

## REVIEW ARTICLE

# Supervised membrane swimming: small G-protein lifeguards regulate PIPK signalling and monitor intracellular PtdIns(4,5) $P_2$ pools

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Regulation of PIPK (phosphatidylinositol phosphate kinase) and PtdIns(4,5) $P_2$  signalling by small G-proteins and their effectors is key to many biological functions. Through selective recruitment and activation of different PIPK isoforms, small G-proteins such as Rho, Rac and Cdc42 modulate actin dynamics and cytoskeleton-dependent cellular events in response to extracellular signalling. These activities affect a number of processes, including endocytosis, bacterial penetration into host cells and cytolytic granule-mediated targeted cell killing. Small G-proteins and their modulators are also regulated by phosphoinositides through translocation and conformational changes. Arf family small G-proteins act at multiple sites as regulators of membrane trafficking and actin cytoskeletal remodelling, and regulate a feedback loop comprising phospholipase D, phosphatidic acid, PIPKs and PtdIns(4,5) $P_2$ , contributing to enhancement of PtdIns(4,5) $P_2$ -mediated cellular events and receptor signalling. Na<sup>+</sup>, Kir (in-

wardly rectifying K<sup>+</sup>), Ca<sup>2+</sup> and TRP (transient receptor potential) ion channels are regulated by small G-proteins and membrane pools of PtdIns(4,5) $P_2$ . Yeast phosphatidylinositol 4-phosphate 5-kinases Mss4 and Its3 are involved in resistance against disturbance of sphingolipid biosynthesis and maintenance of cell integrity through the synthesis of PtdIns(4,5) $P_2$  and downstream signalling through the Rom2/Rho2 and Rgf1/Rho pathways. Here, we review models for regulated intracellular targeting of PIPKs by small G-proteins and other modulators in response to extracellular signalling. We also describe the spatial and temporal cross-regulation of PIPKs and small G-proteins that is critical for a number of cellular functions.

**Key words:** ADP-ribosylation factor 1/6 (Arf1/Arf6), phosphatidylinositol phosphate kinase (PIPK), PtdIns(4,5) $P_2$ , phosphatidic acid, Rho/Rac/Cdc42, small G-protein.

## INTRODUCTION

PtdIns(4,5) $P_2$  is a major cellular phosphoinositide that exists in discrete and compartmentalized pools in membranes and vesicles. In membranes, PtdIns(4,5) $P_2$  is a precursor to second messengers and is a key signalling and targeting molecule [1]. PtdIns(4,5) $P_2$  is involved in regulation of a number of cellular activities, including cell motility [2–4], gating of ion channels [5,6], exocytosis [7–9] and vesicular trafficking [10,11].

There are two subfamilies of PIPKs (phosphatidylinositol phosphate kinases), types I and II, that generate PtdIns(4,5) $P_2$  from distinct substrate pools. PIPKI (type I PIPKs) use PtdIns4P as a substrate [12], whereas PIPKII (type II PIPKs) use PtdIns5P [12,13]. PIPKI also use PtdIns3P as a substrate, with lower *in vitro* efficacy, and, interestingly, generate PtdIns(3,4,5) $P_3$  by a concerted double phosphorylation [13]. The role of PtdIns3P as substrate for generation of PtdIns(3,4,5) $P_3$  by PIPKI *in vivo* has not yet been defined. The PIPKI and PIPKII subfamilies include three major isoforms,  $\alpha$ ,  $\beta$  and  $\gamma$  (the nomenclature for PIPKI $\alpha$  and PIPKI $\beta$  is reversed in mice). The PIPKI isoforms are also alternatively spliced [14–18]. These PIPK isotypes synthesize

PtdIns(4,5) $P_2$  in diverse cellular compartments and are key regulators of PtdIns(4,5) $P_2$ -mediated cellular signalling events.

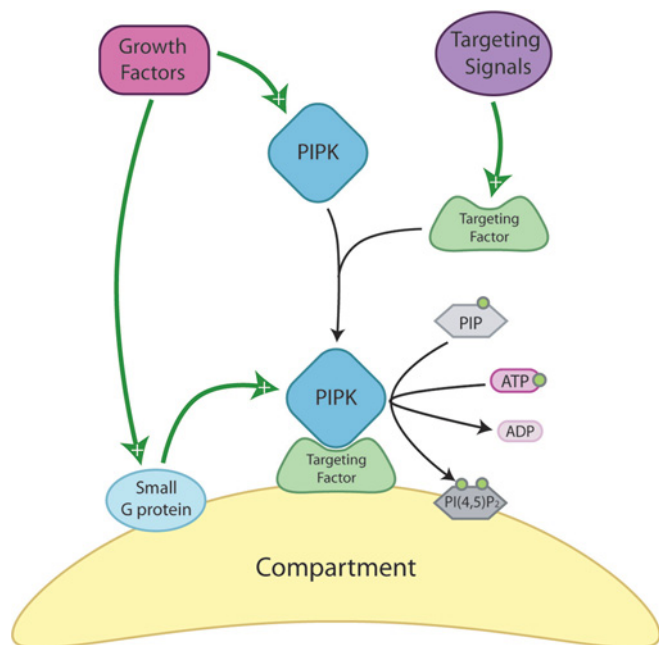
The activation loops of PIPKI and PIPKII differ in a key amino acid residue, a glutamate (PIPKI) or an alanine (PIPKII), that defines the substrate specificity for PtdIns4P compared with PtdIns5P and determines the hydroxy residue on the inositol ring that is phosphorylated [19]. The substrate specificity conferred by the activation loop, as well as differential localization of distinct PIPK isoforms, allows for PtdIns(4,5) $P_2$ -mediated regulation of non-redundant biochemical pathways by specific PIPKs. In this model, targeting of PIPKs by regulated protein–protein interactions controls PtdIns(4,5) $P_2$  generation in a spatial and temporal fashion [19]. There is evidence that specific spatial and temporal generation of PtdIns(4,5) $P_2$  is key for regulation of specific cellular events.

Although the regulation of PIPK spatial targeting is only beginning to be defined, it is understood that stereospecific interactions between a PIPK and its substrate are important. When the specificity-conferring activation loops are swapped between PIPKI and PIPKII, a change of the PIPK intracellular targeting of the overexpressed enzyme that corresponds to that of the PIPK that

Abbreviations used: Arf, ADP-ribosylation factor; Arp2/3, actin-related protein 2/3; DH, Dbl homology; DHR, discs large homology region; EGF, epidermal growth factor; ENaC, epithelial Na<sup>+</sup> channel; ERM, ezrin/radixin/meosin; GAP, GTPase-activating protein; GDI, GDP-dissociation inhibitor; GEF, guanine nucleotide-exchange factor; GIRK, G-protein-activated inwardly rectifying K<sup>+</sup>; GPCR, G-protein-coupled receptor; HEK-293, human embryonic kidney; K<sub>ATP</sub>, ATP-sensitive K<sup>+</sup> channel; Kir, inwardly rectifying K<sup>+</sup> channel; Kv, voltage-gated K<sup>+</sup> channel; LPA, lysophosphatidic acid; PA, phosphatidic acid; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI4K, phosphatidylinositol 4-kinase; PI4P5K, phosphatidylinositol 4-phosphate 5-kinase; PIPK, phosphatidylinositol phosphate kinase; PIPKI, type I PIPK; PIPKII, type II PIPK; PLC, phospholipase C; PLD, phospholipase D; PTX, pertussis toxin; ROCK, Rho kinase; (N)-WASP, (neuronal) Wiskott–Aldrich syndrome protein.

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**Figure 1** Spatial control of PIPK signalling and local PtdIns(4,5) $P_2$  levels through small G-proteins and compartment-specific targeting factors

In response to extracellular signals, PIPKs are targeted to specific cellular localizations, most often membranous structures, by specific targeting molecules where interactions with small G-proteins contribute to increased local PtdIns(4,5) $P_2$  [PI(4,5) $P_2$ ] availability downstream. PIP, PtdIns $P$ .

donated the loop is observed [19]. Clearly, there are additional requirements for PIPK targeting, as PIPKI isoforms with virtually identical specificity loops have dramatically different intracellular locations [20]. In addition to substrates, other targeting factors determine the intracellular location of PIPKs, and some of these factors are likely to be small G-proteins as well as their regulators, which are dynamically regulated by upstream signalling pathways (Figure 1).

