Phosphoinositide signaling and the regulation of membrane trafficking in yeast

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Phosphoinositides are key regulators of diverse cellular processes in eukaryotic cells. Genetic studies in yeast have advanced our understanding of how phosphoinositide-signaling pathways regulate membrane trafficking. Enzymes required for the synthesis (kinases) and turnover (phosphatases) of distinct phosphoinositides have been identified and several downstream effector molecules linked to phosphoinositide signaling have recently been characterized.

IN EUKARYOTIC CELLS, complex regulatory mechanisms must operate to ensure the temporal and spatial specificity of intracellular membrane-trafficking pathways. Several stage-specific transport components have been characterized, including proteins such as SNAREs, Rab GTPases and Sec1 homologs, which maintain the fidelity of the vesicle-docking and -fusion reactions[1]. A key regulatory function has also been demonstrated for phosphatidylinositol (PtdIns or PI) and its phosphorylated derivatives, collectively referred to as phosphoinositides. Unlike the head groups of other phospholipids, the inositol ring of PtdIns can be reversibly phosphorylated at one or a combination of positions (3′, 4′ or 5′). As a result, at least seven unique second-messenger molecules are generated that regulate diverse cellular processes, including growth, differentiation, cytoskeletal rearrangements and membrane trafficking[2,3].

Although present only transiently and in low abundance within cells, phosphoinositides can be highly concentrated within membrane microdomains. Phosphoinositides are, therefore, ideally suited to function as spatially restricted membrane signals because: (1) both the substrate (PtdIns) and products (phosphoinositides) are restricted to the membrane site of modification; (2) the synthesis and turnover of phosphoinositides can be temporally and spatially regulated by a set of lipid kinases, lipid phosphatases and phospholipases; (3) the localization of these kinases and phosphatases to discrete membrane sites restricts signaling to a specific compartment (or a specific domain of a compartment); and (4) the structurally distinct phosphoinositide products can activate unique downstream targets or effectors.

Phosphoinositides were first established as second messengers in signal-transduction pathways by Berridge and co-workers, who showed that agonist-stimulated activation of phospholipase C resulted in cleavage of PtdIns(4,5)P_2 to generate soluble inositol(1,4,5)P_3 [Ins(1,4,5)P_3] and membrane-restricted diacylglycerol (DAG). More recently, phosphoinositide-signaling pathways have also been found to regulate membrane trafficking, raising numerous questions. For example, what signals activate phosphoinositide synthesis and turnover, and how is this restricted to specific membrane domains? What effectors are activated by phosphoinositides and how do these effectors modulate membrane-trafficking pathways?

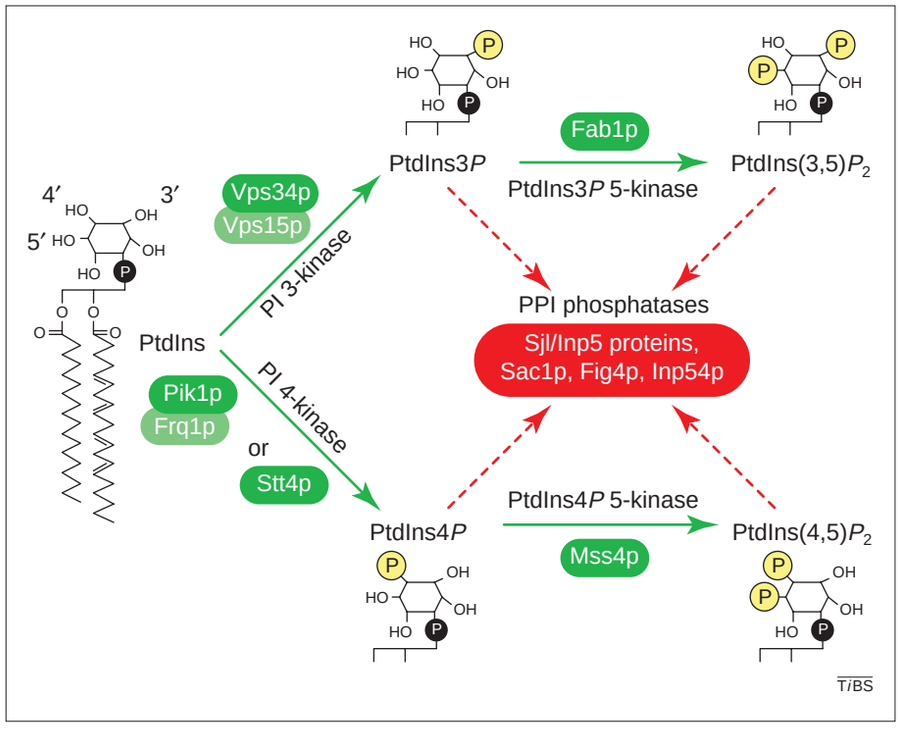

Figure 1

Pathways for the synthesis and turnover of phosphoinositides in yeast. The synthesis pathways mediated by phosphatidylinositol (PtdIns or PI) kinases and PtdIns-phosphate kinases are shown in green; phosphoinositide kinases are dark green and Vps15p and Frq1p (which activate Vps34p and Pik1p, respectively) are light green. For simplicity, both of the PI 4-kinases in yeast, Pik1p and Stt4p, are shown together in the pathway for PtdIns4*P* synthesis. The turnover pathways mediated by polyphosphoinositide (PPI) phosphatases are shown in red. However, because their functional roles *in vivo* have not been extensively characterized, the activities of the PPI phosphatases in yeast are shown collectively.

