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Phosphoinositide signaling and turnover: PtdIns(3)P, a regulator of membrane traffic, is transported to the vacuole and degraded by a process that requires lumenal vacuolar hydrolase activities

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The Golgi/endosome-associated Vps34 phosphatidylinositol 3-kinase is essential for the sorting of hydrolases from the Golgi to the vacuole/lysosome. Upon inactivation of a temperature-conditional Vps34 kinase, cellular levels of PtdIns(3)P rapidly decrease and it has been proposed that this decrease is due to the continued turnover of PtdIns(3)P by cytoplasmic phosphatases. Here we show that mutations in *VAM3* (vacuolar t-SNARE) and *YPT7* (rab GTPase), which are required to direct protein and membrane delivery from prevacuolar endosomal compartments to the vacuole, dramatically increase/stabilize PtdIns(3)P levels *in vivo* by disrupting its turnover. We find that the majority of the total pool of PtdIns(3)P which has been synthesized, but not PtdIns(4)P, requires transport to the vacuole in order to be turned over. Unexpectedly, strains with impaired vacuolar hydrolase activity accumulate 4- to 5-fold higher PtdIns(3)P levels than wild-type cells, suggesting that lumenal vacuolar lipase and/or phosphatase activities degrade PtdIns(3)P. Because vacuolar hydrolases act in the lumen, PtdIns(3)P is likely to be transferred from the cytoplasmic membrane leaflet where it is synthesized, to the lumen of the vacuole. Interestingly, mutants that stabilize PtdIns(3)P accumulate small uniformly-sized vesicles (40–50 nm) within prevacuolar endosomes (multivesicular bodies) or the vacuole lumen. Based on these and other observations, we propose that PtdIns(3)P is degraded by an unexpected mechanism which involves the sorting of PtdIns(3)P into vesicles generated by invagination of the limiting membrane of the endosome or vacuole, ultimately delivering the phosphoinositide into the lumen of the compartment where it can be degraded by the resident hydrolases.

Keywords: endocytosis/endosome/phosphatidylinositol 3-kinase/proteinase/*VPS34*

Introduction

The yeast vacuole is a hydrolytically active organelle that is functionally analogous to the mammalian lysosome (Klionsky *et al*., 1990). Proteins designated Vps (vacuolar protein sorting) mediate the transport of newly synthesized hydrolase precursors from the late Golgi to the vacuole via an intermediate endosome compartment (Vida *et al*., 1993). Mutations within *VPS* genes result in hydrolase mis-sorting and secretion as well as abnormal vacuole morphologies. Based on these mutant phenotypes, >40 *VPS* genes have been subgrouped into six major classes, A–F (Raymond *et al*., 1992). *VPS34* is a class D gene which encodes a phosphatidylinositol 3-kinase (PtdIns 3-kinase) (Herman and Emr, 1990; Schu *et al*., 1993). Class D *vps* mutants exhibit severe defects in hydrolase sorting, contain enlarged vacuoles and cannot grow, or grow poorly, at elevated temperatures (Herman and Emr, 1990; Raymond *et al*., 1992).

Vps34p phosphorylates phosphatidylinositol (PtdIns) at the D-3 position of the inositol ring and represents the only detectable PtdIns 3-kinase activity in *Saccharomyces cerevisiae* (Schu *et al*., 1993). Vps34p is recruited from the cytosol to the membrane-associated serine/threonine protein kinase, Vps15p (Herman *et al*., 1991; Stack *et al*., 1993). Although it is not yet clear how Vps15p is activated, Vps15p protein kinase activity is a prerequisite to the formation of a Vps15p–Vps34p complex (Stack *et al*., 1995). This interaction not only localizes Vps34p to the correct membrane compartment, but also stimulates its PtdIns 3-kinase activity >10-fold (Stack *et al*., 1995). Consistent with the role of Vps15p as an upstream regulator of Vps34p, inactivation of Vps15p results in severe decreases in cellular levels of phosphatidylinositol 3-phosphate [PtdIns(3)P] and also results in the mis-sorting of vacuole hydrolases (Herman *et al*., 1991; Stack *et al*., 1995).

Vps34p is a member of a growing kinase family that phosphorylates PtdIns. These phosphorylated derivatives of PtdIns serve as second messengers which bind effector proteins, recruiting these proteins to specific subcellular localizations and/or influencing their activity (Toker and Cantley, 1997; Vanhaesebroeck *et al*., 1997). For example, 3-phosphorylated derivatives of PtdIns bind p85/p110 phosphoinositide 3-kinase, PDK1, Akt and the AP-2 adaptor complex, accounting, at least in part, for the effects of PI3-kinases on cell growth, apoptosis and membrane trafficking, respectively (Rameh *et al*., 1995; Franke *et al*., 1997; Rapoport *et al*., 1997; Stephens *et al*., 1998). Based on these and other studies, lipid kinases are recognized as an important part of the machinery that controls a diverse array of cellular functions via the formation of various phosphoinositide second messengers. PtdIns(3)P produced by Vps34p is also likely to function as a second messenger, although the relevant target(s) of PtdIns(3)P in yeast has not yet been identified.

