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Phosphoinositides and vesicular membrane traffic

Peter Mayinger

Division of Nephrology & Hypertension and Department of Cell & Developmental Biology, Oregon Health & Science University, Portland, OR 97239

Abstract

Phosphoinositide lipids were initially discovered as precursors for specific second messengers involved in signal transduction, but have now taken the center stage in controlling many essential processes at virtually every cellular membrane. In particular, phosphoinositides play a critical role in regulating membrane dynamics and vesicular transport. The unique distribution of certain phosphoinositides at specific intracellular membranes makes these molecules uniquely suited to direct organelle-specific trafficking reactions. In this regulatory role, phosphoinositides cooperate specifically with small GTPases from the Arf and Rab families. This review will summarize recent progress in the study of phosphoinositides in membrane trafficking and organellar organization and highlight the particular relevance of these signaling pathways in disease.

1. Introduction

Phosphoinositides are phosphorylated intermediates of phosphatidylinositol (PI) and involved in regulating a plethora of cellular processes. Initially discovered in physiological experiments with brain tissue samples and long thought to be solely precursors of soluble and membrane bound second messengers such as inositol-trisphosphate and diacylglycerol, phosphoinositides have now been recognized to play central roles in regulating a wide array of physiological process at intracellular membranes [1]. Phosphorylation of the phosphatidylinositol headgroup gives rise to seven distinct phosphoinositide species with distinct regulatory functions. Phosphoinositides are minor constituents of phospholipid bilayers and show continuous turnover. Phosphatidylinositol-3,4-bisphosphate (PI(3,4)P₂) and phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P₃) are short-lived signaling molecules synthesized in response to extracellular stimuli and involved in cell proliferation and survival [2]. The levels of phosphatidylinositol-3-phosphate (PI(3)P), phosphatidylinositol-4-phosphate (PI(4)P), phosphatidylinositol-3,5-bisphosphate (PI(3,5)P₂) and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) can also be regulated by external cues, however, these phosphoinositides show a relatively stable distribution at specific membranes under normal cell growth conditions [1].

Several studies suggest that PI(3)P is concentrated at early endosomes, PI(4)P at Golgi membranes, PI(3,5)P₂ at late endocytic compartments and PI(4,5)P₂ at the plasma membrane [1]. The compartment-specific distribution of phosphoinositides is coupled to the selective recruitment of effector proteins that bind specifically to individual phosphoinositide species. Recognition of phosphoinositides is mediated by a variety of well-

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Correspondence: mayinger@ohsu.edu.

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defined modular domains that are present in a subset of cytosolic and membrane proteins [3, 4]. In a growing number of cases it has been found that phosphoinositide-binding proteins additionally interact with activated small GTPases from the Ras protein superfamily. In particular, unique combinations of phosphoinositides and GTP-bound versions of Arf and Rab GTPases, play a major role in a coincidence detection mechanism that ultimately controls the timing and selectivity of effector recruitment to membranes [5]. This review will focus on the function of individual phosphoinositides in controlling vesicular transport and how this regulation interconnects with other dynamic processes at membranes. The particular relevance of these molecules for human disease will be highlighted.

2. PI(3)P regulates membrane dynamics at early endosomes, phagosomes and autophagosomes

Phosphoinositides phosphorylated at the 3-position of the inositol headgroup were originally discovered as minor components of total phosphoinositides in fibroblasts and brain tumor cells [6, 7]. Subsequently, three families of PI 3-kinases that utilize distinct phospholipid substrates were characterized [2, 8]. Class I PI 3-kinases phosphorylate PI(4,5)P₂ and PI(4)P to generate short-lived pools of PI(3,4,5)P₃ and PI(3,4)P₂ upon stimulation by growth factors or cytokines and are thus directly involved in regulating cell survival and cell growth [2, 8]. Class II isoforms phosphorylate phosphatidylinositol to PI(3)P and like class I enzymes can be activated upon stimulation of tyrosine kinase receptors and G-protein-coupled receptors [9]. A class III PI 3-kinase that generates PI(3)P was first described in yeast and is encoded by the *VPS34* gene [10]. Vps34 is an essential component of the vacuolar sorting pathway and is the only PI 3-kinase in yeast. Vps34 interacts with the protein kinase Vps15, which is required for membrane targeting of the lipid kinase complex [11, 12]. The pool of PI(3)P that is generated by the Vps34 complex is largely localized to endosomal compartments and essential for protein sorting to the yeast vacuole but plays an additional important role in autophagy [13] [14]. The mammalian homologue of Vps34 is also involved in endocytic sorting and autophagy.