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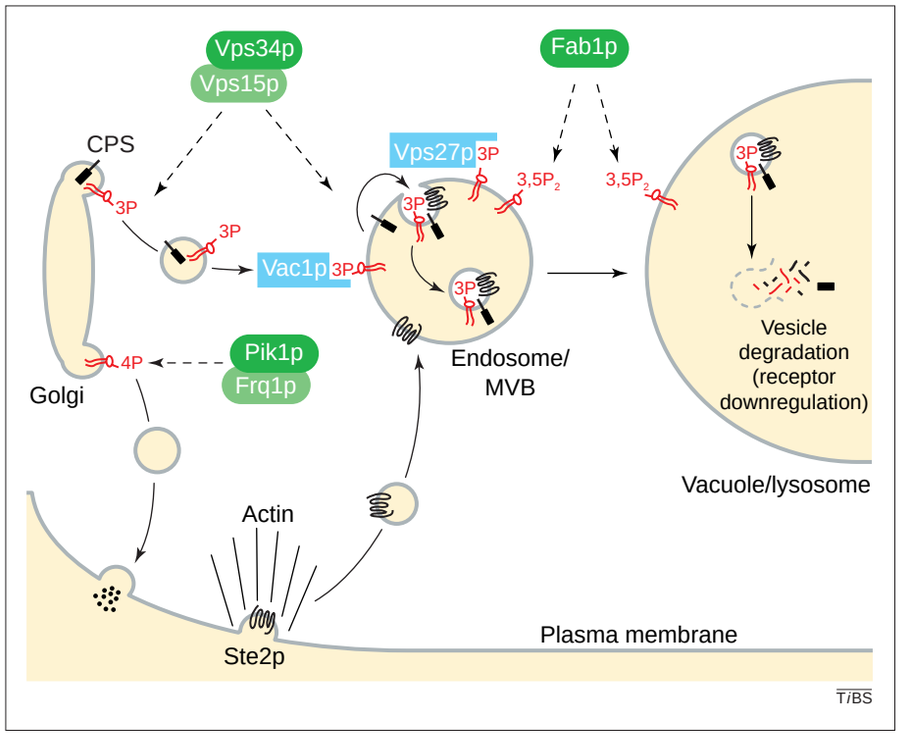

Figure 2

Model for the functional roles of yeast phosphatidylinositol (PtdIns or PI) kinases in membrane trafficking, showing the major routes of transport along the secretory, endocytic, vacuolar protein sorting and multivesicular body (MVB) sorting pathways. The phosphoinositide kinases are as in Fig. 1. Phosphoinositides are shown in red with their specific modification indicated: 3P, PtdIns3*P*; 3,5P_2, PtdIns(3,5)P_2; 4P, PtdIns4*P*. PtdIns3*P* might sequentially recruit and/or activate the FYVE-domain-containing proteins Vac1p and Vps27p (shown in blue). The Fab1 PtdIns3*P* 5-kinase, another FYVE-domain-containing effector of PtdIns3*P*, terminates at least one facet of PI 3-kinase signaling by phosphorylating PtdIns3*P* to produce PtdIns(3,5)P_2. Fab1p signaling via PtdIns(3,5)P_2 synthesis is also required for the MVB sorting pathway, which transports a significant pool of PtdIns3*P* into the vacuole lumen, where it is degraded. The MVB sorting pathway also transports a biosynthetic vacuolar hydrolase, carboxypeptidase S (CPS) and the G-protein-coupled α-factor receptor Ste2p into the vacuole lumen.

In this article, we review insights into the phosphoinositide regulation of membrane trafficking, emphasizing genetic studies in the budding yeast *Saccharomyces cerevisiae*. Together, these studies are beginning to reveal the molecular details of how the regulated synthesis and turnover of phosphoinositides can direct specific vesicle-mediated transport reactions.

PI 3-kinase regulation of vacuolar protein sorting

A direct role for phosphoinositides in membrane trafficking was originally demonstrated by genetic studies of protein transport to the yeast vacuole (or lysosome), an acidified organelle that contains hydrolytic enzymes and serves as a major site for macromolecular turnover. More than 40 vacuolar protein sorting (*VPS*) genes are required for transport from the Golgi apparatus to the vacuole. One of these genes, *VPS34*, was found to encode a PI 3-kinase that specifically phosphorylates PtdIns at the D-3 position of the inositol ring to produce PtdIns3*P* (Ref. 4; Fig. 1). Temperature-sensitive *vps34* mutant cells exhibit an immediate defect in both protein sorting to the vacuole and PI 3-kinase activity when shifted to a non-permissive temperature[5], strongly suggesting that the Vps34 PI 3-kinase plays a direct role in vesicular transport from the Golgi to the vacuole.

