments and cross-links them, and this cross-linking activity is disrupted by both phosphorylation of MARCKS and by Ca<sup>2+</sup>-loaded calmodulin. Most importantly, the effector domain of MARCKS also contains 15 basic amino acid residues that can bind three PI(4,5)P<sub>2</sub> molecules inducing the lipid clustering in the plane of the membrane, as indicated by biochemical and biophysical studies. Physiological levels of MARCKS (10 μM in neuronal tissue) correspond to the estimated cellular PI(4,5)P<sub>2</sub> concentration and, thus, MARCKS can sequester a significant pool of the lipid [142]. The sequestration of PI(4,5)P<sub>2</sub> by MARCKS can be reversed by interaction of the effector domain with Ca<sup>2+</sup>-loaded calmodulin or by phosphorylation of the serine residues of the domain by protein kinase C. Under these conditions, MARCKS detaches from the plasma membrane despite myristoylation of its N-terminus. MARCKS-bound PI(4,5)P<sub>2</sub> is less susceptible to hydrolysis by PLCδ<sub>1</sub>, however, dissociation of the protein from the plasma membrane exposes PI(4,5)P<sub>2</sub> for hydrolysis and, possibly, for interactions with other effector proteins [179, 180]. Some transmembrane receptors, like EGF receptor and membrane-binding proteins structurally resembling MARCKS, have also been proposed to reversibly sequester PI(4,5)P<sub>2</sub> in the plasma membrane in a calcium-dependent manner [180]. Among the latter, neuronal growth-associated protein 43 (GAP43) is of special interest. Unlike MARCKS, GAP43 undergoes dual palmitoylation at the N-terminus, which predisposes the protein for anchoring

in the inner leaflet of rafts. Studies performed on large unilamellar vesicles composed of lipids mimicking the chemical make-up of rafts indicate that palmitoylated GAP43 can facilitate association of PI(4,5)P<sub>2</sub> with rafts. Considering that PI(4,5)P<sub>2</sub> usually has an unsaturated fatty acyl residue at the *n*-2 position of the glycerol backbone, its partition to lipid rafts is unlikely. However, after binding to palmitoylated GAP43, the free energy of transfer of PI(4,5)P<sub>2</sub> from non-raft to raft environment is lowered [181]. GAP43 can act as a reversible PI(4,5)P<sub>2</sub> sink in growth cones on neurons responding to changes in the level of Ca<sup>2+</sup>-loaded calmodulin. In accordance with the in vitro studies, an involvement of MARCKS and GAP43 in the formation of PI(4,5)P<sub>2</sub> clusters engaged in cytoskeleton rearrangement at the leading edge of motile cells was indicated by Caroni's group [182, 183]. Hence, distinct proteins can attract and concentrate PI(4,5)P<sub>2</sub> in clusters in the plane of the plasma membrane to release the lipid reservoir in response to the rise of free Ca<sup>2+</sup> concentration.

## Conclusions

Stimulation of cells with a variety of agents triggers signaling cascades that involve PI(4,5)P<sub>2</sub>. PI(4,5)P<sub>2</sub> contributes to these processes by being converted into second messengers or by controlling the activity of PI(4,5)P<sub>2</sub>-binding proteins. The accumulation of PI(4,5)P<sub>2</sub> at sites of cell stimulation can ensue from two general mechanisms: induction of local PI(4,5)P<sub>2</sub> synthesis by PIP5KI and revealing of PI(4,5)P<sub>2</sub> clusters pre-existing in the plasma membrane. PIP5KI can be anchored at the plasma membrane via electrostatic interactions with PI(4)P and PI(4,5)P<sub>2</sub> being subject to substrate and product inhibition until the appearance of activating factors like PA. PIP5KI can be also recruited and activated at the plasma membrane by proteins including Rac1, talin, the AP-2 complex, and BTK. These interactions allow selective engagement of PIP5KI isoforms. Thus far, the molecular basis underlying such selectivity is only known for the interaction of PIP5KI $\gamma$  with talin and the AP-2 complex. In the case of activation of receptors that utilize assemblies of rafts as "signaling platforms", the synthesis of PI(4,5)P<sub>2</sub> can be confined to these membrane structures. Finally, proteins like MARCKS can sequester PI(4,5)P<sub>2</sub> into clusters in the plane of the plasma membrane of resting cells and release the lipid allowing its interaction with effector proteins in response to changes of free Ca<sup>2+</sup> level accompanying cell activation.

**Acknowledgments** I gratefully thank Prof. Andrzej Sobota, Nencki Institute of Experimental Biology, Warsaw, for support and excellent

discussion. I also thank Prof. Jan Fronk, Warsaw University, for valuable comments on the manuscript.

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