PI(3)P is critical for membrane recruitment of effector proteins that contain specific lipid-binding regions. Important modular domains that recognize PI(3)P are zinc-finger domains, initially detected in the proteins Fab1, YOTB, Vac1 and EEA1, and termed FYVE domains. In addition, Phox homology (PX) domains bind specifically to PI(3)P [3, 15–17]. Many of the proteins containing these domains play an intimate role in vesicular dynamics in the endosomal system. Many FYVE-domain containing proteins are involved in endocytic trafficking. Prominent examples are early endosomal antigen 1 (EEA1) and Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) (Fig. 1). EEA1 is a Rab5 effector required for endosomal tethering and forms homodimers through a coiled-coiled domain [18–20]. EEA1 also interacts with SNARE proteins such as syntaxin-6 and syntaxin-13 at early endosomes [21, 22]. The FYVE domain of EEA1 exhibits low affinity binding to Rab5-GTP, which enables selective recruitment of EEA1 to endosomes containing both Rab5 and PI(3)P [23, 24]. Higher affinity binding, which is required for efficient tethering and SNARE complex formation, requires a specific N-terminal zinc finger motif in EEA1 [25]. The coincidence detection of a phosphoinositide and an activated small GTPase is a frequently observed mechanism for controlling timing and specificity of membrane recruitment of effector proteins involved in vesicular traffic. Importantly, Rab5 binds also to Vps34/Vps15 complexes and is required to recruit the PI 3-kinase complex to early endosomal membranes [26, 27]. Thus, Rab5 acts both upstream and in concert with PI(3)P to regulate endosomal membrane dynamics.

In contrast to EEA1, Hrs binding to early endosomes occurs independently of Rab5, but is dependent on its FYVE domain [28]. Hrs is involved in the endosomal sorting of

ubiquitinated membrane proteins. Hrs contains a clathrin-binding domain and also binds directly to ubiquitin and promotes the endosomal sorting of ubiquitinated membrane proteins [29]. Hrs also associates with phagosomes in a PI(3)P-dependent manner that is required for the maturation of phagosomes and their fusion with late endosomes [30].

Another important group of PI(3)P effectors are PX-domain-containing members of the sorting nexin family (SNX) that function in endosomal membrane dynamics (Fig. 1) [31]. Several SNX proteins contain a Bin, Amphiphysin, Rvs-homology (BAR) domain that triggers dimerization and forms a stable curved structure, which allows these proteins to associate with highly curved membranes and to drive membrane deformation [32]. Not all PX domains of SNX proteins interact with PI(3)P. For example, SNX9 has relatively broad phosphoinositide-binding specificity and is involved clathrin-mediated endocytosis [33].

In both yeast and mammalian cells, class III PI3Ks play an important additional role in autophagy during starvation. In yeast, it was shown that Vps34 forms specific complexes with different sets of cofactors, which specify unique regulatory pathways. Vps34 associates in specific complexes with Vps15, Atg6 and either Vps38 or Atg14 (Fig. 1) [14]. Vps38-containing complexes function in vacuolar protein sorting whereas Atg14-specific complexes are required for autophagy [14]. The mammalian homologue of Atg6 is the protein beclin-1, which also interacts with Bcl2, and plays a prominent role in autophagy [34, 35]. Another binding partner of beclin-1 is the protein encoded by the UV radiation resistance-associated gene (UVRAG) that is involved in regulating hVps34 in autophagy [36]. Mammalian PI(3)P-binding effectors in autophagy include the protein Alf1 that functions in the elimination of aggregated proteins by macroautophagy [37, 38]. In addition, the recently discovered FYVE and coiled-coil domain-containing protein 1 (FYCO1) is a novel PI(3)P effector required for microtubule-based transport of autophagic vesicles [39]. Recent evidence suggests that mutations in FYCO1 are linked to congenital cataracts in certain human populations [40].

Recent evidence suggests that human VPS34 has additional functions in metabolic signaling via mammalian target of rapamycin 1C (mTOR1C), which involves PI(3)P-dependent recruitment of phospholipase D1 (PLD1) to lysosomes in response to amino acids [41–43].

PI(3)P is dephosphorylated by several classes of lipid phosphatases (Fig. 1). Sac1 domain-containing phosphatases show enzymatic activity towards a PI(3)P, PI(4)P and PI(3,5)P₂ [44]. In yeast, deletion of the *SAC1* gene, which encodes a transmembrane lipid phosphatase and represents the founding member of this protein family, display elevated levels of PI(3)P and PI(4)P [44–46]. Although a physiological role of Sac1 in regulating PI(4)P levels has been clearly established, which will be discussed below, the relevance of this protein in PI(3)P regulation is unclear. In contrast, mammalian myotubularin (MTM) and myotubularin-related (MTMR) lipid phosphatases play established roles in the turnover of PI(3)P and PI(3,5)P₂. The MTM/MTMR protein family consists of 15 members, six of which are catalytically inactive [47, 48]. MTM1 localizes to early endosomes and associates directly with the Vps34/Vps15 complex [49]. Whether Vps34/Vps15 and MTM1 are simultaneously active is unclear, but it appears likely that the association of the lipid kinase with its antagonizing phosphatase plays an important role in the tight spatial and temporal control of PI(3)P. MTM2 interacts with Rab7, a late endosomal marker, and probably plays a role in late endosomal dynamics [50]. Mutations in MTMs and MTMRs causes severe neurological diseases in humans such as X-linked myotubular myopathy and Charcot–Marie–Tooth (CMT) disease, characterized by loss of muscle tissue and touch sensation [47, 48]. Complex formation between active and inactive members of the MTM/MTMR family appears to be critical for their proper function. The relevance of these complexes is highlighted by recent findings showing that mutations in both the phosphoinositide 3-

phosphatase MTMR2 and in its catalytically inactive binding partner MTM13 cause type 4B CMT [51, 52].