Vps34p is recruited from the cytosol to the membrane and activated by the *VPS15* gene product, a membrane-associated serine/threonine protein kinase that catalyses an autophosphorylation reaction[5,6]. Vps15p kinase activity is required for it to associate with Vps34p, and membrane recruitment activates (by more than ten times) the PI 3-kinase activity of Vps34p (Ref. 5). Like *vps34* mutant cells, inactivation of Vps15p kinase activity causes a severe decrease in cellular levels of PtdIns3*P* and in mis-sorting of vacuolar hydrolases[5], indicating that Vps15p serves as an upstream regulator of Vps34p. Biochemical studies have localized Vps15p and Vps34p to Golgi- or endosome-enriched membrane fractions[6], suggesting a functional role for PtdIns3*P* in membrane trafficking from the Golgi to the endosome (Fig. 2).

The human homolog of Vps34p has been cloned and is sensitive to nanomolar concentrations of the fungal metabolite wortmannin, a potent inhibitor of PI 3-kinases[7]. *In vivo*, wortmannin impairs the biosynthetic transport of cathepsin D and the endocytic transport of platelet-derived growth factor (PDGF) receptor to the lysosome[8–10]; PDGF-receptor trafficking is also impaired in cells microinjected with antibodies against human Vps34p[11]. The human homolog of Vps15p has also been cloned and found to stimulate the PI 3-kinase activity of human Vps34p[12]. Thus, synthesis of PtdIns3*P* through the activation of a Vps15p–Vps34p kinase complex in human cells appears to regulate membrane trafficking to the lysosome in a manner similar to that seen in yeast.

Effectors of Vsp34 PI 3-kinase signaling: FYVE-domain proteins

How does PtdIns3*P* synthesis direct vesicle-mediated protein transport? Recent genetic and biochemical studies in both yeast and mammalian cells have uncovered a set of effector molecules that bind directly to PtdIns3*P*. One of these, the human early endosome autoantigen 1 (EEA1) protein, is a peripheral membrane protein that interacts with the active form of the Rab5 GTPase. EEA1 must associate with membranes to stimulate homotypic endosome fusion *in vitro*[13]. Membrane association of EEA1 is blocked by wortmannin[14], suggesting that EEA1 binds to one or more 3′-phosphorylated phosphoinositides. Membrane binding of EEA1 also requires a conserved RING domain of ~70 amino acids that has been termed the FYVE domain, named after the first letters of four proteins that were initially found to contain this domain[15]. Like other RING domains, the FYVE domain binds two Zn^{2+} atoms, which are coordinated by eight cysteine or histidine residues. However, a highly conserved basic amino acid patch surrounds the third cysteine residue of all FYVE domains (Fig. 3a), distinguishing it from other RING domains[15].

In yeast, genetic and biochemical studies have indicated that a number of *VPS*-gene products function together with the Vps15p–Vps34p complex at the Golgi-to-endosome stage of vacuolar