The discovery of specific cytoplasmic lipases and phosphatases which antagonize the activity of lipid kinases reveals the requirement of the cell to downregulate and modify signals mediated by phosphoinositides. For example, cleavage of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] to distinct second messengers is carried out by phospholipase C in response to tyrosine-

kinase- and G-protein-coupled receptor activation (Rhee, 1991; Sternweis and Smrcka, 1992). In addition, type II 5-phosphatases like synaptojanin and OCRL may also play roles in the turnover of PtdIns(4,5)P_2 (Attree *et al*., 1992; McPherson *et al*., 1996). However, to date, 3-phosphorylated phosphoinositides have not been found to be good substrates of phospholipase C (Serunian *et al*., 1989; Woscholski *et al*., 1995). Instead, a family of proteins, including SHIPs, which exhibit phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase activity *in vitro* have been isolated (Woscholski and Parker, 1997). In addition, cytoplasmic 3-phosphatase activities have been detected (Woscholski *et al*., 1995) and purified (Caldwell *et al*., 1991). However, their precise role in the turnover of PtdIns(3)P is still unclear.

In *S.cerevisiae*, it is likely that a specific turnover mechanism exists for PtdIns(3)P because PtdIns(3)P levels decrease significantly within 10 min after inactivation of Vps34p (Stack *et al*., 1995). Here, we present evidence demonstrating that PtdIns(3)P is degraded by an unexpected mechanism. We have found that interruption of endosome-to-vacuole transport leads to elevations in PtdIns(3)P levels by disrupting the turnover of PtdIns(3)P. Turnover of PtdIns(3)P is also dependent upon vacuolar hydrolase activity, strongly suggesting that PtdIns(3)P degradation occurs within the hydrolytic environment of the vacuole/lysosome lumen. We propose that a significant pool of PtdIns(3)P shifts from a cytoplasmic membrane leaflet where PtdIns(3)P is produced, to the lumen of the vacuole where it can be exposed to and turned over by active vacuolar enzymes. We present evidence indicating that lipids in the cytoplasmic leaflet of the membrane are transferred to the lumen of the vacuole through the invagination and budding of vesicles into the lumen of the vacuole or prevacuolar endosomes which then fuse with the vacuole. Inactivation of Vps34p results in the impaired fusion of endosomal transport intermediates with the vacuole. Therefore, PtdIns(3)P is not only a cargo of the endosome, but may also regulate this late step in the vacuolar protein transport pathway.

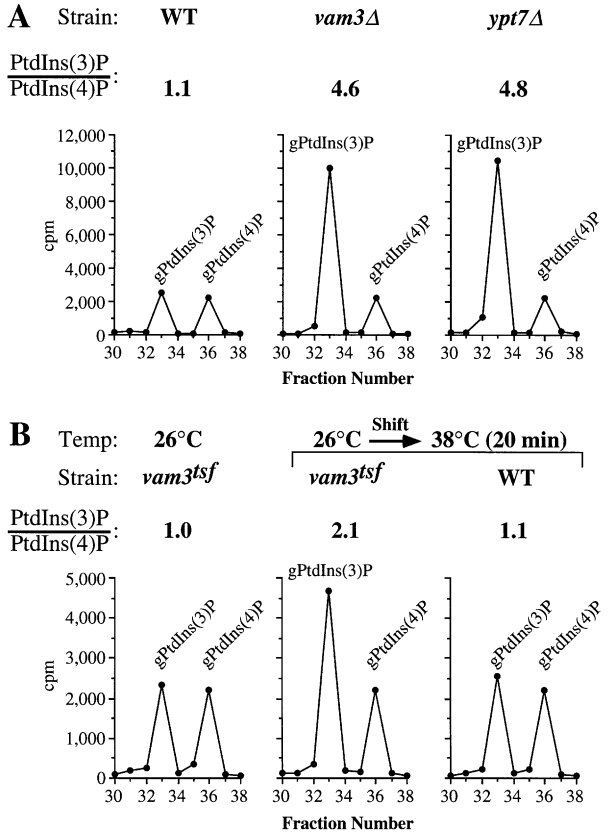

Fig. 1. Cellular PtdIns(3)P levels increase in *vam3* and *ypt7* mutant strains. (**A**) Wild-type cells, *vam3*Δ cells and *ypt7*Δ cells were labeled with myo-[2-^{3}H]inositol for 12 h, lysed and extracted with chloroform/methanol. After deacylation, lipids were separated by HPLC yielding peaks corresponding to PtdIns(3)P and PtdIns(4)P. (**B**) Wild-type cells and two aliquots of *vam3^{tsf}* cells were labeled at 26°C with myo-[2-^{3}H]inositol for 12 h. One aliquot of *vam3^{tsf}* cells was maintained at the permissive temperature of 26°C while the second aliquot was shifted to the nonpermissive temperature of 38°C for the final 20 min of labeling. Wild-type cells were also shifted to 38°C for the final 20 min of labeling. Following cell lysis, lipids were extracted in chloroform/methanol, deacylated and analyzed by HPLC. Deacylated PtdIns(3)P and PtdIns(4)P peaks are indicated. The data presented are representative of multiple experiments.