Individual PIPK isoforms have been identified as downstream effectors of small G-protein signalling that produce PtdIns(4,5) $P_2$  at specific cellular sites [20,21]. Functional coupling of small G-proteins and PIPKs regulates the generation of PtdIns(4,5) $P_2$  critical to regulation of cytoskeletal-dependent functions, including platelet activation and membrane ruffle formation required for cell migration [22–24]. The synthesis of PtdIns(4,5) $P_2$  by PIPKs has the potential to be regulated by a feedback loop involving small G-proteins, PLD (phospholipase D) and its reaction product, PA (phosphatidic acid) [25–29].

Emerging evidence supports the concept that PtdIns(4,5) $P_2$  is generated in highly specific pools or by channelling PtdIns(4,5) $P_2$  directly to effectors [30]. In this model, the interaction of PIPKI isoforms with components for targeting to specific cellular compartments modulates highly specific PtdIns(4,5) $P_2$  synthesis and channelling to effectors. For example, PIPKI $\gamma$ 661 targeting to focal adhesions by an association with talin results in spatially generated PtdIns(4,5) $P_2$  that strengthens the binding of talin to  $\beta$ -integrin [30]. Understanding how PIPKs are regulated by interactions with their binding partners, as well as the mechanisms by which their product, PtdIns(4,5) $P_2$ , exerts its effects on cellular processes is critical to understanding the co-ordinated assembly of cellular signalling complexes and pathways. In this review, we describe and critically examine significant findings in the field surrounding regulation of PIPKs by small G-proteins and their

associated regulators, as well as the roles of the PIPKs and PtdIns(4,5) $P_2$  in co-ordinated stimuli-driven signalling.

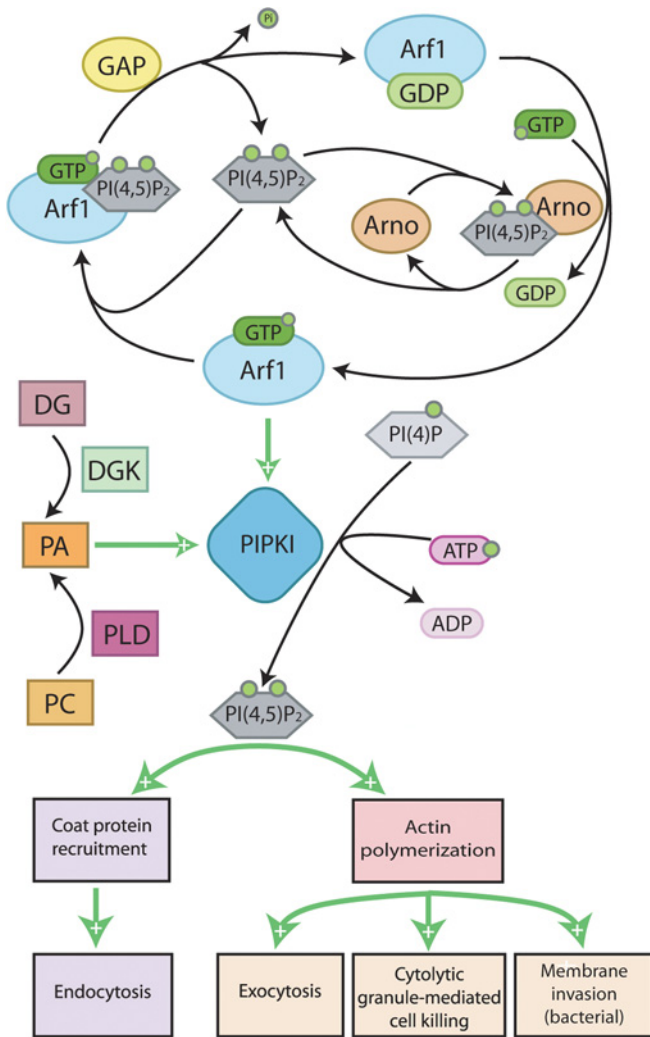
## SMALL G-PROTEINS

The superfamily of small G-proteins can be classified into five subfamilies: Ras, Rho, Arf (ADP-ribosylation factor), Rab and Ran. Commonly referred to as monomeric G-proteins, members of this superfamily share monomeric structure and contain GTPase domains ranging in size from 20 to 40 kDa. Monomeric G-proteins act as molecular switches to spatially and temporally modulate a variety of cellular functions through diverse signalling networks, including activities or targeting of PIPKs [31]. Small G-proteins are shuttled between membranes and the cytosol, and their ability to modulate downstream signalling processes depends on the finely tuned regulation of their activation and inhibition through interactions with regulators. Through regulation of downstream signalling processes, small G-proteins co-ordinate activities of effector molecules to elicit specific physiological responses to stimuli.

### Regulation of the Rho and Arf families of small G-proteins

Most small G-proteins can be found in the cytosol or on membranes and are modified by C-terminal lipid conjugation, which is essential for binding to membranes, interactions with regulators and signalling to downstream effectors [32,33]. Biochemical activities of the Rho family of small G-proteins are subject to modulation by GEFs (guanine nucleotide-exchange factors) and GAPs (GTPase-activating proteins), as well as by GDIs (GDP-dissociation inhibitors) [34–37]. Through interactions with these factors, Rho family members are regulated by catalytic and membrane association cycling [31,37]. Rho GEFs contain PH (pleckstrin homology) and DHR (discs large homology region) domains, which are important for regulation of their activities through interactions with factors including phosphoinositides [34,38]. The Dbl family of GEFs, which contain DH (Dbl homology)/PH tandem repeats, have been established as the major canonical Rho protein GEFs [39]. One member of this family, Tiam1, is recruited to membranes and is activated by PtdIns(3,4,5) $P_3$  binding to its PH domain [40]. More recently, the CZH family of Rho GEFs, lacking tandem DH/PH domains, has been characterized [34]. One member of this family, DOCK180, binds to PtdIns(3,4,5) $P_3$  via its DHR-1 domain and couples signalling to GTP loading of Rac via its DHR-2 domain [41]. There is a feedback mechanism modulating signalling between Rho family small G-proteins and phosphoinositides. For example, Rac GEFs are stimulated by PtdIns(4,5) $P_2$  and PtdIns(3,4,5) $P_3$ , products of Rac activation of PIPKI and PI3K [39,40,42]. Activities of Rho family GAPs are regulated by protein phosphorylation, lipid binding and protein–protein interaction [36]. For instance, one member of the Rac-specific GAPs, chimaerin, is regulated *in vitro* by diacylglycerol and its analogues, phorbol esters, as well as by phosphatidylinositol lipids [36].

Arf protein cycling is also mediated by specific GEFs and GAPs, but is not subject to regulation by GDIs (Figure 2) [31]. Regulation of Arf proteins by GEFs and GAPs is modulated by interactions of GEFs, GAPs and Arf proteins themselves with PtdIns(4,5) $P_2$  [43–45]. For example, Arno is an Arf GEF whose activation of Arf1 depends on its binding to PtdIns(4,5) $P_2$  and subsequent translocation to the plasma membrane, as shown in Figure 2 [44,45]. Arf GAP activity depends on PtdIns(4,5) $P_2$  binding directly to Arf, also illustrated in Figure 2 [43]. Collectively, regulation of the Rho and Arf families of small G-proteins



**Figure 2** Arf protein signalling through PIPKs affects both cellular trafficking and motility

Arf1 and Arf6 signal through PIPKs to control intracellular trafficking and actin polymerization. A positive-feedback loop involving PtdIns(4,5) $P_2$  [PI(4,5) $P_2$ ], PLD and its product PA intensify Arf effects on PtdIns(4,5) $P_2$  levels. See the text for detailed pathway descriptions. DG, diacylglycerol; DGK, DG kinase; PC, phosphatidylcholine; PI(4)P, PtdIns4P.

by phosphoinositides occurs by two major mechanisms: alteration of activity and physical translocation.

## Rho

Members of the Rho family of small G-proteins are important for regulation of the formation of actin stress fibres and focal adhesion complexes [46]. The specific role of Rho in these processes is in actin filament reorganization, and, accordingly, cells treated with Rho inhibitors show significant disruption of stress fibres [46].