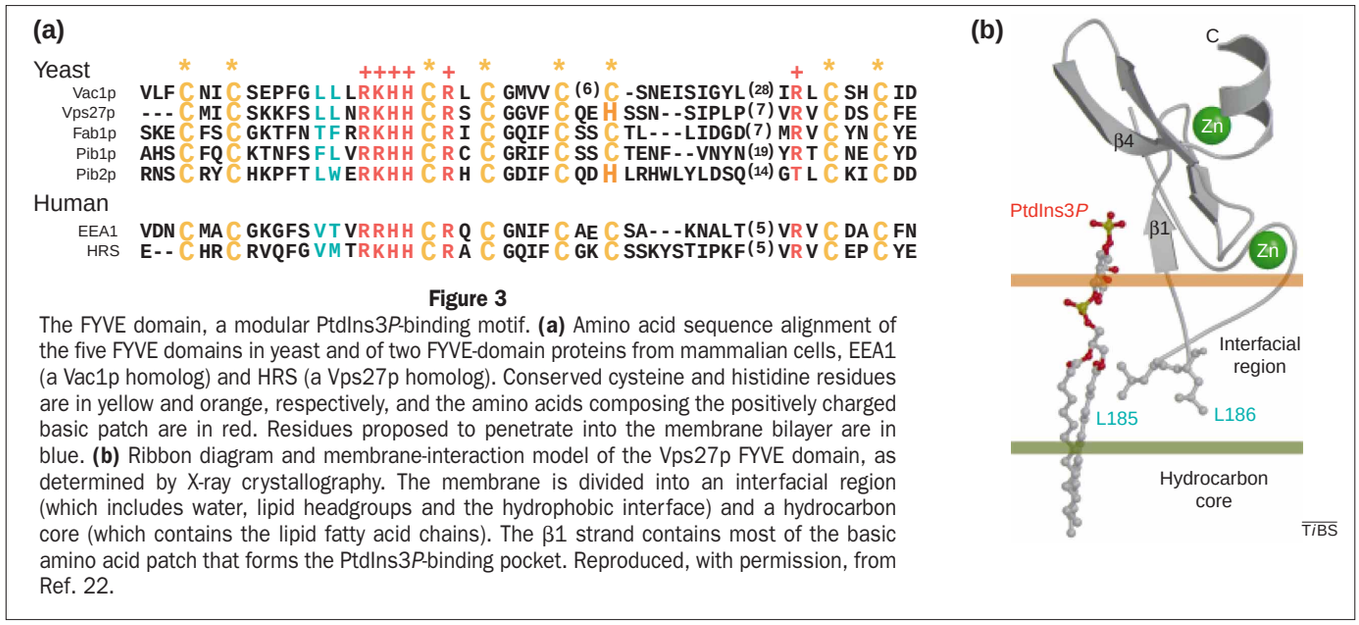

Figure 3

The FYVE domain, a modular PtdIns3*P*-binding motif. **(a)** Amino acid sequence alignment of the five FYVE domains in yeast and of two FYVE-domain proteins from mammalian cells, EEA1 (a Vac1p homolog) and HRS (a Vps27p homolog). Conserved cysteine and histidine residues are in yellow and orange, respectively, and the amino acids composing the positively charged basic patch are in red. Residues proposed to penetrate into the membrane bilayer are in blue. **(b)** Ribbon diagram and membrane-interaction model of the Vps27p FYVE domain, as determined by X-ray crystallography. The membrane is divided into an interfacial region (which includes water, lipid headgroups and the hydrophobic interface) and a hydrocarbon core (which contains the lipid fatty acid chains). The β1 strand contains most of the basic amino acid patch that forms the PtdIns3*P*-binding pocket. Reproduced, with permission, from Ref. 22.

protein transport. One of these, Vac1p/Vps19p, is a structural homolog of EEA1. Like EEA1, Vac1p contains a FYVE domain and is found as a peripheral membrane protein. Vac1p interacts with PtdIns3*P* and the Rab5-GTPase homolog Vps21p. Vac1p also interacts with Pep12p, an endosomal t-SNARE, and with the Sec1-protein-family member Vps45p[16,17]. These multivalent interactions coordinated by Vac1p might ensure the orderly progression of vesicle tethering, SNARE-complex assembly and vesicle fusion (Fig. 4). Point mutations of the conserved cysteine residues disrupt vacuolar protein sorting[18], which is consistent with the FYVE domain of Vac1p playing an important role in this process.

In addition to Vac1p, the FYVE domain is present in four other yeast proteins (Fig. 3a), including Vps27p, another peripheral membrane protein associated with endosomal compartments. The FYVE domains of mammalian EEA1 and of yeast Vac1p, Vps27p, Fab1p and Pib1p have all been shown to bind to liposomes that contain PtdIns3*P* but not to liposomes that contain other phosphoinositides, showing that the FYVE domain specifically binds to PtdIns3*P* *in vitro*[19–21]. In addition, green-fluorescent protein (GFP) fused to the FYVE domain of EEA1 was shown to bind to intracellular membranes in yeast[19]. This binding depended on the PI 3-kinase activity of Vps34p, showing that the GFP–FYVE-domain construct is a useful *in vivo* reporter for membrane compartments that contain PtdIns3*P* (Ref. 19).

Recent structural studies have firmly established the FYVE domain as a modular PtdIns3*P*-binding motif. The crystal structure of the Vps27p FYVE domain has been determined to 1.15 Å resolution[22] and the FYVE domain of EEA1 has been analysed by nuclear magnetic resonance (NMR) spectroscopy[23]. Both studies revealed a structure composed of two pairs of antiparallel β strands that are stabilized through the coordination of two Zn^{2+} atoms by the conserved cysteine and histidine residues. Most of the positively charged amino acids that compose the basic patch are contained within the β1 strand and form a shallow groove that can specifically accommodate the negatively charged phosphate in the head group of PtdIns3*P* (Fig. 3b). Based on the crystal structure of the Vps27p FYVE domain, the basic pocket is too small for PtdIns bis- or trisphosphate, which explains the absence of binding to polyphosphoinositides.

The side-chain orientation of amino acids lining this pocket forms an almost-perfect binding site for the 3′-phosphate of PtdIns3*P* but is incompatible with binding the 4′-phosphate of PtdIns4*P* (Ref. 22). NMR analysis of the EEA1 FYVE domain also showed that binding of PtdIns3*P* is preferred over PtdIns5*P* or PtdIns (Ref.23). Modeling of the Vps27p FYVE domain suggested that Leu185 and Leu186 could interact nonspecifically with the hydrophobic region of the phospholipid bilayer (Fig. 3b), which would help to orient the FYVE domain and to stabilize its interaction with the membrane[22]. This was also suggested for the EEA1 FYVE domain, which is consistent with the finding that mutation of this region disrupted interaction with membranes *in vivo*[23].