Results

Inactivation of the vacuolar t-SNARE, Vam3p, or the rab GTPase, Ypt7p, results in elevated PtdIns(3)P levels

Vam3p (vacuolar t-SNARE) and Ypt7p (yeast rab7 GTPase) are essential factors in endosome-to-vacuole trafficking (Wichmann *et al*., 1992; Darsow *et al*., 1997). We have found that the mutation of *VAM3* or *YPT7* has strong effects on steady-state PtdIns(3)P levels *in vivo*. To assay PtdIns(3)P levels, SEY6210 (wild-type), TDY2 (*vam3*Δ) and WSY99 (*ypt7*Δ) cells were labeled with myo-[2-^{3}H]inositol for 12 h, lysed, and the cellular lipids extracted with chloroform/methanol. Lipids were then deacylated, separated by HPLC (see Materials and methods), and the amount of radioactivity in each fraction collected was quantified. This system clearly separates PtdIns(3)P from phosphatidylinositol 4-phosphate [PtdIns(4)P] (Schu *et al*., 1993). Wild-type cells had ~2200 c.p.m. of ^{3}H incorporated into both PtdIns(3)P (fraction 33) and PtdIns(4)P (fraction 36), yielding a PtdIns(3)P: PtdIns(4)P ratio of 1.1 (Figure 1A). Deletion of *VAM3* or *YPT7* resulted in significant increases in PtdIns(3)P levels, without affecting PtdIns(4)P levels, which translate to 4.6- and 4.8-fold increases, in the PtdIns(3)P: PtdIns(4)P ratio, respectively.

Previous work has described the isolation of a temperature-sensitive allele of the *VAM3* gene which, at the nonpermissive temperature, inhibits the transport of hydrolases to the vacuole (Darsow *et al*., 1997). As an additional means of compromising Vam3p function, without causing secondary effects which may result from the deletion of *VAM3*, PtdIns(3)P levels were also measured in TDY2 + *pVAM3-6.416* (*vam3^{tsf}*) cells briefly shifted to the nonpermissive temperature. At the permissive temperature (26°C), *vam3^{tsf}* cells exhibited wild-type PtdIns(3)P levels (Figure 1B). However, *vam3^{tsf}* cells grown at 26°C for 12 h and then shifted to the nonpermissive temperature (38°C) for 20 min prior to the assay, exhibited a 2-fold increase in PtdIns(3)P and no change in PtdIns(4)P levels. Wild-type cells shifted to 38°C for 20 min did not exhibit a significant elevation in PtdIns(3)P. *VAM3* and *YPT7* are considered a subset of class B *VPS*

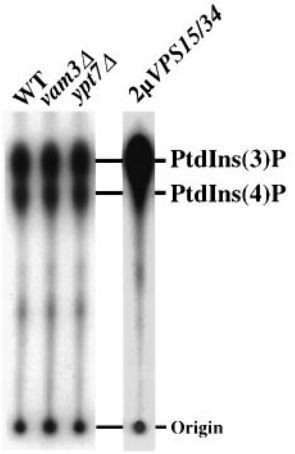

Fig. 2. Vps34p PtdIns 3-kinase activity is not affected in *vam3*Δ or *ypt7*Δ mutant strains. Protein extracts were prepared from wild-type cells and strains deleted for *VAM3* or *YPT7*. PtdIns 3-kinase activity present was measured by incubating 2 μg of protein extract with [γ-^{32}P]ATP and sonicated PtdIns for 5 min at 25°C. The lipid products of this reaction were resolved by TLC and analyzed by autoradiography (equal film exposures are shown). PtdIns(3)P and PtdIns(4)P are indicated.

genes (Darsow *et al*., 1997). We also tested whether inactivation of *VPS33*, a class C *VPS* gene that functions at a late stage of vacuolar protein sorting (Banta *et al*., 1990), had an effect on PtdIns(3)P levels. Similar to the *vam3^{tsf}*, the LBY317 + *pVPS33-8.416* (*vps33^{tsf}*) strain when shifted to the nonpermissive temperature for 15 min resulted in a 2-fold increase in PtdIns(3)P levels (data not shown). These data indicate that the mutation of either *VAM3*, *YPT7* or *VPS33* results in increased PtdIns(3)P levels, perhaps by disrupting trafficking to the vacuole.