3. PI(3,5)P₂ controls late endosomal dynamics

PI(3,5)P₂ was first described in yeast, where its synthesis is strongly induced by osmotic stress, but was also discovered as a minor lipid component in non-stimulated mammalian cells [53, 54]. The synthesis of PI(3,5)P₂ requires PI(3)P as precursor and therefore depends on a functional Vps34 protein [53]. Studies in yeast identified Fab1 as the kinase responsible for synthesizing PI(3,5)P₂ (Fig. 2) [55, 56]. Fab1 is activated by osmotic stress and is involved in regulating vacuolar size and morphology [55, 56]. Fab1 contains a FYVE domain that is important for interaction with PI(3)P and for localization to the endosomal system and the vacuole [56]. The mammalian Fab1 homologue PIKfyve displays similar binding to PI(3)P and is also present in the endosomal/lysosomal system [57, 58]. PI(3,5)P₂ appears to play multiple functions in late endosomal dynamics (Fig. 2). The PI(3,5)P₂ effector Atg18 (also termed Svp1 and Aut10) localizes to the vacuole and is involved in a retrograde recycling pathway from the vacuole to the Golgi [59]. Atg18 is also required for autophagosome maturation and for the targeting of cytosolic proteases to the vacuole (Cvt pathway) [60]. Atg18 and its paralogue Atg21 are members of a family of novel family of novel beta-propeller proteins with phosphoinositide binding properties and with a few exceptions present in all eukaryotes [61]. Atg18 is thought to regulate membrane recycling and a recent report showed that Atg18 interacts with the myosin adaptor Vac17 [62]. It is therefore possible that Atg18 regulates interaction of membranes with cytoskeletal elements during retrograde transport.

In yeast, hypertonic shock induces a transient increase in cytosolic Ca²⁺ that depends on Yvc1p, a vacuolar membrane protein with homology to transient receptor potential (TRP) channels [63, 64]. A recent report now demonstrated that this Ca²⁺ release requires PI(3,5)P₂ production [65]. In addition, the homologous mammalian endolysosome-localized mucolipin transient receptor potential (TRPML) channel is activated by binding to PI(3,5)P₂ [65]. These results suggest that TRPML channels are PI(3,5)P₂ effectors that control Ca²⁺-dependent membrane dynamics in response to extracellular stimuli.

Other membrane trafficking regulators that may bind to PI(3,5)P₂ are certain members of the epsin family that contain an epsin NH2-terminal homology (ENTH) domain (Fig. 2) [66, 67]. A recent report indicated that Ent3 and Ent5 bind to PI(3,5)P₂ *in vitro* via their ENTH domain and interact with Vps27, a protein that recognizes ubiquitinated cargo [68]. This protein complex is important for ubiquitin-dependent sorting into multivesicular bodies [69]. However, Ent3 and Ent5 also interact with clathrin and the clathrin adaptors Gga2 and AP-1, and are involved in regulating clathrin coat assembly at the Golgi and at endosomes [70] and further studies will be required to determine whether these Ent proteins are physiologically relevant PI(3,5)P₂ effectors.

The levels of PI(3,5)P₂ are regulated by a unique mechanism (Fig. 2). Fab1 (PIKfyve in mice) forms a protein complex that is essential for regulating its enzymatic activity. One of the interaction partners of Fab1 in this complex is Vac14 (also known as ArPIKfyve in mice), which acts as scaffold protein and also interacts with Atg18 (Fig. 2) [71, 72]. Importantly, Vac14 binds to the PI(3,5)P₂-specific phosphatase Fig4 and the presence of Fig4 in the complex is important for efficient PI(3,5)P₂ synthesis, thus tightly coupling PI(3,5)P₂ synthesis and turnover [71–75].

Deficiencies in PI(3,5)P₂ synthesis causes severe neurological phenotypes in mammals. Spontaneous mutations in the Fig4 gene in the “pale tremor” mouse result in a significant reduction in PI(3,5)P₂ levels [76]. Similar reduced levels of PI(3,5)P₂ are present in mice

with mutated Vac14 [77], which highlights the importance of these factors in the enzymatic complex that synthesizes PI(3,5)P₂. In both cases, the deficiency in PI(3,5)P₂ levels leads to severe neurodegeneration and early lethality. Mutations in FIG4 were also identified in human patients with type 4J CMT, clinically manifested with characteristic motor and sensory neurological defects [76, 78, 79]. Turnover of PI(3,5)P₂ is also catalyzed by phosphatases from the myotubularin family, in particular MTM1, MTMR1, MTMR2 and MTMR6 [80]. Different from Fig4 that acts as a 5-phosphatase and generates PI(3)P, myotubularins dephosphorylate PI(3,5)P₂ at the 3-position of the inositol headgroup to produce PI(5)P [80]. As mentioned above, mutations in MTMR2 cause type 4B CMT and it is possible that aberrant PI(3,5)P₂ levels play a role in this disorder [80].