These structural studies of the FYVE domain, together with the functional analyses of Vac1p and EEA1 described above, suggest that PtdIns3*P* functions as a compartment-specific membrane signal that acts sequentially to recruit or to activate distinct FYVE-domain-containing effector proteins (Vac1p followed by Vps27p and Fab1p) that then direct membrane-transport steps in the vacuolar protein-sorting pathway (Fig. 2). A high local concentration of PtdIns3*P* on the membrane might also drive Vac1p/EEA1-complex formation to stabilize vesicle docking before membrane fusion (Fig. 4).

Another phosphoinositide-binding motif that has been well characterized is the pleckstrin-homology (PH) domain, a structurally conserved module of ~100 amino acids that is found in a wide variety of proteins. In contrast to FYVE domains, PH domains have a broad range of phosphoinositide-binding specificities[3]. In mammalian cells, both the dynamin GTPase (required for endocytosis) and CAPS (a protein required for calcium-regulated exocytosis) contain PH domains that bind to PtdIns(4,5)P_2 (Ref. 3). PH domains are also found in several yeast proteins (Table 1) but none of these have been linked directly to membrane trafficking.

PtdIns3*P* 5-kinase regulation of the MVB sorting pathway

Interestingly, the FYVE-domain-containing protein Fab1p functions as a PtdIns3*P* 5-kinase, phosphorylating PtdIns3*P* to produce PtdIns(3,5)P_2 (Fig. 1)[24,25]. Thus, Fab1p has a unique regulatory role as both an effector

protein of PtdIns3*P* and a kinase that inactivates PI 3-kinase signaling. Unlike PtdIns3*P* synthesis by Vps34p, however, Fab1p kinase activity is not essential for membrane trafficking to the vacuole. Nevertheless, the loss of Fab1p function causes a dramatic increase in vacuole size as well as a reduction in vacuolar hydrolytic activity, caused in part by a defect in acidification of the vacuole lumen[24]. Synthesis of PtdIns(3,5)P_2 is thus required for maintenance of normal vacuolar morphology and function.

PtdIns(3,5)P_2 might be required to control membrane-turnover pathways. Membrane proteins targeted for vacuolar degradation [including activated epidermal growth factor (EGF) receptors in mammalian cells and G-protein-coupled pheromone receptors in yeast] are sorted within multivesicular bodies (MVBs), which are formed by invagination of the endosomal membrane[26,27]. MVBs fuse with the vacuole to deliver their contents for degradation by the lipases and proteases within this organelle[28] (Fig. 2).

Membrane vesicles have been detected within the vacuole lumen of mutant yeast cells that have reduced vacuolar hydrolase activity[29]; however, their numbers are dramatically reduced in cells lacking Fab1p kinase activity[24]. In addition, *fab1* mutant cells have a selective defect in the sorting of cargo into the vacuole lumen: this is instead delivered to the outer limiting membrane of the vacuole[27]. Without PtdIns(3,5)P_2 synthesis, the delivery of additional membrane to the vacuole surface could, at least partially, contribute to the increase in vacuole size seen in *fab1* mutant cells.

Wortmannin inhibits the formation of specialized MVBs involved in antigen processing and presentation (MHC-class-II compartments), resulting in the accumulation of swollen endosomes[30]; this is consistent with a role for 3′-phosphorylated phosphoinositides in the regulation of MVBs. A mammalian homolog of Fab1p, PIKfyve, has recently been identified and shown to synthesize PtdIns(3,5)P_2 *in vitro*[31], but the role of PIKfyve in membrane trafficking has not yet been investigated.

As both a FYVE-domain-containing effector of PI 3-kinase signaling and a PtdIns3*P* 5-kinase, Fab1p functions at a pivotal regulatory step in the spatial and temporal control of protein sorting to the vacuole. Conversion of PtdIns3*P* to PtdIns(3,5)P_2 would terminate the recruitment or activation, or both, of other FYVE-domain-containing effector proteins that bind to PtdIns3*P*. Simultaneously, Fab1p activity would initiate a new PtdIns(3,5)P_2 signaling pathway. Fab1p might therefore function as a molecular switch that both inactivates the endosome as an acceptor compartment for Golgi-derived biosynthetic transport and also signals MVB formation and endosome maturation before docking and fusion with the vacuole.

As in the case of Vps34p, the PtdIns3*P* 5-kinase signaling of Fab1p might activate downstream effectors that interact directly with PtdIns(3,5)P_2 to stimulate endosome maturation and MVB sorting. Potential candidate effectors include one or more of a subset of Vps proteins that, like Fab1p, are needed for transport via the MVB sorting pathway[27]. PtdIns(3,5)P_2 could also be needed for membrane efflux from the vacuole. A recycling pathway from the vacuole to prevacuolar compartments requires the *VAC7* gene product, a transmembrane protein identified in a screen for mutants with defective vacuole inheritance[32]. Like *fab1* mutant cells, *vac7* mutants have enlarged vacuoles and fail to synthesize PtdIns(3,5)P_2 (Ref. 24), suggesting that Vac7p can regulate PtdIns(3,5)P_2 synthesis or turnover.