Deletion of VAM3 and YPT7 do not influence Vps34p PtdIns 3-kinase activity

Vps34p is the only detectable PtdIns 3-kinase in *S.cerevisiae* (Schu *et al*., 1993). This suggests that one possibility by which mutations of *VAM3* and *YPT7* may increase steady-state PtdIns(3)P levels is by increasing Vps34p activity. Alternatively, deletion of these genes may impair the turnover of PtdIns(3)P. To differentiate between these possibilities, protein extracts from wild-type, *vam3*Δ and *ypt7*Δ cells were assayed for relative levels of PtdIns 3-kinase activity (see Materials and methods). When incubated with PtdIns and [γ-^{32}P]ATP, 2 μg of protein extracts from wild-type, *vam3*Δ and *ypt7*Δ strains all contained equal levels of PtdIns 3-kinase activity, suggesting that mutations in *VAM3* and *YPT7* do not increase steady-state PtdIns(3)P levels by stimulating Vps34p (Figure 2). In contrast, 2 μg of protein extract from cells co-overexpressing the *VPS15* and *VPS34* genes exhibited >10-fold increases in PtdIns 3-kinase activity under identical assay conditions and with the same film exposure (Figure 2). This suggests that deletion of *VAM3* and *YPT7* inhibits the turnover of PtdIns(3)P rather than increasing its synthesis.

Vacuolar hydrolase activity is required for PtdIns(3)P turnover

Since endosomal transport intermediates fail to fuse with the vacuole and accumulate in the cytoplasm of *vam3* and *ypt7* mutants (Schimmoller and Riezman, 1993; Darsow *et al*., 1997), our data indicate that endosome-to-vacuole trafficking may be required for PtdIns(3)P turnover. One interpretation of these findings is that PtdIns(3)P could be degraded at the vacuole. This implies that deletion of either *VAM3* or *YPT7* may elevate PtdIns(3)P levels by spatially separating PtdIns(3)P in a prevacuolar compartment (e.g. endosome) from its turnover enzymes. If this hypothesis is correct, then the hydrolytic activity of the vacuole might be required for the turnover of PtdIns(3)P.

PEP4, *PRB1* and *PRC1* encode the vacuolar proteinase A (PrA), proteinase B (PrB) and carboxypeptidase Y (CPY) hydrolases, respectively. PrA and PrB are important to the overall hydrolytic activity of the vacuole as they function to cleave proteolytically a variety of inactive vacuolar zymogens, including proteases (like CPY) and phosphatases (like alkaline phosphatase), to their mature, active forms (Klionsky and Emr, 1989; Hirsch *et al*., 1992; van den Hazel *et al*., 1992). Vacuolar enzymes can be separated into two broad classes: (i) PrA and PrB which activate other vacuolar enzymes; and (ii) the remaining proteases, lipases and phosphatases which, upon activation by PrA and/or PrB, carry out the bulk metabolism of their relevant substrates (Klionsky *et al*., 1990; van den Hazel *et al*., 1996).

In order to test the possibility that PtdIns(3)P is turned over in a vacuolar hydrolase-dependent manner, steady-state PtdIns(3)P levels were measured in cells that are deficient in vacuolar hydrolase activity. Representative data from multiple analyses are shown in Figure 3. While wild-type cells had normal levels of PtdIns(3)P, TVY614 cells (*pep4*Δ*/prb1*Δ*/prc1*Δ), a strain simultaneously deleted for *PEP4*, *PRB1* and *PRC1*, had a >5-fold stabilization of PtdIns(3)P (Figure 3). Vps34p PtdIns 3-kinase activity was not affected in the *pep4*Δ*/prb*Δ*/prc1*Δ strain (data not shown). This suggests that PtdIns(3)P turnover occurs in a hydrolase-dependent manner and that either PrA, PrB or CPY, or a combination of these hydrolases, participate in the activation of an enzyme(s) involved in PtdIns(3)P turnover. Given the role of PrA and PrB in the activation of vacuolar enzymes, it is likely that PrA and/or PrB, but not hydrolases like CPY or carboxypeptidase S (CPS) (Spormann *et al*., 1991), would be important to the maturation of PtdIns(3)P turnover proteins at the vacuole. Consistent with this, we found that PtdIns(3)P levels also increased 3.5-fold in TVY1 (*pep4*Δ) and 4-fold in TVY2 (*prb*Δ) cells lacking only PrA or PrB, respectively. This demonstrates that both PrA and PrB activity are important factors in PtdIns(3)P turnover. Deletion of *PRC1* or *CPS1* did not result in a change in PtdIns(3)P levels demonstrating that not all protease activities are required for the turnover of PtdIns(3)P. When we extend our analysis to fraction 75, wild-type and *prb1*Δ cells also exhibited peaks of similar magnitude (~400 c.p.m.) which co-eluted with [^{3}H]PtdIns(4,5)P_2 standards (data not shown). Therefore, decreases in vacuolar hydrolase activity specifically affect PtdIns(3)P since neither PtdIns(4)P (Figure 3) or PtdIns(4,5)P_2 levels were influenced in *prb1*Δ cells.