PI(4)P is a central regulator of Golgi function

PI(4)P is the substrate for PIP 5-kinases and was initially considered solely an intermediate in the biosynthesis of PI(4,5)P₂. More recently, many PI(4)P effectors have been identified and it has been established that PI(4)P itself is essential for cell function, which is independent from its role in PI(4,5)P₂ signaling (Fig. 3). PI(4)P is highly enriched in the Golgi complex, where it plays a direct role in controlling trafficking and Golgi morphology [81].

PI(4)P is synthesized by type II and type III PI 4-kinases that are unrelated in primary sequences and have distinct structural and enzymatic properties [82]. Mammalian cells have four PI4Ks, namely PI4KII α , PI4KII β , PI4KIII α , and PI4KIII β (Fig. 3) [82]. PI4KII β localizes to endosomes and translocates to the plasma membrane after growth factor stimulation [83]. PI4KIII α is localized at the ER, but synthesizes a plasma membrane pool of PI(4)P possibly at plasma membrane/ER contact sites [84]. Both PI4KII α and PI4KIII β localize to Golgi membranes and generate Golgi PI(4)P. PI4KII α is not restricted to the Golgi and is also present at the ER, the plasma membrane and late endosomal compartments [83, 85, 86]. PI4KII α is modified by palmitoylation at a cysteine-based motif within the catalytic domain, which is important for membrane association and targeting to the Golgi [87, 88]. The activity of PI4KII α is regulated by cholesterol levels and biochemical fractionation experiments suggest that this enzyme may preferentially associate with lipid-raft domains [88, 89]. Specific enrichment of PI4KII α at cholesterol-rich Golgi regions may explain the spatial segregation of PI4KII α and PI4KIII β within the Golgi that was observed in polarized cells [90].

Targeting of PI4KIII β to the Golgi requires complex formation with neuronal calcium sensor-1 (NCS-1), a myristoylated co-factor that is also involved in modulating Ca²⁺-dependent processes in neuronal cells [91–93]. NCS-1 binds to a variety of proteins involved in signal transduction, including ion channels, G-protein-coupled receptors and inositol trisphosphate receptors, but the exact role of this protein in neurotransmission and synaptic plasticity remains to be determined [94]. In yeast, the PI4KIII β homolog Pik1 forms a similar complex with the myristoylated co-factor Frq1 [95, 96]. Recruitment of PI4KIII β to the Golgi is regulated by the small GTPase Arf1 that interacts with both PI4KIII β and NCS-1 [92, 97]. The enzymatic activity of PI4KIII β at the Golgi is controlled by protein kinase D (PKD). Phosphorylation of PI4KIII β by PKD1 or PKD2 triggers recruitment of 14-3-3 proteins, which prevents dephosphorylation and causes sustained activity of PI4KIII β [98, 99]. Yeast Pik1 is phosphorylated by unknown kinases and phosphorylated Pik1 binds to 14-3-3 proteins like its mammalian counterpart. [100]. 14-3-3 binding to Pik1 regulates the distribution of Pik1 between cytosol, nucleus and Golgi membranes [100]. In *Drosophila melanogaster*, the PI4KIII β homolog four wheel drive (Fwd) binds and recruits the recycling endosomal Rab11 to Golgi membranes during cytokinesis, which is required for proper cell division [101]. Overall these results indicate that Golgi PI 4-kinases generate functionally

distinct pools of PI(4)P, perhaps in response to specific physiological stimuli, and future work will be required to dissect these different functions.

Golgi PI(4)P cooperates with activated Arf1 to regulate protein and lipid sorting at this organelle. The clathrin adaptor protein complex 1 (AP-1) and γ -ear-containing, ADP-ribosylation factor-binding (Gga) proteins bind directly to PI(4)P at the Golgi (Fig. 3) [102–104]. Efficient Golgi association of these clathrin adaptors requires additional binding to Arf1-GTP [102–104]. In yeast, Golgi PI(4)P can regulate the sequential activation of GTPases from the Rab family. The Rab GEF Sec2 binds simultaneously to Golgi PI(4)P and the Rab GTPase Ypt32 [105]. Sec2 can also activate the Rab GTPase Sec4, which in turn recruits the exocyst effector Sec15, but this can only occur at post-Golgi membranes with low PI(4)P, which allows Sec15 to replace Ypt31 on Sec2 [105]. Another effector in this mechanism is Myo2, a myosin responsible for transporting secretory vesicles to sites of cell growth [106]. Myo2 binds to PI(4)P at late Golgi membranes and also interacts with Ypt31 and Sec4 and thus receives inputs from both phosphoinositides and Rab proteins for membrane association and facilitating vesicular transport [107].