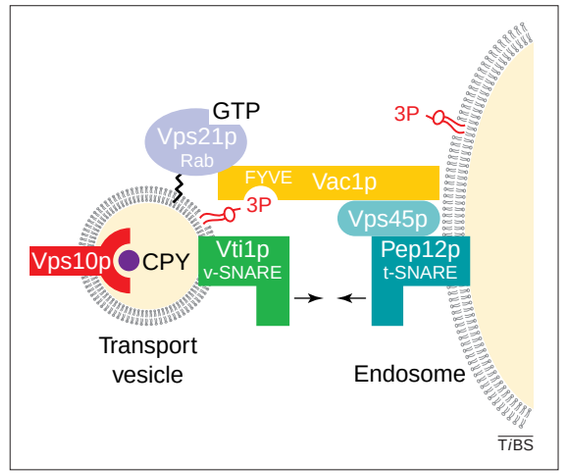

Figure 4

Vac1p functions as an effector of phosphatidylinositol (PI) 3-kinase and Rab GTPase signaling to regulate transport vesicle docking and fusion. Vac1p binds to PtdIns3*P* (via its FYVE domain) and to the activated, GTP-bound form of the Vps21p Rab5 homolog. Vac1p also interacts with Pep12p, the endosomal t-SNARE and with Vps45p, a Sec1 protein homolog required for SNARE-complex formation. Vac1p therefore functions as a multivalent adaptor protein that requires both PI 3-kinase and Rab GTPase signals for productive assembly of the docking and fusion complex. Although Vac1p is shown as binding to PtdIns3*P* on the transport vesicle, the endosomal pool of PtdIns3*P* might be important for binding or activating Vac1p, or both. The Vac1p-mediated assembly of this transport apparatus ensures the orderly progression of vesicle tethering, formation of the complex between t-SNARE (Pep12p) and v-SNARE (Vti1p), and vesicle fusion, which results in sorting of the carboxypeptidase Y (CPY) receptor, Vps10p, as well as other cargo proteins from the Golgi to the prevacuolar endosome. PtdIns3*P*, phosphatidyl inositol 3-phosphate.

Terminating phosphoinositide signaling: phosphoinositide phosphatases

To ensure their temporal and spatial specificity, phosphoinositide signaling pathways must be inactivated by a set of phosphoinositide turnover mechanisms. Consistent with this regulation, cellular levels of PtdIns3*P* are rapidly depleted when a temperature-sensitive *vps34* mutant is inactivated[5], indicating that the Vps34 PI 3-kinase directs the

Table 1. Relationships between phosphatidylinositol kinase signaling pathways and downstream effector functions

	PtdIns3*P*	PtdIns(3,5)P_2	PtdIns4*P*	PtdIns4,5P_2
Binding domain	RING-FYVE (~70 aa)	?	?	PH? (~100 aa)
Candidate effector proteins[a]	Vac1p, Vps27p, Fab1p	?	?	Pld1p, Bem2p/3p Boi1p/2p, Bud4p, Cdc24p, Rom2p, Stt4p
Membrane trafficking function	Vacuolar protein sorting	MVB sorting, vacuole size	Secretion	Actin cytoskeleton, endocytosis?

[a]Effector proteins in yeast indicated as having pleckstrin-homology (PH) domains have not been directly demonstrated to bind to phosphoinositides or shown to function directly in membrane trafficking. Abbreviation: PtdIns, phosphatidylinositol.

synthesis of a transient second-messenger molecule.

Conversion to PtdIns(3,5)P_2 represents one mechanism for inactivating PtdIns3*P* (Fig. 1) but not all PtdIns3*P* is phosphorylated by Fab1p[24], suggesting that other mechanisms operate in parallel to inactivate PI 3-kinase-mediated signaling. Surprisingly, elevated levels of PtdIns3*P* have been found in yeast mutants with defective vacuolar hydrolase activity, indicating that PtdIns3*P* is also turned over by vacuolar degradation[29]. MVBs are likely to transport a significant portion of PtdIns3*P* into the vacuole (Fig. 2), as turnover of this pool of PtdIns3*P* is blocked by late-acting *vps* mutations that also prevent the fusion of MVBs with the vacuole[29]. Because the recruitment of FYVE-domain-containing effector proteins occurs at the cytoplasmic face of the membrane, sorting of PtdIns3*P* into the internal membrane vesicles of MVBs is also an effective mechanism for attenuating PI 3-kinase signaling.

The termination of other phosphoinositide signaling pathways that regulate membrane trafficking appears to require cytoplasmic phosphatases. A role for inositol lipid phosphatases in vesicular transport was initially suggested by the finding that synaptojanin (a protein in mammalian nerve cells that, with dynamin, is thought to participate in synaptic-vesicle endocytosis and recycling) is an inositol-polyphosphate 5-phosphatase[33] (Inp5). Yeast cells have three genes that encode Inp5 proteins, also referred to as synaptojanin-like (Sjl) proteins (Table 2). As for synaptojanin in neurons, Sjl1p/Inp51p and Sjl2p/Inp52p in yeast appear to function in endocytosis[34,35].