We also tested a strain deleted for the *VMA4* gene, which encodes a component of the vacuolar ATPase (Ho *et al*., 1993). Mutation of *VMA4* inactivates the vacuolar ATPase and prevents vacuole acidification (Morano and Klionsky, 1994). Studies indicate that certain vacuolar enzymes, including PrA, are sensitive to increases in pH

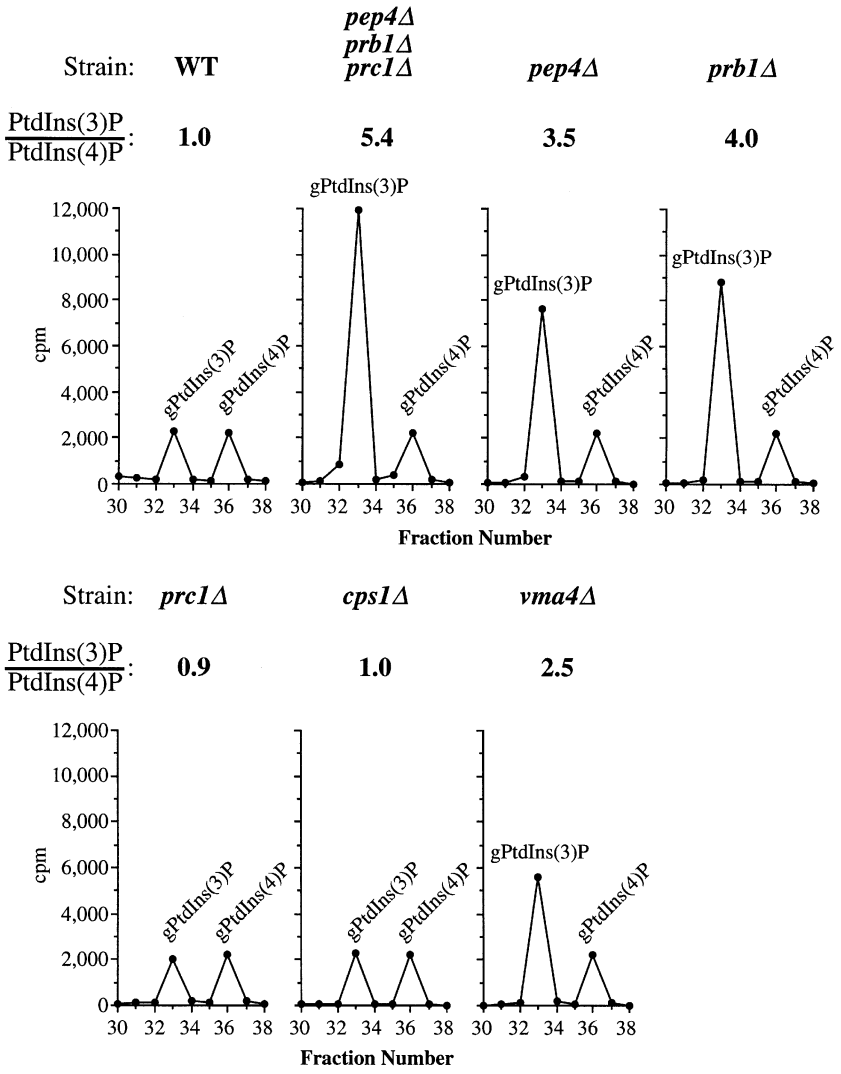

Fig. 3. Cellular PtdIns(3)P levels increase in strains that are deficient in vacuolar hydrolase activity. The indicated strains, including TVY6 (*prc1*Δ) and CCY241 (*cps1*Δ), were labeled for 12 h with myo-[2-^{3}H]inositol, lysed and extracted in chloroform/methanol. Lipids were deacylated and separated by HPLC. Representative data from several experiments are shown. PtdIns(3)P and PtdIns(4)P are indicated.

(Sorensen *et al*., 1994), and thus are 30–40% less active (Morano and Klionsky, 1994). Consistent with this moderate effect on vacuolar hydrolase activity, deletion of *VMA4* caused a 2.5-fold stabilization of PtdIns(3)P. Together, these results indicate that PtdIns(3)P is delivered to the vacuole and turned over by a PrA- and PrB-dependent pathway. Because PtdIns(3)P is synthesized in the cytoplasmic leaflet of the membrane (Herman *et al*., 1991), and PrA and PrB are active in the lumen of the vacuole, our data suggest that it might be necessary to transfer PtdIns(3)P to the interior of the vacuole in order for it to be exposed to and turned over by active hydrolases.

A critical step in shifting lipids of the cytoplasmic leaflet to the lumen of an organelle may entail the incorporation of such lipids into membrane invaginations. Unlike wild-type cell vacuoles, which appear as dark, electron-dense compartments when viewed by electron microscopy (Banta *et al*., 1988), an interesting feature of the vacuoles in *vma4*Δ cells is that they are electron-transparent. This revealed the presence of intravacuolar vesicles (Figure 4A and B). These vesicles are uniform in appearance, being 40–50 nm in diameter and having a single limiting membrane. Membrane tubules, which when cross-sectioned should sometimes appear as elongated structures, were not observed in the vacuoles of *vma4*Δ cells prepared by this methodology (see Materials and methods). Like wild-type cells, *prb1*Δ cells exhibited electron-dense vacuoles that mask their intralumenal contents (data not shown). It is possible that these vesicles are derived from vacuolar invaginations or endosomal multivesicular bodies (MVBs) which are thought to fuse with lysosomal/vacuolar compartments, depositing vesicles into the lumen of the lysosome/vacuole (Futter *et al*., 1996; Cowles *et al*., 1997b). Interestingly, the *vam3^{tsf}* mutant stabilized PtdIns(3)P (Figure 1B) and also accumulates prevacuolar MVBs (Darsow *et al*., 1997). If PtdIns(3)P is a component of these vesicles then PtdIns(3)P, together with these vesicles, could be degraded within the lumen of the vacuole (see Discussion).