In mammalian cells, Golgi phosphoprotein 3 (GOLPH3) is a PI(4)P effector required for recruiting the unconventional myosin MYO18A. Depletion of GOLPH3 results in trafficking defects and aberrant Golgi morphology [108]. GOLPH3 is an oncoprotein involved in regulating mTOR ([109], which suggests an interesting functional link between the control of Golgi function and regulation of cell growth. The yeast homologue of GOLPH3 is Vps74, a protein required for Golgi enzyme trafficking and localization. [110]. Vps74 binds to PI(4)P and also interacts with Golgi glycosylation enzymes and regulates their recruitment into retrograde COP-I transport vesicles [110–112].

Another functionally distinct group of PI(4)P effectors at the Golgi is comprised of proteins involved in non-vesicular transport of lipids (Fig. 3). Ceramide transfer protein (CERT), four-phosphate adaptor proteins (FAPPs) and oxysterol-binding proteins (OSBP) have PH domains that bind to both PI(4)P and Arf1-GTP. CERT shuttles ceramide that is synthesized at the ER to the Golgi, where it is used to synthesize sphingolipids [113]. FAPP2 binds to glucosylceramide and is required for glycosphingolipid biosynthesis [114, 115]. Interestingly, FAPP proteins play also a role in anterograde trafficking from the Golgi [116–118]. Although it is not entirely clear how FAPP2 functions in these different processes, recent studies show that FAPP2 can dimerize, which triggers membrane tubulation *in vitro* [119, 120]. FAPP2 may therefore coordinate local sphingolipid distribution at the Golgi and promote assembly of trafficking structures. [118–120].

OSBP and OSBP related proteins (ORP) play a role in both lipid homeostasis and membrane traffic. It is possible that some of these proteins catalyze sterol transfer between different compartments at specific membrane contact sites, which may be important for organellar integrity and trafficking [121–123]. In yeast, the homologous Osh proteins are involved in regulating the localization of Rho GTPases Arf1, Cdc42, Rho1 and the Rab GTPase Sec4 [124, 125]. It is also possible that OSBPs and ORPs function as sterols and phosphoinositide sensor at organellar contact sites. Recent studies in yeast provided new exciting insights into the mechanism by which yeast Osh proteins may control lipid homeostasis. A recent report showed that Osh3 interacts simultaneously with the transmembrane adaptor Scs2 at the ER and binds to plasma membrane PI(4)P at sites that are in close proximity of the cortical ER [126]. This pool of PI(4)P is synthesized by the PI 4-kinase Stt4 that forms a stable complex with two cofactors, Ypp1 and Efr3, at spatially restricted membrane regions [127]. In turn Osh3 binds and activates Sac1, a lipid phosphatase that is mainly retained at the ER in proliferating cells [126]. Biochemical assays determined that activation by Osh3 allows Sac1 to act *in trans* at closely apposed lipid membranes and such a mechanism could

account for Sac1-dependent regulation of Stt4-dependent PI(4)P pools at the plasma membrane [126]. In an alternative mechanism, Osh4 (also known as Kes1) may play a central role in PI(4)P homeostasis [128]. Osh4 also interacts with Sac1 and is implicated in regulating trafficking from the Golgi [124, 129]. A recent study showed that Osh4 can bind to PI(4)P in exchange for sterols *in vitro* and may thus function in delivering the phosphoinositide substrate to the Sac1 phosphatase [130]. Although, the source of the PI(4)P pool that is recognized by Osh4 is unknown, there is evidence that Osh4 associates with exocytic vesicles and is required for their docking at the plasma membrane during polarized growth [131]. Whether Osh4 can deliver PI(4)P from these docking sites at the plasma membrane to the ER for degradation by Sac1 remains to be determined.

Local regulation of sphingolipids and PI(4)P may be important for other aspects of Golgi membrane lipid regulation as well. Drs2, a flippase required for regulating phosphatidylserine distribution between membrane bilayer leaflets is activated by PI(4)P at the Golgi [132]. PI(4)P binds to a C-terminal regulatory domain that resembles a split PH domain [132]. In addition, ArfGEF Gea2 binds to the same C-terminal domain and also stimulates flippase activity [132]. In yeast, lipid flippases are regulated by the protein kinase Fpk1 [133]. Interestingly, the upstream kinase Ypk1 that inhibits Fpk1 is negatively regulated by complex sphingolipids [133]. These findings underline the central role of PI(4)P and sphingolipid dynamics in Golgi membrane remodeling.