Mammalian synaptojanin and the three yeast Inp5 proteins have a C-terminal domain containing two consensus sequences (HDVIFWLGDLNYRI and PAWTDRILY) that are signature motifs of inositol 5-phosphatases[36]. The 5-phosphatase domain from Sjl3p/Inp53p has been shown *in vitro* to convert PtdIns(4,5)P_2 to PtdIns4*P* but does not appear to use PtdIns4*P*, PtdIns3*P* or PtdIns(3,5)P_2 as a substrate[37].

In addition to having a C-terminal 5-phosphatase domain, mammalian synaptojanin and the Inp5 proteins in yeast contain an N-terminal domain that is also found in the yeast Sac1 protein (Table 2). This Sac1 domain contains the consensus sequence $Cx_5R(T/S)$, which is common to many other protein and inositol-polyphosphate phosphatases[37].

Table 2. Kinases and phosphatases involved in phosphoinositide synthesis and turnover in yeast

Protein	Function	Number of amino acids	Domain structure
Phosphoinositide kinases			
Vps34p	PI 3-kinase	875	
Pik1p	PI 4-kinase	1066	
Stt4p	PI 4-kinase	1900	
Fab1p	PtdIns3*P* 5-kinase	2278	
Mss4p	PtdIns4*P* 5-kinase	779	
Polyphosphoinositide phosphatases			
Sjl1p/Inp51p	PPI Ptase, 5-Ptase	946	
Sjl2p/Inp52p	PPI Ptase, 5-Ptase	1183	
Sjl3p/Inp53p	PPI Ptase, 5-Ptase	1107	
Inp54p	PPI 5-Ptase	384	
Sac1p	PPI Ptase	623	
Fig4p	PPI Ptase	879	

PI kinase domain; PIP kinase domain; Sac1 domain (PPI Ptase); PPI 5-Ptase domain; FYVE domain; PH domain; transmembrane domain

Abbreviations: PH, pleckstrin-homology; PI and PtdIns, phosphatidylinositol; PPI, polyphosphoinositide; Ptase, phosphatase

In contrast to the 5-phosphatase domain, however, purified recombinant Sac1 domains from both Inp53p and synaptojanin can dephosphorylate PtdIns4*P*, PtdIns3*P* and PtdIns(3,5)P_2 but not PtdIns(4,5)P_2 (Ref. 37). Thus, both a PtdIns(4,5)P_2 5-phosphatase activity and a polyphosphoinositide-phosphatase activity are coupled in the synaptojanin-like proteins, which could be critical for localized phosphoinositide turnover.

However, not all phosphoinositide phosphatases in yeast exhibit this apparent dual functionality. Inp54p contains a 5-phosphatase domain but not a Sac1 domain (Table 2) and, although Inp51p contains both Sac1 and 5-phosphatase domains, its Sac1 domain has multiple substitutions of conserved residues important for catalytic activity[36,37]. Similarly, neither Sac1p nor Fig4p (another protein in yeast that contains a Sac1 domain) contain a C-terminal 5-phosphatase domain (Table 2).

Mutations in *SAC1* have been found to suppress a deletion of *SEC14*, enabling cells to maintain Golgi secretory function in the absence of Sec14p PtdIns or phosphatidylcholine (PtdCho) binding and transfer activity[38]. How might cells overcome the requirement for *SEC14*, a gene that is normally essential for viability? In addition to the *sac1* mutation, a loss of Sec14p function can be bypassed by mutations that block PtdCho biosynthesis via the cytosine 5′-diphosphate (CDP)–choline pathway[39]. In both cases, the Sec14p bypass requires phospholipase D (PLD) activity, which hydrolyses PtdCho to yield phosphatidic acid (PA)[40,41]. Dephosphorylation of PA produces DAG, which can then be utilized by the CDP–choline pathway, suggesting that DAG consumption via this route limits Golgi secretory activity and contributes to the lethality observed in *sec14* mutant cells.

The role of DAG in secretory-vesicle formation, however, has been controversial. Although overexpression of a bacterial DAG kinase (which converts DAG back to PA) prevents cells from bypassing the *sec14* mutation[42], increased DAG levels are not observed in either *sac1* mutant cells[43] or cells with mutations that block the CDP–choline pathway[40]. Furthermore, DAG kinase added to permeabilized mammalian cells stimulated PLD-dependent secretory-vesicle formation more than did adding a PtdIns-specific phospholipase C that generates DAG (Ref. 44). Thus, increased levels of PA, and not DAG, might be responsible for maintaining secretory competence in the absence of Sec14p function in yeast.