A major pool of PtdIns(3)P is turned over at the vacuole

To address whether the vacuole represents a major site for PtdIns(3)P turnover or whether the vacuole is respons-

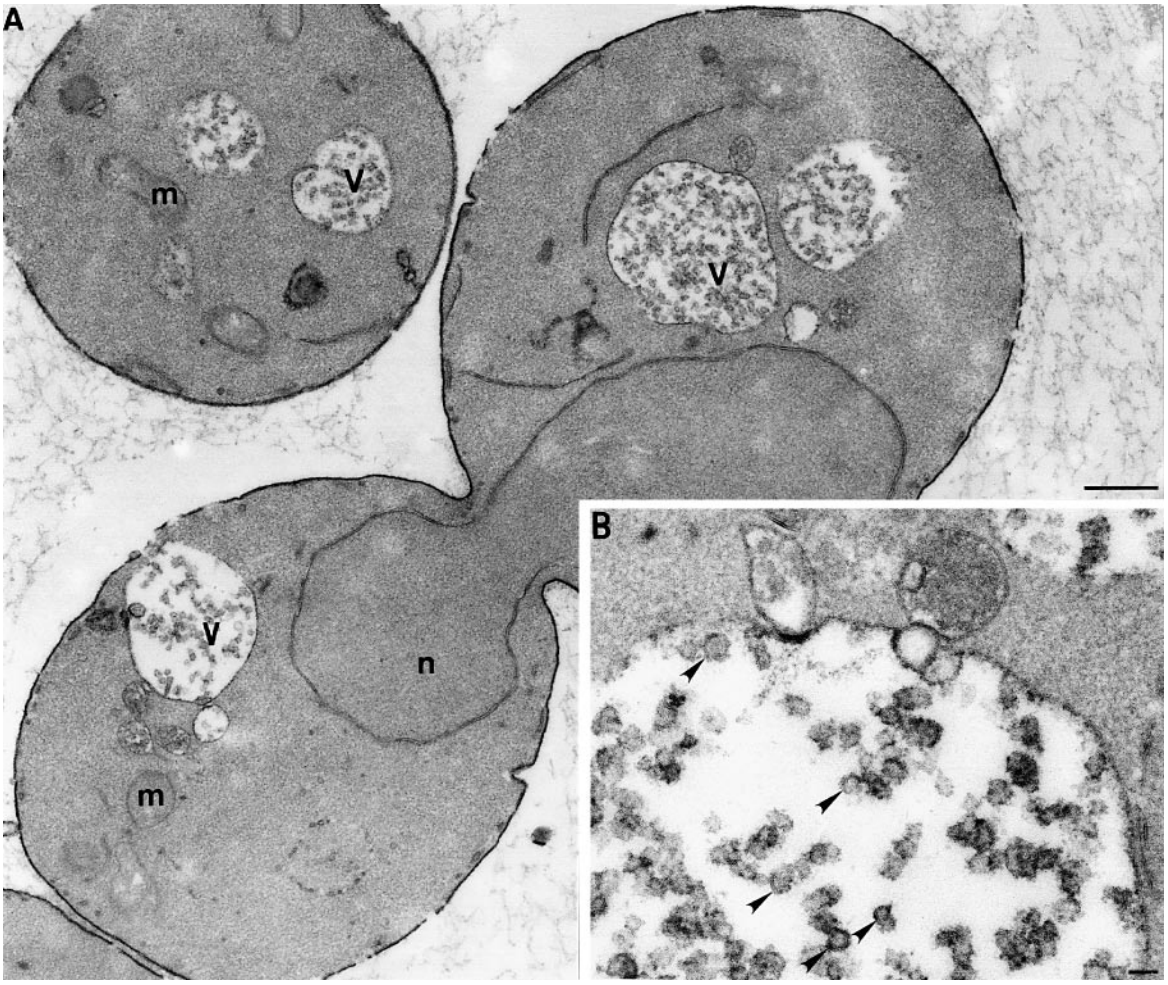

Fig. 4. Electron microscopic analysis of *vma4*Δ mutant cells reveals intravacuolar vesicles. *vma4*Δ mutant cells were grown to mid-log phase, fixed and visualized by electron microscopy. Vesicles are indicated by arrowheads. (**A**) Whole-cell image (bar = 0.5 μm). (**B**) High magnification image of vacuolar vesicles (bar = 0.1 μm). V, vacuole; M, mitochondria; N, nucleus.

ible for degrading only a small portion of the total pool of PtdIns(3)P produced by Vps34p, PtdIns(3)P levels were measured in *vps34^{tsf}/vam3^{tsf}* double-mutant cells. At the restrictive temperature, both production of PtdIns(3)P and the transport of PtdIns(3)P to its site of turnover at the vacuole is expected to be blocked, revealing the contribution of the vacuolar degradative pathway to total PtdIns(3)P turnover.