A wide variety of downstream effectors of Rho proteins have been identified, and their regulatory signalling mechanisms have been characterized. Several PIPKs have been identified as Rho protein interactors and effectors, with PIPKI specifically serving a critical role in Rho-mediated signalling. GTP[S] (guanosine 5'-[ $\gamma$ -thio]triphosphate) stimulation of PIPKI activity in C3H 10T $_{1/2}$  mouse fibroblast and HEK-293 (human embryonic kidney) cell lysates is blocked by the Rho inhibitor *Clostridium botulinum* C3 exoenzyme [47,48]. Addition of GTP-activated recombinant RhoA increases PIPKI activity in the same system, providing

additional evidence to support the hypothesis that Rho modulates PIPKI activity [47,48]. Furthermore, although microinjection of C3 exoenzyme alone into cells reduces PtdIns(4,5) $P_2$ -mediated PDGF- (platelet-derived growth factor) and thrombin-induced  $Ca^{2+}$  mobilization, addition of constitutively active Rho reverses this defect, indicating that Rho modulates PtdIns(4,5) $P_2$  synthesis and modulates PIPK activity [47]. Consistent with this evidence, inactivation of Rho GTPases by *Clostridium difficile* toxin B and C3 exoenzyme reduces total cellular PtdIns(4,5) $P_2$  levels by 40–50% in HEK-293 cells [49]. The reduction in total cellular PtdIns(4,5) $P_2$  supply results in an inhibition of receptor-mediated inositol phosphate formation by PLC (phospholipase C) and PtdIns(4,5) $P_2$ -sensitive PLD [49,50].

Ren et al. [51] established that PIPKI physically associates with Rho independently of Rho being in its active, GTP-bound, form. Although direct interaction was not verified in these experiments, only the GTP-bound form of Rho was found to have the capacity to activate PIPKI, suggesting a model in which Rho-GTP activates a downstream effector that is also required for the activation of PIPKI [51]. This model was supported by identification of ROCK (Rho kinase) as a downstream effector of Rho-GTP that regulates the controlled synthesis of PtdIns(4,5) $P_2$  by PIPKI in HEK-293 cells [48,52]. The existence of a RhoA/ROCK/PIPKI signalling pathway is supported further by the observation that PIPKI $\alpha$ -mediated stress-fibre formation was blocked and PtdIns(4,5) $P_2$  synthesis was inhibited by the ROCK inhibitor Y-27632 [(+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide] in CV-1 monkey kidney fibroblast cells [53] and human lens epithelial cells [54]. How these events coordinate with the modulation of focal adhesions by the PIPKI $\gamma$  remains to be defined [30,55].

PIPKI has also been indicated as a downstream effector of the RhoA–ROCK signalling unit during neurite retraction; overproduction of kinase-deficient mutant PIPKI $\alpha$  blocks both LPA (lysophosphatidic acid)-induced and ROCK-induced neurite retraction in N1E-115 neuroblastoma cells, while Y-27632, a ROCK-specific inhibitor, is unable to block the overexpressed PIPKI $\alpha$ -induced neurite remodelling effects. This suggests that PIPKI $\alpha$  plays a role as a downstream effector of the RhoA/ROCK pathway and serves as the mechanism to couple LPA signalling to neurite retraction [56]. More recently, Yang et al. [22] showed that platelet PIPKI is regulated by Rho and ROCK. In their system, Rho-GTP and ROCK-GTP activate PIPKI translocation to the actin cytoskeleton, making it available to initiate actin assembly [22].

In contrast with the above model, evidence exists that PIPKs are direct effectors of Rho signalling independently of ROCK [57]. ERM (ezrin/radixin/moesin) proteins are normally activated and recruited to the plasma membrane, where they serve to cross-link actin filaments to the plasma membrane [58]. Experimental evidence indicates that ERM protein phosphorylation and the resultant formation of microvilli are up-regulated by constitutively active Rho, but not by ROCK. In addition, ERM phosphorylation induced by introduction of constitutively active RhoA was found to be protected against suppression by the Y-27632, a ROCK-specific inhibitor. PIPKI $\alpha$  expression alone, however, is sufficient for induction of ERM phosphorylation and microvilli formation in NIH 3T3 cells [57]. These results indicate that PIPKI is a direct downstream effector of Rho independently of ROCK signalling.

## Rac

Rac has a number of established roles in cell signalling, including regulation of lamellipodium formation, membrane ruffling and

actin polymerization [59,60]. Similarly to Rho, several lines of evidence demonstrate a link between Rac and PIPKI [42,61–64]. Rac has also been shown to interact with PIPKI regardless of whether it is bound to GDP or to GTP. The lack of GTP dependence in Rac binding to PIPKI is consistent with the location of the interaction site between Rac and PIPKI, which is separated from the Rac effector domain [31,42,61]. This suggests the possibility that activation of Rac by a GDP-into-GTP conversion results in a conformational change in Rac that allows its effector domain to interact with, and more efficiently activate, already bound PIPKI. The relevance of this interaction was demonstrated in a permeabilized platelet system, where the addition of a constitutively active mutant Rac was shown to activate PtdIns(4,5) $P_2$ -mediated F-actin (filamentous actin) uncapping and actin polymerization [65]. Building on these findings [65], overexpression of PIPKI $\alpha$  and overexpression of constitutively active Rac had similar effects on actin filament uncapping and assembly [62]. Together these results provide salient evidence that PIPKI $\alpha$  is an important mediator of Rac-dependent actin assembly. However, overexpression of the PIPKs can be misleading, as this may saturate normal specific targeting mechanisms.

Different PIPKI isoforms have been found to localize to a number of distinct subcellular locations. Endogenous PIPKI $\alpha$  localizes to the nucleus and membrane ruffles [20,66], whereas PIPKI $\beta$  is found primarily at cytosolic perinuclear vesicular structures, and one splice variant of PIPKI $\gamma$  is targeted to focal adhesions [66]. Despite differences in localization, all three PIPKI isoforms physically associate with both Rho and Rac1 [64]. This direct association is guanine-nucleotide-independent and results in increased cellular PtdIns(4,5) $P_2$  levels, and presumably PIPK activity, in HEK-293 cells [64]. Additionally, indirect regulation of PIPKI activity by Rac has also been proposed. Both Rac and Rho have been shown to mediate thrombin receptor activation and thus translocation and activation of PIPKI $\alpha$ . This mediated translocation is dependent on the small G-protein's GTP-bound state. Experiments with dominant-negative and constitutively active mutants of Rac and Rho indicate that the regulation of PIPKI by Rac is indirect in nature and requires engagement of activated Rho. *In vivo*, this interaction results in membrane-translocated and activated PIPKI $\alpha$  becoming available to phosphorylate PtdIns4P to generate PtdIns(4,5) $P_2$  on the plasma membrane in response to Rac and Rho signalling [67].

### Cdc42

Cdc42 has been reported to regulate filopodia formation through localized actin polymerization and function as a sensor at the leading edge of motile cells [68,69]. It has been implicated in up-regulation of PtdIns(4,5) $P_2$  synthesis by all three PIPKI isoforms independently of direct physical interaction between Cdc42 and PIPKI [63,64]. Cdc42 influences actin polymerization through downstream effector, N-WASP (neuronal Wiskott–Aldrich syndrome protein), which is activated by increased local levels of PtdIns(4,5) $P_2$  resulting from Cdc42 activation of PIPKI. N-WASP then activates the Arp2/3 (actin-related protein 2/3) complex to initiate actin filament branching and elongation [70].

All members of the Rho family of small G-proteins increase local PtdIns(4,5) $P_2$  levels through up-regulation of PIPK activity, although only Rac and Rho associate directly with PIPKI isoforms. Local phosphoinositide levels, especially PtdIns(4,5) $P_2$  and PtdIns(3,4,5) $P_2$  in turn affect activity and localization of Rho family members and their effectors. This signalling loop is critically important for co-ordinated regulation of actin stress fibres and focal adhesion complexes.

### Arf

Mammalian cells contain six Arf small G-proteins that are categorized into three classes on the basis of primary structure [31,71]. Arf1 and Arf6 are the most extensively characterized members of the Arf family of small G-proteins. Multiple regulated roles for Arf1 and Arf6 have been identified in the regulation of membrane trafficking and actin structural remodelling [71–75]. Activity of the Arf family of GTP-binding proteins is generally mediated through interactions with effector proteins, as shown in Figure 2. All Arf proteins have been shown to be capable of activating PIPKs as well as PLD, implying that many of the Arf protein functions may be dependent on the products of PIPKI and PLD activation, namely PtdIns(4,5) $P_2$  and PA [23,76,77].