The Golgi is characterized by continuous influx and exit of membranes and proteins, which requires a specific control system to prevent the random equilibration of PI(4)P pools. The major factor in controlling the steady state distribution of PI(4)P is the evolutionary conserved lipid phosphatase Sac1 that was originally discovered by genetic analyses in yeast [134, 135]. Sac1 is a type II transmembrane protein with a large cytosolic domain containing the catalytic lipid phosphatase motif [44]. In both yeast and mammals, Sac1 shuttles between ER and Golgi membranes [136]. In proliferating mammalian cells, Sac1 is mainly localized at the ER and at cisternal Golgi region, which creates a steep gradient of PI(4)P across the Golgi resulting in a particularly high concentration at the TGN (trans-Golgi network) [137]. The relevance of this distribution is not entirely clear, but deficiency in Sac1 function causes early embryonic lethality in mice and leads to aberrant Golgi structures, mitotic spindle defects and Golgi enzyme mislocalization in cultured cells [137, 138]. It is therefore possible that the PI(4)P gradient at the Golgi provides directionality for Golgi enzyme localization and anterograde trafficking. Sac1 is also required for downregulation of secretion after starvation and during quiescence. This mechanism was first discovered in yeast, where Sac1 accumulates at the Golgi and downregulates PI(4)P when cells run out of carbon sources and proliferation is terminated [139, 140]. These starvation conditions also cause dissociation of PI 4-kinase Pik1 from the Golgi [100, 139]. When starved cells are stimulated with glucose, Sac1 rapidly translocates back to the ER and Pik1 reassociates with Golgi membranes [100, 139]. A similar mechanism is also present in mammalian cells. Mammalian Sac1 orthologues have the ability to oligomerize during serum starvation, which triggers recruitment into COP-II vesicles and accumulation of Sac1 at the TGN [141, 142]. Downregulation of Golgi PI(4)P in quiescent cells produces a reduction in secretory capacity [141]. Stimulation with growth factors induces rapid shuttling of Sac1 back to the ER [141]. The nutrient and growth-factor regulated traffic of Sac1 is therefore an important mechanism to synchronize secretion and Golgi function with growth and proliferation.

PI(4,5)P₂ regulates membrane dynamics at the plasma membrane

PI(4,5)P₂ is enriched at the plasma membrane and regulates many distinct processes at this location [143]. Small fractions of PI(4,5)P₂ were also detected at the Golgi and the nuclear

envelope [144]. PIP5K activity appears to be associated with Golgi membrane, however, the enzyme responsible for this activity was not identified [145].

Plasma membrane PI(4,5)P₂ is produced by two distinct classes of lipid kinases (Fig. 4). Type I PIP kinase phosphorylates PI(4)P and this reaction is the major route for plasma membrane PI(4,5)P₂ biosynthesis [146]. However, PI(4,5)P₂ can also be generated from PI(5)P by type II PIP kinase [146]. In yeast cells, all PI(4,5)P₂ is generated by Mss4 utilizing two distinct PI(4)P pools that are generated by Pik1 and Stt4 at the Golgi and the plasma membrane respectively [147, 148]. Interestingly, *pik1* and *stt4* mutants have distinct phenotypes either in endocytosis or in actin cytoskeletal arrangement that are likely caused by PI(4,5)P₂ deficiency [148], indicating that PI(4,5)P₂ generated at the plasma membrane may form functionally distinct pools. This idea is supported by a study that showed anisotropic distribution of plasma membrane PI(4,5)P₂ in yeast cells during mating that depends on Stt4 but is independent of Pik1 [149].

PI(4,5)P₂ is important for classical signal transduction either via phospholipase C-mediated cleavage into inositol-trisphosphate and diacylglycerol or via PI 3-kinase-dependent phosphorylation to PI(3,4,5)P₃ [2, 150]. PI(4,5)P₂ is also important for interactions between the plasma membrane and the cytoskeleton [150]. PI(4,5)P₂ plays additional critical roles in regulating exocytosis and endocytosis. During exocytosis, plasma membrane PI(4,5)P₂ functions in immobilizing secretory granules and in controlling docking of synaptic vesicles prior to fusion [151, 152]. Several PI(4,5)P₂ binding proteins play a role in this process including CAPS (calcium-activated protein for secretion), Syt1 and rabphilin (Fig. 4) [152].

PI(4,5)P₂ is also directly involved in regulating clathrin-mediated endocytosis. Many of the clathrin adaptors required for endocytosis such as AP-2, AP180 and epsin bind directly to PI(4,5)P₂ (Fig. 4) [153–155]. The initial PI(4,5)P₂-dependent plasma membrane recruitment of these factors is stabilized by additional interactions with transmembrane cargo proteins containing tyrosine based sorting motifs [156]. Plasma membrane PI(4,5)P₂ is also important for regulating actin dynamics during the endocytic internalization reaction [157]. After internalization, PI(4,5)P₂ is rapidly turned over by lipid phosphatases mainly from the synaptojanin family, which is essential for the disassembly of clathrin coats at endocytic vesicles (Fig. 4) [158]. Deletion of synaptojanin-1 in mice causes accumulation of clathrin-coated vesicles and leads to neurological defects and perinatal lethality [159]. Zebrafish synaptojanin-1 mutants have a less severe phenotype and show defective synaptic transmission at hair-cell synapses [160]. In contrast, a mutation in synaptojanin-2 results in progressive hearing loss and hair cell degeneration in mice [161]. In yeast, deletion of synaptojanin-like phosphatases causes defects in actin organization, endocytosis, and clathrin-mediated sorting between the Golgi and endosome that correlate with an accumulation of PI(4,5)P₂ at the cell periphery [162]. Though these studies show species-dependent differences in phenotypes, it is clear that turnover of PI(4,5)P₂ by synaptojanin lipid phosphatases is instrumental for rapid recycling of clathrin coated endocytic vesicles.