Accordingly, PtdIns(3)P levels were measured in *vps34^{tsf}*, *vam3^{tsf}* and in AWY1 (*vps34^{tsf}/vam3^{tsf}*) double-mutant cells after shifting these strains to the nonpermissive temperature for 0, 10 and 20 min. At the permissive temperature of 26°C, *vps34^{tsf}* cells, *vam3^{tsf}* cells and the *vps34^{tsf}/vam3^{tsf}* double-mutant all had relatively normal PtdIns(3)P levels (Figure 5, 0 min). As previously observed, cells expressing the *vps34^{tsf}* allele exhibited ~25% decreases in PtdIns(3)P, even at the permissive temperature (Stack *et al*., 1995). After a brief, 10-min shift to the nonpermissive temperature of 38°C, there was a 2-fold decrease in PtdIns(3)P levels in *vps34^{tsf}* cells while *vam3^{tsf}* cells exhibited a 1.7-fold increase in PtdIns(3)P (Figure 5, 10 min). Thus, significant decreases in PtdIns(3)P were observed within 10 min after shifting the *vps34^{tsf}* strain to the nonpermissive temperature, suggesting that PtdIns(3)P turnover and the maturation of

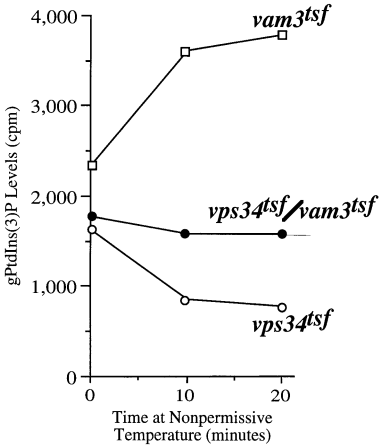

Fig. 5. PtdIns(3)P levels are stabilized in cells that are simultaneously compromised in PtdIns 3-kinase activity and PtdIns(3)P transport to the vacuole. PtdIns(3)P levels were assayed in *vps34^{tsf}* cells, *vam3^{tsf}* cells and in *vps34^{tsf}/vam3^{tsf}* double mutants. *vps34^{tsf}*, *vam3^{tsf}* and *vps34^{tsf}/vam3^{tsf}* strains were labeled for 12 h with myo-[2-^{3}H]inositol at the permissive temperature, 26°C, and shifted to the nonpermissive temperature of 38°C for 0, 10 or 20 min. Lipids extracted from cells after lysis were deacylated and resolved by HPLC. Levels of deacylated PtdIns(3)P are shown as a function of time at the nonpermissive temperature.

precursor CPY (half-life, 6–8 min) occur at similar rates (Klionsky *et al*., 1988). PtdIns(4)P levels were not affected upon temperature shift in this experiment (data not shown). However, even after 20 min at 38°C, PtdIns(3)P levels remained nearly constant in the *vps34^{tsf}/vam3^{tsf}* double-mutant cells (Figure 5, 20 min). The same was true after 40 min at the nonpermissive temperature, the longest temperature-shift carried out (data not shown). Together, these data indicate that upon inactivation of the Vps34 PtdIns 3-kinase, the *vam3^{tsf}* mutant transport block stabilized the bulk of PtdIns(3)P. The vacuole, therefore, represents a major site for PtdIns(3)P turnover in yeast.

Vacuolar transport of PtdIns(3)P requires the CPY sorting pathway but not the alkaline phosphatase, late secretory or early endocytic pathways

Multiple transport pathways to the vacuole have been documented. The CPY pathway traffics hydrolases such as CPY and CPS to the vacuole but not alkaline phosphatase (ALP), which instead relies on a distinct ALP pathway (Cowles *et al*., 1997b; Piper *et al*., 1997). The Ste6 protein (plasma membrane **a**-factor transporter), in contrast, follows the secretory pathway to the plasma membrane and then the endocytic pathway to the vacuole where it is degraded (Kolling and Hollenberg, 1994). The subset of class B genes represented by *VAM3* and *YPT7* are required for a late stage along many of these transport routes (Wichmann *et al*., 1992; Darsow *et al*., 1997). Therefore, it is possible that PtdIns(3)P traffics via the CPY pathway, the ALP pathway, the endocytic pathway or a combination of these pathways to the vacuole. To determine more precisely which of the Vam3p- and Ypt7p-dependent transport pathways are required to deliver PtdIns(3)P to the vacuole, we investigated whether specific blocks in a subset of these membrane-trafficking pathways could influence endogenous PtdIns(3)P levels.