The primary localization of Arf1 in cells is at the Golgi membrane, where it reversibly associates via GDP–GTP cycling and functions in the recruitment of coat proteins for intracellular trafficking [74,78]. Arf1 has also been shown to be required for Golgi body structure and function. Interestingly, PtdIns(4,5) $P_2$  availability appears to be critical for the role of the Golgi compartment in membrane trafficking [79]. Current lines of evidence indicate that Arf1 stimulates PtdIns(4,5) $P_2$  synthesis by mediating the recruitment of PI4K $\beta$  (phosphatidylinositol 4-kinase  $\beta$ ) and PIPKI from the cytosol to the Golgi complex [80]. Jones et al. [79] reported that PIPKI isoforms directly interact with Arf1 and this interaction regulates PtdIns(4,5) $P_2$  synthesis in a guanine nucleotide-dependent manner, thus identifying PIPKI as a direct downstream effector of Arf1-GTP. This Arf1-mediated PIPKI activation has been shown to be stimulated by PA derived from PLD activation [81].

Arf6 is primarily associated with the plasma membrane, where it is involved in the formation of PtdIns(4,5) $P_2$  and modulation of the cortical actin cytoskeleton [73,78,82]. Arf6 is required for Rac attachment to plasma membrane ruffles, as well as changes in actin structure at the plasma membrane through Rac translocation and activation [83]. Expression of the Arf6-specific GEF EFA6 in cells generates membrane protrusions and ruffles through activation of Arf6, a Rac-dependent activity [84,85].

Arf6 transforms membrane topography by altering membrane lipid composition, and it has been shown to co-localize with, bind to, and activate PIPKI at the plasma membrane [7,23,86]. Arf-GTP binds to and activates PLD, which cleaves phosphatidylcholine to generate PA [76,77], whereas PtdIns(4,5) $P_2$  stimulates PLD activity and PA stimulates PIPKI activity [25–29]. These combined regulatory loops result in enhanced production of PtdIns(4,5) $P_2$  and PA, lipid messengers that mediate Arf's role in recruiting additional proteins to compartments involved in membrane trafficking and sites of cortical actin cytoskeleton reorganization [72,73,75,82,87–89]. In addition, these lipid messengers may also alter membrane structure and curvature, supplying a partial explanation for Arf6 regulation of membrane trafficking [10,73,82].

In terms of membrane trafficking, Arf6 has been shown to regulate PtdIns(4,5) $P_2$ -dependent exocytosis in MIN6 pancreatic cells [82] and PC12 neuroendocrine cells [7]. Furthermore, expression of a constitutively active Arf6 mutant in PC12 cells results in an accumulation of Arf6 mutant protein, PtdIns(4,5) $P_2$ , and PIPKI $\gamma$  on endosomal membranes and their corresponding depletion from the plasma membrane and inhibits  $Ca^{2+}$ -dependent exocytosis [7]. Arf6-mediated depletion of plasma membrane PtdIns(4,5) $P_2$  is thought to be due to the re-targeting of PIPKI $\gamma$ , a major PIPKI isoform in neuroendocrine cells, to endosomal compartments. This PIPKI $\gamma$  translocation and associated plasma membrane PtdIns(4,5) $P_2$  depletion dampens the PtdIns(4,5) $P_2$ -dependent  $Ca^{2+}$ -dependent exocytosis response. Direct evidence

of Arf6's regulation of PIPKI $\gamma$  comes from the observation that Arf6 physically associates with PIPKI $\gamma$  and is able to stimulate human growth hormone exocytosis in PC12 cells in a Ca<sup>2+</sup>-dependent manner [7]. Association of PIPKI $\gamma$  and Arf6 has been shown to depend on the phosphorylation status of PIPKI $\gamma$ , rather than the guanine nucleotide-binding status of Arf6; the association of PIPKI $\gamma$  with membrane-bound Arf6 may be important for targeting the enzyme to its membrane substrate, PtdIns4P, for spatial generation of PtdIns(4,5)P<sub>2</sub> necessary for further signalling.

Another line of evidence regarding Arf6 regulation of PIPKI $\gamma$  reported by Krauss et al. [86] found that GTP-bound Arf6 and PIPKI $\gamma$  co-localize upon transfection into COS7 kidney fibroblast cells. Arf6 is known to stimulate PtdIns(4,5)P<sub>2</sub> production on synaptic membranes, possibly via activation of PIPKI $\gamma$  and subsequent clathrin/AP-2 (activator protein 2) coated pit assembly [86]. In this manner, Arf6 is able to regulate endocytosis in synaptic membranes by localized synthesis of PtdIns(4,5)P<sub>2</sub> through activation of PIPKI $\gamma$ .

Honda et al. [23] have suggested that Arf6 activates PIPKI $\alpha$  directly in concert with PA in membrane ruffles. In an *in vitro* reconstitution assay containing purified Arf6, PIPKI $\alpha$  and PA, they demonstrated that PA and Arf6 synergistically up-regulate PIPKI $\alpha$  activity. In a subcellular localization experiment in HeLa cells, PIPKI $\alpha$  was found to translocate to membrane ruffles with Arf6 upon AIF<sub>4</sub><sup>-</sup> and EGF (epidermal growth factor) stimulation of G-proteins, although both proteins normally localize throughout the cytoplasm and plasma membrane. The observed translocation of PIPKI $\alpha$  could be completely inhibited by expression of a dominant-negative Arf6 mutant [23]. PA is a known activator of all PIPKI isoforms and, upon EGF stimulation, overexpressed PLD2, which produces PA, is translocated to the ruffling membrane where it co-localizes with PIPKI $\alpha$ . The PA produced by PLD in ruffles then functions with Arf6 as a co-activator of PIPKI $\alpha$  [23,92]. Additionally, Arf6 stimulates actin dynamics through activation of PIPKI $\alpha$  and regulates growth cone motility in the developing nervous system [90]. These results suggest that Arf6 is a physiological downstream activator of PIPKI $\alpha$  [23,90].

Another body of evidence supporting a functional link between Arf activation and PIPKI has recently been reported. Both *Chlamydia trachomatis* [91] and *Yersinia pseudotuberculosis* [92] bacteria have been shown to utilize the Arf6 GTPase-PIPKI signalling unit to penetrate host epithelial cells. The proposed mechanism is initiated by the recruitment to and activation of Arf6 and PIPKI at the site of bacterial entry into the host cell. There, the resultant locally produced PtdIns(4,5)P<sub>2</sub> causes extensive actin remodelling to allow for more efficient entry at the invasion site, highlighting further how activation of small G-proteins can affect localized PtdIns(4,5)P<sub>2</sub>, leading to spatially defined cellular changes [92].

Functional coupling of Arf6 and PIPKI has also been implicated in immune cell function [93]. In this capacity, Arf6 has been indicated to have a role in Fc $\gamma$ R1IIIA lymphocyte-mediated cytotoxicity. Specifically, aggregation of the Fc $\gamma$ R1IIIA receptor for the Fc portion of IgG is thought to induce Arf6 activation, which subsequently activates PIPKI. This is demonstrative of an Arf6-mediated targeting mechanism that regulates spatially generated *de novo* synthesis of PtdIns(4,5)P<sub>2</sub>. In natural killer cells, IgG receptor activation mediates signalling through the concerted effort of Arf-PIPKI signalling, generating a localized pool of PtdIns(4,5)P<sub>2</sub> necessary for marking and modifying membrane microdomain rafts for specific structural and functional modifications required for granule docking and fusion [10,93].