PI(4,5)P₂ is also dephosphorylated by the 5-phosphatase OCRL1 that localizes to early endosomes and Golgi compartment. Mutations in OCRL1 cause Lowe syndrome, which is characterized by a triad of clinical symptoms consisting of congenital cataracts, mental retardation and renal proximal tubular dysfunction [163]. OCRL1 interacts with clathrin, the adaptor protein APPL and several proteins from the Rab GTPase family and is involved in the endocytic pathway and in trafficking between endosomes and the trans-Golgi network [164–167]. A recent report shows that OCRL deficiency causes accumulation of PI(4,5)P₂ in early endosomes and induces an increase in endosomal F-actin, which may be the cause for the observed defects in early endosomal dynamics and in receptor recycling at the plasma membrane [168].

6. PI(5)P plays distinct roles in nuclear signaling and in membrane dynamics

PI(5)P represents probably the least characterized phosphoinositide (Fig. 5). Although improved methods for the quantitative analysis of PI(5)P from cell extracts and subcellular fractions using HPLC are now available [169], it is unclear where this phosphoinositide localizes in live cells. PI(5)P appears to play a regulatory role in the nucleus and was shown to bind to the PHD finger motif in the nuclear protein inhibitor of growth protein-2 (ING2) [170]. This protein is involved in controlling histone acetylation and p53 during cellular stress [171].

The mechanisms for PI(5)P biosynthesis are not well characterized. Theoretically, PI(5)P can be generated either by dephosphorylation of PI(3,5)P₂ and PI(4,5)P₂ or by phosphorylation of PI (Fig.5) [172]. The most established pathway is the generation of PI(5)P from PI(3,5)P₂ by the 3-phosphatase myotubularin [173]. Two lipid phosphatases, termed type I and type II PIP 4-phosphatase, have been described and these enzymes convert PI(4,5)P₂ to PI(5)P in biochemical assays [174]. Both enzymes localize to endosomal and lysosomal membranes in cultured epithelial cells and it appears likely that these phosphatases contribute to the physiological regulation of PI(5)P. Finally, *in vitro* assays suggest that both type I PIP 5-kinase and PIKfyve can use PI as a substrate for PI(5)P synthesis [58, 175, 176], but it remains controversial whether these reactions occur in live cells [172].

Interestingly, several bacterial enzymes convert plasma membrane PI(4,5)P₂ to PI(5)P *in vivo*. *Shigella flexneri* is a bacterial pathogen that uses a type 3 secretion system to inject virulence factors into host cells to facilitate invasion [177]. Among these factors is invasion plasmid gene D (IpgD) a protein containing domains homologous to mammalian inositol 4-phosphatases. IpgD-mediated conversion of PI(4,5)P₂ to PI(5)P induces membrane and actin cytoskeletal rearrangements that are required for the invasion process [178]. Recent evidence suggest that PI(5)P inhibits selectively the degradation of epidermal growth factor receptor (EGFR) by delaying its trafficking to late endosomal and lysosomal compartments, which in turn enhances signaling through the pathways downstream of EGFR in particular via Akt [179]. This mechanism for bacterial invasion is also found in other bacteria. *Salmonella typhimurium* contains the IpgD homolog SigD, which appears to function in the invasion process by breakdown of PI(4,5)P₂ coupled to PI(5)P production [180].

Stimulation of cultured adipocytes causes a transient increase in PI(5)P levels, which promotes Akt phosphorylation and GLUT4 translocation to the plasma membrane [181, 182]. Together with the results obtained for bacterial pathogen-mediated control of PI(5)P, these findings suggest that this phosphoinositide plays an important but as yet incompletely defined role in membrane trafficking and dynamics.

7. Perspectives and conclusions

Phosphoinositides are intimately involved in controlling virtually all vesicular transport pathways. Recent evidence suggests that specific phosphoinositides act in concert with small GTPases mainly from the Arf1 and Rab family. A combinatorial code consisting of compartment-specific sets of phosphoinositides and activated Arfs and Rabs is responsible for high affinity recruitment of effector proteins to specific sites. In addition, phosphoinositides control membrane-cytoskeletal interactions, ion transporters and signal transduction at the plasma membrane, all of which is critical for proper control of membrane dynamics and organellar function. How the localization of certain phosphoinositides at specific compartments is regulated is not well understood. One limitation in the

experimental approach to elucidate these question involves the probes that are commonly used to visualize phosphoinositides by fluorescence microscopy. It is likely that highly dynamic and functionally important phosphoinositide pools exist that cannot be detected using these tools. A given phosphoinositide can be rapidly converted to another phosphoinositide species, which either terminates or changes its physiological role. It is clear that lipid kinases and phosphatases play a paramount role in this regulation. However, the localization of these enzymes does not always explain the localization profile of the various phosphoinositides and future work will be required to resolve these questions. Finally, even small disturbances in the turnover or steady state levels of phosphoinositides lead to specific human disorders and it will be a future challenge to define how these lipid-based pathways intersect with other cellular regulation systems.