A role for DAG in Golgi secretory function appeared to gain support from the finding that *sac1* mutant cells accumulated sixfold higher levels of mannosyldiinositolphosphorylceramide [M(IP)$_2$C], an inositol sphingolipid[42]. Because two molecules of DAG are produced for every molecule of M(IP)$_2$C synthesized, this apparent observation explained why a loss of Sac1p function could lead to elevated DAG production. More recent studies, however, have showed that this inositol lipid had been misidentified[42] and that *sac1* mutant cells actually accumulate PtdIns4*P* (Refs. 43,45).

The increased levels of PtdIns4*P* observed upon loss of Sac1p function in *sec14* mutant cells is consistent with the recent finding that the Sac1 domain has PtdIns4*P*-phosphatase activity[37]. Nevertheless, the connection between PtdIns4*P* synthesis and Sec14p function is still unclear. For example, PLD activity remains essential for the *sac1* mutation to cause a bypass of the Sec14p requirement[45], indicating that increases in PtdIns4*P* levels cannot be solely responsible for relieving the secretory defects observed in *sec14* mutant cells. In addition, expression of a mutant *sec14* allele that binds PtdCho but not PtdIns can rescue a deletion of the *SEC14* gene independently of PLD activity[46]. This surprising observation not only confirms that the PtdIns binding and transfer activity of Sec14p can be dispensable (which could have been assumed because of the ability to isolate mutants that bypass a *SEC14* deletion) but also indicates that PtdCho-bound Sec14p can function effectively without PLD-mediated production of PA or DAG. Because PtdCho-bound Sec14p has been found to inhibit the CDP–choline pathway (and, hence, DAG consumption) directly[47], it remains possible that DAG plays a significant role in Golgi secretory-vesicle formation.

PI 4-kinase regulation of secretion

A role for PtdIns4*P* synthesis in regulating secretion is supported by a recent analysis of phosphoinositide levels in temperature-sensitive *sec14* mutant cells, in which a specific decrease in the level of PtdIns4*P* was demonstrated upon inactivation of Sec14p[48]. This finding is consistent with the observation that PtdIns-bound Sec14p stimulates PtdIns4*P* synthesis *in vivo*[46]. Binding or transfer of PtdIns by Sec14p therefore appears to have a role in PtdIns4*P* synthesis, which might directly affect Golgi secretory function.

Recent studies strongly suggest that the pool of PtdIns4*P* affected by Sec14p function is synthesized by Pik1p, one of two PI 4-kinases in yeast (Table 2). Decreased PtdIns4*P* synthesis in temperature-sensitive *pik1* mutant cells has been found to correlate with a defect in protein secretion (Ref. 48; A. Audhya and S. Emr, unpublished). In addition, overexpression of Pik1p was found to alleviate the temperature-sensitive growth defects observed in *sec14* mutant cells, whereas overexpression of the other PI 4-kinase in yeast, Stt4p, had no effect[48].

Examination of *pik1* mutant cells by electron microscopy revealed the accumulation of 'Berkeley bodies', unusual membrane structures that are thought to represent abnormal Golgi cisternae[49]. Consistent with a defect in Golgi morphology and transport function, *pik1* mutant cells were also found to have a partial defect in protein transport from the Golgi to the vacuole (Ref. 49; A. Audhya and S. Emr, unpublished). Recent studies have shown that the mammalian homolog of Pik1p, PI 4-kinase β and an unidentified PtdIns4*P* 5-kinase can be recruited onto isolated Golgi membranes by ARF, which stimulates the synthesis of PtdIns4*P* and PtdIns(4,5)P_2[50]. Indirect immunofluorescence analysis has localized Pik1p at the Golgi in yeast, although a significant amount was also found within the nucleus[49]. Localization of Pik1p at the Golgi might depend upon its interaction with Frq1p, a small myristoylated calcium-binding protein that stimulates the PI 4-kinase activity of Pik1p[51].

Future directions

Genetic studies in yeast are beginning to reveal how distinct pathways for synthesis and turnover of phosphoinositides can regulate different membrane-trafficking pathways in eukaryotic cells. We now have a general understanding of how PtdIns3*P* synthesis controls vesicle-mediated protein transport to the vacuole. Recent studies have also uncovered roles for PtdIns(3,5)P_2 and PtdIns4*P* in MVB sorting and secretion, respectively.

However, many important questions remain concerning phosphoinositide regulation of membrane trafficking. For example, what effectors function downstream of Fab1 PtdIns3*P* 5-kinase and Pik1 PI 4-kinase signaling? Does the other PI 4-kinase in yeast, Stt4p, have a role in membrane trafficking? In mammalian cells, PtdIns4,P_2 synthesis is required for regulated fusion of secretory granules with the plasma membrane and for clathrin-mediated endocytosis[3] but does PtdIns(4,5)P_2 have a role in vesicular transport in yeast? Which of the phosphoinositide phosphatases regulate vesicular transport and how is their localization and activation controlled? The role of phosphoinositide turnover in membrane trafficking is also largely unexplored. If the past is any indication, then studies in yeast should continue to provide answers to many of these questions.

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