Two members of the Arf family of small G-proteins, Arf1 and Arf6, play important roles in both membrane trafficking and actin structural remodelling. A number of pathways in these processes

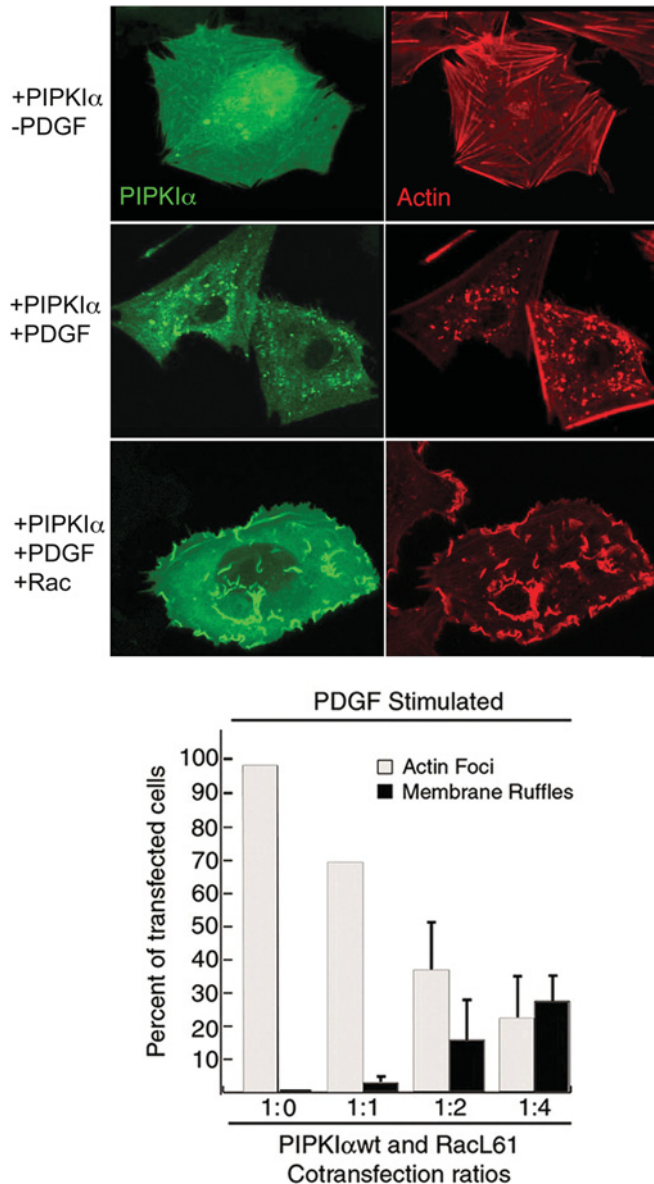
have been linked directly to PIPKs or to their products, most notably PtdIns(4,5)P<sub>2</sub>. Downstream signalling effects of Arf activation of PIPK activity include endocytosis, exocytosis, actin polymerization and membrane ruffle formation. How these events are spatially regulated and the identity of the upstream signals remains an important area of investigation.

### Spatial and temporal regulation of PIPKs by small G-proteins

There exists evidence indicating the presence of discrete compartmentalized cellular pools of PtdIns(4,5)P<sub>2</sub> [10,94]. These pools are functionally and spatially distinct and are influenced by differentially targeted PIPKI isoforms. For example, PIPKI $\gamma$ 635 is reported to be responsible for generation of PtdIns(4,5)P<sub>2</sub> pools that can be mobilized by GPCR (G-protein-coupled receptor)-linked Ca<sup>2+</sup> signalling to control activities such as histamine receptor activation [95]. mouse PIPKI $\beta$  and PIPKI $\gamma$  are responsible for generating PtdIns(4,5)P<sub>2</sub> pools for exocytosis of large dense core vesicles in chromaffin cells [8,9], and PIPKI $\gamma$ 661 targets to focal adhesion sites [30]. The targeting of different PIPK isoforms to various subcellular locations is regulated by small G-protein signalling. Precise spatial and temporal synthesis of PtdIns(4,5)P<sub>2</sub> is critical for modulation of various cellular responses such as platelet aggregation, endocytosis, exocytosis and cell migration events including lamellipodia formation, focal adhesions and membrane ruffling [1].

Early studies have shown that the addition of low concentrations of thrombin up-regulates PtdIns(4,5)P<sub>2</sub> synthesis [96] and increases PIPK activity associated with cytoskeletal functions [97]. Hinchliffe et al. [98] reported that the integrin-mediated signalling associated with platelet aggregation promotes translocation of PIPKII to the cytoskeleton where it regulates a cytoskeleton-associated pool of PtdIns(4,5)P<sub>2</sub>. Thrombin activation of PIPKI $\alpha$  is via Rac [62], and PIPKI $\alpha$  is targeted to specific sites in the plasma membrane where rapid actin assembly takes place in a Rac- or Rac/Rho-dependent manner [62,67]. Locally produced PtdIns(4,5)P<sub>2</sub> regulates actin polymerization through activation of WASP family proteins in platelets. WASP family proteins activate actin assembly via Arp2/3 (actin-related proteins 2/3), and act to gate this actin assembly process dependent upon local PtdIns(4,5)P<sub>2</sub> concentrations [99,100]. Recently, Yang et al. [22] demonstrated that platelet PIPKI is tightly regulated by Rho GTPase by illustrating that inhibition of Rho completely blocks trafficking and activation of PIPKI in thrombin receptor-stimulated platelets. The activated Rho-ROCK signalling unit induces the activation and actin cytoskeleton recruitment of PIPKI to generate PtdIns(4,5)P<sub>2</sub>. It is unclear, however, how GTP-bound Rho targets PIPKI to the platelet membrane cytoskeleton.

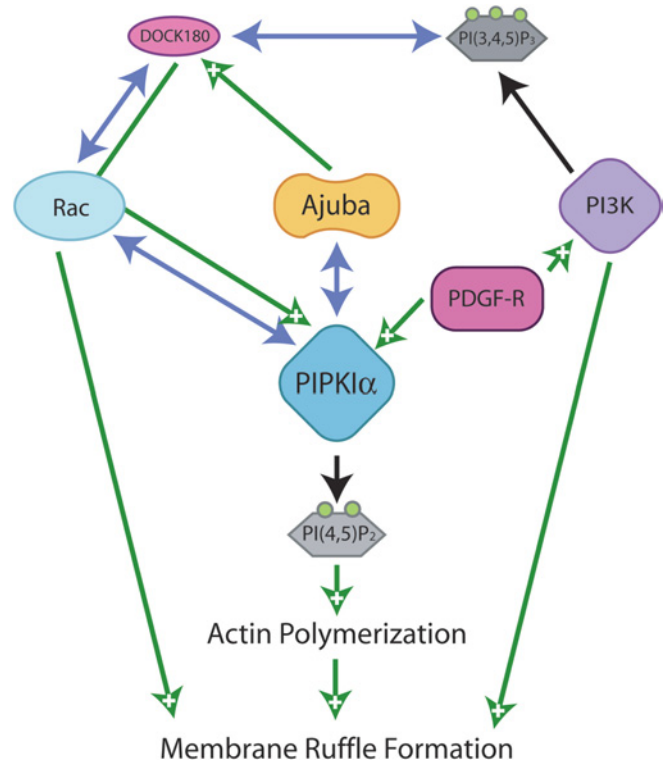
Rac also plays a role in spatial targeting of PIPKI $\alpha$  to the plasma membrane. There are two distinct subcellular localizations of Rac and PIPKI $\alpha$ , on the membrane and in the cytosol [22,67,101]. It is thought that these subcellular locales are not mutually exclusive pools and that shuttling can occur between them. In addition, Rac1 has been shown to be associated with PIPKI $\alpha$ , forming a Rac1-PIPKI $\alpha$  signalling complex in both locations [20,61]. In this complex, membrane-associated Rac activates PIPKI $\alpha$  to produce PtdIns(4,5)P<sub>2</sub>. Membrane ruffle formation induced by PDGF is increased dramatically by increasing ratios of active Rac to PIPKI $\alpha$  in MG63 cells, indicating that the association of PIPKI $\alpha$  with Rac and the signalling of activated Rac are essential in localized actin remodelling to form cell membrane ruffles (Figure 3). This phenomenon is specific to PIPKI $\alpha$ , as Rac1 has been shown to associate with PIPKI $\alpha$ , but not PIPKI $\beta$ , in membrane ruffles upon PDGF receptor stimulation in MG63 cells [20]. From this, it is supposed that simultaneous and synchronized



**Figure 3** Co-expression of PIPKI $\alpha$  and Rac results in disruption of PIPKI $\alpha$ -induced actin foci and instead increased membrane ruffle formation

MG63 cells were transiently transfected with epitope-tagged PIPKI $\alpha$  with or without epitope-tagged Rac. At 24 h post-transfection, cells were stimulated with PDGF or control treatment, fixed in 4% (w/v) paraformaldehyde and immunostained to visualize PIPKI $\alpha$  and actin. Immunofluorescence data show that PDGF treatment reorganizes singly expressed PIPKI $\alpha$  to reorganized actin focus structures, while PDGF treatment of cells co-expressing both PIPKI $\alpha$  and Rac results in formation of actin-containing membrane ruffles and translocation of PIPKI $\alpha$  to ruffle structures. This restructuring has been found to be dose-dependent. Quantification of actin morphology is shown in the lower panel, and indicates that focus and membrane ruffle formations are dose-dependent according to the ratio of PIPKI $\alpha$  to active Rac (RacL61). These data show that an increased ratio of Rac to PIPKI $\alpha$  results in a skewing of actin morphology from focus formation towards ruffle formation in response to PDGF stimulation.