Highlights

> Phosphoinositide lipids are critical regulators of membrane traffic. > Important roles in exocytosis, endocytosis, endosomal traffic and Golgi function. > Additional roles in autophagy and organellar dynamics. > Relationship to human disease is discussed.

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Fig. 1.

Regulation of PI(3)P and its role in early endosomal traffic and autophagy. PI(3)P is synthesized by class III PI 3-kinase Vps34 in yeast and mammals. Vps34 forms specific complexes with co-factors that specify its function in either endosomal dynamics or in autophagy. In addition, PI(3)P plays a role in metabolic signaling via PLD1 and mTORC1. Several phosphatases belonging to the myotubularin (MTM) and myotubularin-related protein (MTMR) family can dephosphorylate PI(3)P. Mammalian lipid kinases are marked by orange boxes, lipid phosphatases are in green boxes. Yeast homologs are indicated in lighter shades of the same colors. Effector proteins are in blue boxes.

Fig. 2.

Regulation of PI(3,5)P₂ and its function in late endosomal dynamics and in autophagy. PI(3,5)P is generated by the FYVE domain-containing lipid kinase Fab1. Synthesis and turnover of PI(3,5)P₂ are tightly coupled. Yeast Fab1 is only active when it is in a complex with the scaffold protein Vac14 that also interacts with Vac7 and with the PI(3,5)P₂ phosphatase Fig4. A similar complex is also found in mammals containing the lipid kinase PIKfyve, the scaffold protein Vac14 (also termed ArPIKfyve) and the lipid phosphatase Fig4 (also termed Sac3). PI(3,5)P₂ plays multiple roles in late endosomal dynamics, autophagy, MVB sorting and Ca²⁺ mediated membrane fusion. Mammalian lipid kinases or kinase complexes are marked by orange boxes, lipid phosphatases are in green boxes.

Alternative names for certain enzymes are in parentheses. Yeast homologs are indicated by lighter shades of the same colors. Effector proteins are in blue boxes.

Fig. 3.

Regulation of PI(4)P and its essential function at the Golgi. In yeast, PI(4)P is synthesized by two essential PI 4-kinases, the Golgi-localized Pik1-Frq1 complex and plasma membrane Stt4. In mammalian cells, PI4KII α and PI4KIII β generate Golgi PI(4)P, whereas PI4KII β and PI4KIII α are involved in plasma membrane PI(4)P synthesis. Turnover and spatial control of PI(4)P is achieved by the evolutionary conserved lipid phosphatase Sac1 that also responds to nutrients and growth signals. Many effectors at the Golgi bind PI(4)P and regulate anterograde trafficking from the Golgi, resident enzyme recycling, lipid dynamics and sphingolipid biosynthesis. Mammalian lipid kinases or kinase complexes are marked by orange boxes, lipid phosphatases are in green boxes. Alternative names for certain enzymes

are in parentheses. Yeast homologs are indicated by lighter shades of the same colors. Effector proteins are in blue boxes.

Fig. 4.

Regulation of PI(4,5)P₂ and its function in vesicular traffic. In yeast, PI(4,5)P₂ is generated by the PIP 5-kinase Mss4. In mammals, PI(4,5)P₂ is synthesized by type I and type II PIP kinases either from PI(4)P or from PI(5)P, respectively. PI(4,5)P₂ is enriched at the plasma membrane, where it regulates clathrin-mediated endocytosis and exocytosis of synaptic vesicles and secretory granules. Rapid turnover of PI(4,5)P₂ by lipid phosphatases from the synaptojanin family is important for rapid recycling of endocytic vesicles. Early endosomal trafficking and recycling is also regulated by the lipid phosphatase OCRL1. PI(4,5)P₂ regulates many additional processes including the actin cytoskeleton, ion channels, and signal transduction at the plasma membrane, which is not depicted in the figure. Mammalian

lipid kinases are marked by orange boxes, lipid phosphatases are in green boxes. Yeast homologs are indicated by lighter shades of the same colors. Effector proteins are in blue boxes.

Fig. 5.

Regulation of PI(5)P and its role in nuclear signaling and membrane dynamics. PI(5)P can be synthesized by MTM and MTMR phosphatases via dephosphorylation of PI(3,5)P₂. *Salmonella* and *Shigella* bacterial pathogens express well characterized lipid phosphatases (IpgD and SigD) that convert PI(4,5)P₂ to PI(5)P upon injection into the host cell which is important for the invasion mechanism. Recently, mammalian type I and II PIP 4-phosphatases have been identified that generate PI(5)P *in vitro* using PI(4,5)P₂ as substrate. Biochemical assay suggest that PIKfyve and type I PIP kinase can directly phosphorylate PI to create PI(5)P, but it is unclear whether this reaction is relevant for the physiological regulation of PI(5)P. PI(5)P binds to the nuclear adaptor ING2 that regulates chromatin

rearrangement in response to stress. But there is mounting evidence that PI(5)P may play an important role in regulating membrane dynamics in both endocytic and exocytic sorting. Mammalian lipid kinases are marked by orange boxes, lipid phosphatases are in green boxes. Bacterial lipid phosphatases are in cyan boxes. Effector proteins are in blue boxes.