activation of PIPKI $\alpha$  and Rac is required for efficient membrane ruffle formation, which is a critical process in cell migration [20]. Recently, the LIM protein Ajuba has been identified as a positive regulator of PIPKI $\alpha$  and Rac as well as a negative regulator of PIPKI $\beta$  [3]. Ajuba signals through both PIPKI $\alpha$  and Rac to contribute to the formation of membrane ruffles in response to growth factor signalling. This supports the model illustrated in Figure 4,



**Figure 4** Interaction between Ajuba and PIPKI $\alpha$  in conjunction with Rac signalling increases membrane ruffle formation

Co-ordinated signalling from extracellular signals through phosphoinositide phosphate kinases PIPK and PI3K in concurrence with interaction with Ajuba alters the localization of PIPKI $\alpha$  to sites of membrane ruffle formation where concurrent translocation of and signalling through Rac allows their collective effect of increased membrane ruffle formation. PDGF-R, PDGF receptor; PI3K, phosphoinositide 3-kinase; PI(3,4,5)P $_3$ , PtdIns(3,4,5)P $_3$ ; PI(4,5)P $_2$ , PtdIns(4,5)P $_2$ .

where the PIPKI $\alpha$  is spatially targeted, possibly by an interaction with Ajuba, and then modulated by Rac signalling as well as other signalling downstream of PDGF.

Several characteristics of both small G-proteins and their binding partners are implicated in translocation of small G-proteins to the plasma membrane. For example, Rac is prenylated at a C-terminal site, making it more suitable for cell membrane association [32,33]. In the cytosol, Rho GDI interacts with Rac through its prenylated moiety as well as its switch I and II domains [102,103]. Rac has also been shown to exist in a multimeric signalling complex with lipid kinases in the cytosol, complexed with PIPKI in addition to Rho GDI and diacylglycerol kinase [61]. Activation via upstream signalling by integrins, lipids and kinases results in extraction of Rho GDI, which is necessary for shuttling of the Rac–lipid kinase complex to the target site of PtdIns(4,5)P $_2$  production, as has been proposed previously [37,61,104–106]. In a similar scheme of modification, Rho family GTPases have C-termini that are positively charged and resemble phosphoinositide-binding sequences [107]. These modifications are critically important to correct localizations of these small G-proteins, as maintenance of subcellular pools of PtdIns(4,5)P $_2$  is dependent on precise spatial and temporal regulation of PIPKs by these activators.

As previously mentioned, Arf proteins regulate both PLD and PIPKs [23,76,79,108,109]. PLD isoforms PLD1 and PLD2 have been shown to generate PA via hydrolysis of phosphatidylcholine and through this production activate PIPKI both *in vitro* and

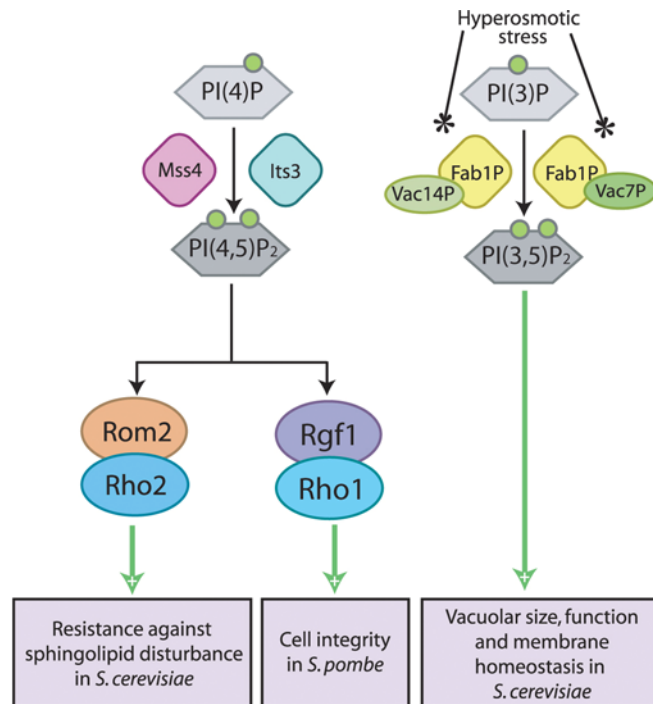
*in vivo* [25,26]. Both mammalian PLD1 and PLD2 have an absolute requirement for stimulation by  $\text{PtdIns}(4,5)P_2$  and functional PH domains to regulate their activity and localization [110]. Given these functional links, the possibility of a direct interaction between PLD isoforms and PIPK $\alpha$  has been postulated [27]. Both PLD1 and PLD2 have been shown to interact with murine PIPK $\alpha$ , and PLD2 has been demonstrated further to be able to recruit mPIPK $\alpha$ . In porcine aortic endothelial cells, mPIPK $\alpha$  localizes to the plasma membrane, cytosol and microtubule-like structures when overexpressed singly. Co-expression with PLD2 results in a relocation of mPIPK $\alpha$  to the submembranous PLD2-positive patch, indicating that PLD2 is able to recruit mPIPK $\alpha$  to this compartment [27].

It has been proposed that the  $\text{PtdIns}(4,5)P_2$  generated by PIPK acts as a cofactor for PLD activation [28]. If this is correct, then it is plausible that the co-localization observed between overexpressed mPIPK $\alpha$  and PLD2 mentioned above would lead to a rapid local increase in both PA and  $\text{PtdIns}(4,5)P_2$  concentrations caused by the positive-feedback loop between increases in  $\text{PtdIns}(4,5)P_2$  concentration and PA formation by PLD and subsequent PIPK activation. Both PA and  $\text{PtdIns}(4,5)P_2$  have been suggested to be important in generation of membrane curvature, vesicle budding and the recruitment and activation of proteins involved in coating of vesicles [10,111–114]. This hypothesis is supported by several different lines of inquiry. First, it has been reported that PLD2-derived PA stimulates PIPK $\gamma$ 635, and that the subsequent generation of  $\text{PtdIns}(4,5)P_2$  drives the initial stages of cell adhesion by regulating cell-surface integrins [29]. This model stresses the importance of PA-dependent generation of  $\text{PtdIns}(4,5)P_2$  by PIPK $\gamma$ 635, specifically at the plasma membrane. Secondly, it has been demonstrated that there is a comparatively high degree of co-localization between PLD2 and PIPK $\gamma$ 635 at the plasma membrane of cells in suspension or during the initial stages of adhesion [29]. This spatial and temporal proximity gives credence to the hypothesis that these factors cooperate within the same signalling cascade to affect the initial stages of cell adhesion.

Distinct  $\text{PtdIns}(4,5)P_2$  pools accumulate and modulated spatially and temporally in cells through regulated PIPK activity. These pools appear to be highly transient, and small G-proteins play roles both in translocation of PIPKs and their effectors to synthesis sites and in their direct stimulation and alter local levels of  $\text{PtdIns}(4,5)P_2$  production in response to extracellular and intracellular stimuli. Small G-protein-mediated changes in  $\text{PtdIns}(4,5)P_2$  pools are important for regulated membrane trafficking and a number of processes involved in cell motility.

### PIPks and small G-proteins in yeast

Mss4, the product of the budding yeast *Saccharomyces cerevisiae* *Mss4* gene, shares extensive sequence homology with the mammalian PIPKs [15,18] and has been shown to be the major PI4P5K (phosphatidylinositol 4-phosphate 5-kinase) in budding yeast most similar to mammalian PIPKs [115]. However, unlike mammalian PIPKs, Mss4 shows dual substrate specificity *in vitro* and is able to convert both  $\text{PtdIns}4P$  into  $\text{PtdIns}(4,5)P_2$ , and  $\text{PtdIns}3P$  into  $\text{PtdIns}(3,5)P_2$  [116]. Mss4 has been revealed to control organization of the actin cytoskeleton as part of a signalling pathway involving yeast Rho GTPases, including Rho2, as illustrated in Figure 5 [116]. Recent data suggest that defects in  $\text{PtdIns}(4,5)P_2$  synthesis reduce Mss4 kinase activity and alter its subcellular localization by preventing recruitment of Rom2 to the membrane, which results in reduced Rho2 activation [117]. The PH domain of Rom2 serves an essential function *in vivo* [118], as it allows regulation of the Rom2/Rho2 signalling pathway to



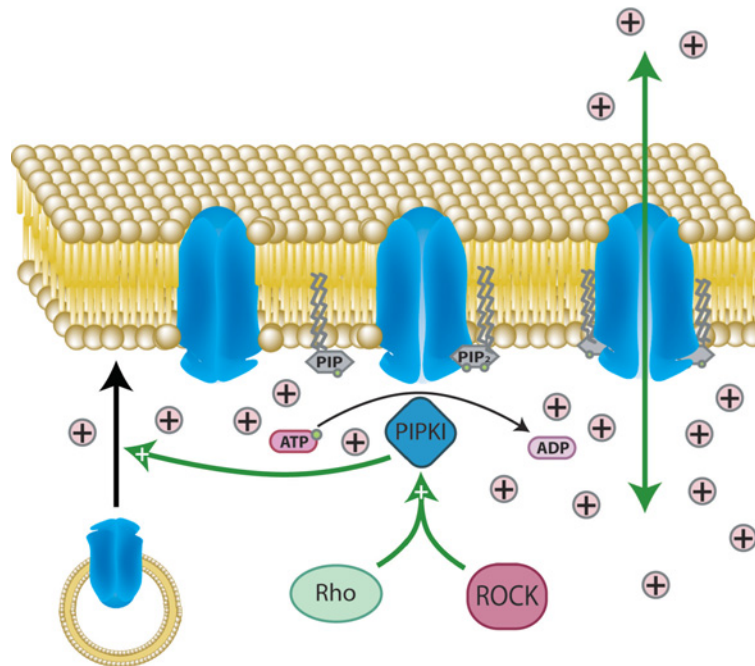
**Figure 5** Yeast PIPKs signal through small G-proteins to mediate structural remodelling and membrane integrity

Similarly to mammalian PIPK signalling pathways, yeast PIPK isoforms convert  $\text{PtdIns}3P$  [ $\text{PI}(3)P$ ] and  $\text{PtdIns}4P$  [ $\text{PI}(4)P$ ] into  $\text{PtdIns}(3,5)P_2$  [ $\text{PI}(3,5)P_2$ ] and  $\text{PtdIns}(4,5)P_2$  [ $\text{PI}(4,5)P_2$ ] respectively to modulate cell integrity and membrane homeostasis through pathways gated by small G-protein signalling.

resist disturbance of sphingolipid biosynthesis by  $\text{PtdIns}(4,5)P_2$ , as shown in Figure 5 [117]. Rho1 and Rho2 are key proteins that are also implicated in construction of cell wall and cell polarity processes in *S. cerevisiae* [119] and *Schizosaccharomyces pombe* [120,121].

The fission yeast *Schizosaccharomyces pombe* *its3<sup>+</sup>* gene product, Its3, is the budding yeast Mss4 homologue that has been identified as yeast PI4P5K. It has been shown to be required for completion of cytokinesis in concert with calcineurin [122]. Additionally, recent research indicates that the Its3 PI4P5K is involved in regulating fission yeast cell integrity via regulation of the Rgf1, Rho1 and protein kinase C signalling pathway as well as the PLC1-mediated PKC-independent pathway through production of  $\text{PtdIns}(4,5)P_2$  (Figure 5) [123]. Rgf1, a specific Rho1 GEF is involved in formation of cell wall and septum [124], and co-ordination of cell polarization with cell wall biogenesis [125] in fission yeast.

The yeast *FAB1* gene product, Fab1p, was initially proposed as a PI4P5K [126], but now defines a phosphatidylinositol 3-phosphate 5-kinase that synthesizes  $\text{PtdIns}(3,5)P_2$  using  $\text{PtdIns}3P$  as a substrate [127]. Complementation analysis of PIPK-deficient yeast mutants indicates that Fab1p and its murine homologue p235 (PIKfyve) comprise a distinct class of type III PIPKs that can exclusively generate  $\text{PtdIns}(3,5)P_2$  in eukaryotic cells [128]. It has been proposed that Fab1p and its upstream regulator Vac7p form a signalling complex that regulates vacuolar size and membrane homeostasis via production of  $\text{PtdIns}(3,5)P_2$  in *S. cerevisiae* [127,129]. As an extension to this observation, Fab1p is also activated by Vac14p, another novel vacuolar protein, upon hyperosmotic stress and rapidly synthesizes  $\text{PtdIns}(3,5)P_2$ , which induces changes in vacuolar volume to smaller, more highly



**Figure 6** Coupling of Kir family channel transport to membrane PtdIns(4,5)P<sub>2</sub> availability

In the absence of bound PtdIns(4,5)P<sub>2</sub>, Kir family channels are closed. PIPKI conversion of PtdIns4P into PtdIns(4,5)P<sub>2</sub> and subsequent PtdIns(4,5)P<sub>2</sub> channel binding is required for the conformational change from the closed to the open state. Additionally, PIPKI activity and increased membrane concentrations of PtdIns(4,5)P<sub>2</sub> have been shown to be linked to increases in channel membrane insertion.

fragmented vacuoles in *S. cerevisiae* [130,131]. PtdIns(3,5)P<sub>2</sub> is essential for vesicle recycling from vacuole/lysosomal compartments and for protein sorting in the multivesicular body in *S. cerevisiae* [132–134]. More recently, it has been proposed that levels of both PtdIns(3,5)P<sub>2</sub> and PtdIns(4,5)P<sub>2</sub> regulate intracellular acidification status in response to environmental pH changes in *S. cerevisiae* [135].

There is interplay between PIPK and small G-protein signalling pathways in yeast similarly to what has been established in mammalian systems. Although established signalling pathways through Mss4 and Its3 exert effects on cell membrane composition and permeability, it is unknown how Fab1p and PtdIns(3,5)P<sub>2</sub> play into signalling pathways to alter vacuolar size, function and membrane homeostasis. It is likely that at least some of these effects are linked to small G-protein signalling pathways considering the precedent in mammalian systems.

#### PIPK REGULATION OF ION CHANNELS: IS THERE A ROLE FOR SMALL G-PROTEINS?

Following initial reports that the activities of the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger and K<sub>ATP</sub> channel (ATP-sensitive K<sup>+</sup> channel) in giant cardiac membrane patches were affected by changes in membrane PtdIns(4,5)P<sub>2</sub> availability [5], a tremendous number of channel activities have been reported to be regulated by phosphoinositides [6]. Small G-proteins have also been implicated in the regulation of membrane ion channel activity, and it is thought that these two types of channel regulation may be interconnected through small G-protein modulation of PIPK activity.

#### Na<sup>+</sup> channels

ENaC (epithelial Na<sup>+</sup> channel) is the limiting factor responsible for Na<sup>+</sup> reabsorption in distal tubule epithelia. ENaC opening has

been linked to membrane PtdIns(4,5)P<sub>2</sub> availability through EGF receptor activation, where the resultant decrease in PtdIns(4,5)P<sub>2</sub> levels has been shown to decrease Na<sup>+</sup> transport [136]. Further reports indicate that PtdIns(4,5)P<sub>2</sub> depletion mediates inhibition of ENaC by P2Y<sub>2</sub> receptor activation, increasing the body of evidence that PtdIns(4,5)P<sub>2</sub> regulates Na<sup>+</sup> channel activity [137]. As in Kir channels, PtdIns(4,5)P<sub>2</sub> is thought to increase individual channel open probabilities by direct receptor binding and activation or open state stabilization in ENaCs [138]. In addition to this mechanism, PtdIns(4,5)P<sub>2</sub> levels are also thought to contribute to channel membrane insertion, thus increasing Na<sup>+</sup> transport by increasing total transporting channels [139].

Small G-proteins RhoA and K-Ras have both been linked to ENaC receptor activation through phosphoinositide signalling. The RhoA–ROCK signalling pathway increases membrane PtdIns(4,5)P<sub>2</sub> levels through stimulation of PIPKI activity, an effect shown to both increase open probabilities of existing ENaC channels and promote additional channel insertion into the membrane as shown in Figure 6 [139]. K-Ras induces ENaC opening, not through PtdIns(4,5)P<sub>2</sub>, but through up-regulation of PI3K activity and PtdIns(3,4,5)P<sub>3</sub> generation at the membrane [140]. Like PtdIns(4,5)P<sub>2</sub>, PtdIns(3,4,5)P<sub>3</sub> has also been shown to bind directly to a specific ENaC-binding site [141]. This binding has been linked to increased channel open probabilities [141] and provides another mechanism by which small G-proteins regulate ENaC activity.

#### K<sup>+</sup> channels

The Kir channel family is the one of the earliest and most extensively studied channel families regarding direct interactions between positively charged residues in channel cytoplasmic domains and the anionic inositol head groups of phosphoinositides



[142,143]. This family includes Kir2/IRK Kir3/GIRK (G-protein-activated inwardly rectifying K<sup>+</sup>), Kir6/K<sub>ATP</sub> and Kv (voltage-gated K<sup>+</sup>) type channels. The inwardly rectifying K<sup>+</sup> channel, Kir3, mediates postsynaptic inhibitory effects of various neurotransmitters in the brain and atrium via GPCRs linked to PTX (pertussis toxin)-sensitive G<sub>i/o</sub> family. Kir6 plays a role as a glucose sensor in pancreatic β-cells using association of the Kir subunit with a sulfonylurea receptor.

Kir channel activity is intimately coupled to PtdIns(4,5)P<sub>2</sub> availability, as channel opening has been found to be dependent upon PtdIns(4,5)P<sub>2</sub> [144,145]. Different members of the Kir channel family have differential sensitivity to PtdIns(4,5)P<sub>2</sub>. Current research has supported the concept that channels with higher affinity for PtdIns(4,5)P<sub>2</sub> overall exist more in open conformations, whereas channels with low PtdIns(4,5)P<sub>2</sub> affinity have lower openness probabilities [142] GIRK and Kir2.3 display lower relative affinities for PtdIns(4,5)P<sub>2</sub> than do Kir2.1 and Kir1. This presumably has physiological relevance for channel regulation, as a variety of transmembrane signalling systems in diverse cellular environments are able to modulate PtdIns(4,5)P<sub>2</sub> concentrations [6,146].

Surprisingly, the small G-protein Rho has been shown to inhibit Kir2.1 channel activity by an unknown mechanism [146a], possibly through alteration of channel membrane targeting or recycling [147]. Rho is a known activator of PIPKs, and through this mechanism increases local membrane PtdIns(4,5)P<sub>2</sub> levels. This indicates that Kir2.1 channel inhibition by Rho is through an alternative pathway, possibly the MAPK (mitogen-activated protein kinase) pathway, which has been implicated previously in channel inhibition [148]. The Kv1.2 channel has been shown to be negatively regulated by Rho as well, and, in this case, GTP-bound RhoA binds directly to the channel to inhibit its activity [149].

### Ca<sup>2+</sup> channels

It has been reported that PtdIns(4,5)P<sub>2</sub> is required for regulation of P/Q type Ca<sup>2+</sup> channels [150]. More recently, PtdIns(4,5)P<sub>2</sub> has also been shown to be required for stabilization and activation of N-type Ca<sup>2+</sup> channels [151]. Muscarinic receptor-mediated suppression of Ca<sup>2+</sup> channel activity has been shown to be inhibited by expression of PI(4)K in *Xenopus* oocytes heterologously expressing Ca<sup>2+</sup> channels in an inside-out patch experiment. As indicated by said experiment, PI4K serves to replenish PtdIns4P, the substrate for PtdIns(4,5)P<sub>2</sub> synthesis by PIPK, and supplies PtdIns(4,5)P<sub>2</sub> into lipid microdomains, where this channel is located. Blockade of PI4K by its pharmacological inhibitor, wortmannin, strongly and selectively attenuates recovery from PTX-insensitive muscarinic modulation. This suggests that G<sub>q/11</sub>-mediated depletion of PtdIns(4,5)P<sub>2</sub> contributes to G<sub>q/11</sub>-mediated muscarinic modulation of N-type Ca<sup>2+</sup> channels [151]. Ras family small G-protein Kir/Gem has been indicated in inhibition of voltage-gated Ca<sup>2+</sup> channels through direct interaction with the channel's β subunit [152]. As membrane PtdIns(4,5)P<sub>2</sub> has been shown to influence channel activity, there is a possibility that another small G-protein signalling pathway could, through PIPK activation, alter membrane PtdIns(4,5)P<sub>2</sub> and modulate channel activity.

### TRP (transient receptor potential) family ion channels

Plasma membrane PtdIns(4,5)P<sub>2</sub> is an important regulator of TRP channels as its breakdown appears to underlie desensitization of several TRP channels, including TRPM4, TRPM5, TRPM8 and TRPV1 [153–156]. In contrast with PtdIns(4,5)P<sub>2</sub>-mediated activation or stabilization of channel activities as described above,

there have been reports that PtdIns(4,5)P<sub>2</sub> acts as a negative regulator in some cases and inhibits certain channel activities. PKD2 belongs to the TRP channel superfamily and has been shown to function as an EGF-activated non-selective cation channel in the plasma membrane. Experimental evidence shows that EGF-induced currents were able to be blocked by U73122 [1-(6-{[17-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1H-pyrrole-2,5-dione], a PLC inhibitor. This is strongly indicative that EGF-induced PKD2 activity requires PLC activity, and suggestive that reduction of PtdIns(4,5)P<sub>2</sub> may be linked to activation of PKD2 by EGF. Support for this hypothesis comes from observations that addition of PtdIns(4,5)P<sub>2</sub> blocks EGF-induced channel current [157].

Although a direct role for PtdIns(4,5)P<sub>2</sub> in channel insertion has yet to be elucidated, PtdIns(4,5)P<sub>2</sub> is likely to modulate translocation of the TRPC5 channel to the plasma membrane in addition to playing a role in regulation of TRP channel activity. In HEK-293 cells, Rac1 has been shown to mediate EGF stimulation of PtdIns(4,5)P<sub>2</sub> synthesis through activation of PIPK1α and facilitate translocation of TRPC5-containing vesicles to the plasma membrane [158]. To summarize, Rac1 and its downstream effector PIPK1 are necessary to stimulate TRPC5 insertion into the plasma membrane after EGF stimulation [158].

As with other phosphoinositide-mediated signalling events, small G-proteins and their effectors play important roles in signalling through stimulation of PIPK activity, localized PtdIns(4,5)P<sub>2</sub> production and subsequent changes ion channel activity, as well as, in some cases, ion channel insertion at the plasma membrane.

### SUMMARY AND COMMENTS

Understanding how phosphoinositide signalling and small G-protein signalling are connected is critical for the understanding of the functional dynamics of many cellular signal transduction pathways. In this review, we propose a model for regulated intracellular targeting of PIPKs by small G-proteins and other modulators in response to extracellular signalling. The importance of spatial and temporal regulation of these factors in signal transduction cannot be overstated for *in vivo* signalling events.

Highly co-ordinated signalling networks operate to control functional integrity of PtdIns(4,5)P<sub>2</sub>-mediated cellular events such as cellular cytoskeletal changes, exocytosis and endocytosis. Small G-proteins and their effectors play pivotal roles in controlling these signalling events through the regulation of PIPKs and are themselves regulated by phosphoinositides. Regulation of PIPKs by small G-proteins occurs primarily through recruitment and activation of different PIPK isoforms and resultant localized *de novo* synthesis of PtdIns(4,5)P<sub>2</sub> in spatially and temporally distinct intracellular pools. The term 'pool', commonly used to describe how PtdIns(4,5)P<sub>2</sub> is organized in membranes as a second messenger, does not refer exclusively to free PtdIns(4,5)P<sub>2</sub>. In fact, an unbound PtdIns(4,5)P<sub>2</sub> gradient would be difficult to maintain as there would be rapid diffusion of PtdIns(4,5)P<sub>2</sub> as well as hydrolysis by PtdIns(4,5)P<sub>2</sub> phosphatases. To date, there is no convincing evidence that PtdIns(4,5)P<sub>2</sub> is differentially distributed in discrete concentration pools in a contiguous membrane, although it is conceivable that varied concentrations may occur in membrane vesicles. Pools of PtdIns(4,5)P<sub>2</sub> are probably stabilized by binding to effectors. In this model, the G-protein lifeguards may work more like G-protein plumbers in directing the channelling of PtdIns(4,5)P<sub>2</sub> to specific effectors.

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