Vps4p, a class E Vps protein which encodes an ATPase, mediates trafficking along the CPY pathway and a late step of endocytosis but not the transport of ALP to the vacuole (Munn and Riezman, 1994; Babst *et al*., 1997). Vps4p activity is required for endosome function, and shifting the *vps4^{ts}* strain to the nonpermissive temperature results in the accumulation of endosomal structures which contain CPY (Babst *et al*., 1997). When the *vps4^{ts}* strain was labeled with myo-[2-^{3}H]inositol at the permissive temperature and shifted, for the final 15 min of labeling, to the nonpermissive temperature, we observed a 1.4-fold increase in PtdIns(3)P (Figure 6). This suggests that at least a pool of PtdIns(3)P transits a Vps4p endosome compartment and that the CPY and/or the endocytic pathway are required to transport PtdIns(3)P to the vacuole. To determine if PtdIns(3)P is secreted to the plasma membrane and then endocytosed to the vacuole, we assayed PtdIns(3)P levels in *sec1* and *end4* temperature-sensitive strains. When shifted to the nonpermissive temperature, *sec1* and *end4* mutants block the late secretory and early endocytic pathways, respectively (Novick *et al*., 1980; Raths *et al*., 1993). PtdIns(3)P levels did not increase in either of these strains after a 15 min shift to 38°C (Figure 6), suggesting that PtdIns(3)P does not follow this route to the vacuole. Inactivation of the AP-3 adaptor complex through the deletion of *APM3* blocks transport of ALP to the vacuole but does not block transport via

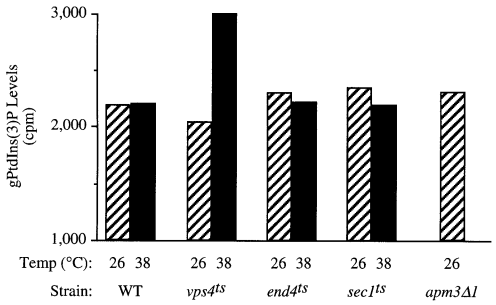

Fig. 6. Cellular PtdIns(3)P levels in *vps4^{ts}*, *end4^{ts}*, *sec1^{ts}* and *apm3Δ1* strains. Two samples of wild-type, *vps4^{ts}*, RH268-1C (*end4^{ts}*) and *sec1^{ts}* cells were labeled for 12 h with myo-[2-^{3}H]inositol at 26°C. One sample of each strain was then shifted to 38°C for the final 15 min of labeling while the other sample was maintained at 26°C. GOY3 (*apm3Δ1*) cells were also labeled for 12 h at 26°C. All strains were then subjected to glass bead lysis in the presence of acidified chloroform/methanol. The extracted lipids were deacylated and analyzed by HPLC. Representative PtdIns(3)P counts recovered in multiple experiments are indicated.

the CPY pathway (Cowles *et al*., 1997a). PtdIns(3)P levels did not change in the *apm3Δ1* strain (Figure 6). Collectively, these results indicate that a significant pool of PtdIns(3)P is trafficked by the CPY pathway from a CPY-containing endosome to the vacuole. In contrast, PtdIns(3)P transport does not depend on the ALP, late secretory or early endocytic pathways. This is consistent with the known functional role of Vps34p in the CPY pathway but not the ALP pathway (Stack *et al*., 1995).

Vps34p PtdIns 3-kinase activity is required for efficient delivery of endocytic cargo to the vacuole

While the PtdIns 3-kinase activity of Vps34p is known to be critical for the trafficking of newly synthesized hydrolases from the Golgi to the vacuole (Herman and Emr, 1990; Stack *et al*., 1995), the specific site(s) of PtdIns(3)P function in the vacuolar transport pathway has not been defined. The data presented here indicate that a significant pool of PtdIns(3)P is trafficked from endosomal compartments to the vacuole, making PtdIns(3)P, at least briefly, a cargo component of endosomal membranes. These results raise the possibility that PtdIns(3)P plays a role in endosome function. To address the possibility that Vps34p activity is required for endosome-to-vacuole trafficking, the endocytosis of FM4-64 and Ste6p was monitored in the *vps34^{tsf}* strain after shifting to the nonpermissive temperature.

FM4-64 is a fluorescent lipophilic molecule which intercalates into the plasma membrane when added exogenously to yeast cells (Vida and Emr, 1995). The endocytic progress of FM4-64 to the vacuole can then be monitored by fluorescence microscopy (Wendland *et al*., 1996). Wild-type and *vps34^{tsf}* cells were grown at the permissive temperature (26°C) and pre-shifted for 10 min to the nonpermissive temperature of 37°C. The cells were then pulse-labeled with FM4-64 for 8 min and the trafficking of FM4-64 to the vacuole monitored at various chase times in FM4-64-free media. After 10 min of chase, the bulk of FM4-64 reached the vacuole in wild-type cells while *vps34^{tsf}* cells exhibited limited vacuole staining and the accumulation of FM4-64 in punctate spots immediately peripheral to the vacuole (Figure 7A). Even after a 50-min chase, the *vps34^{tsf}* strain still exhibited accumulations

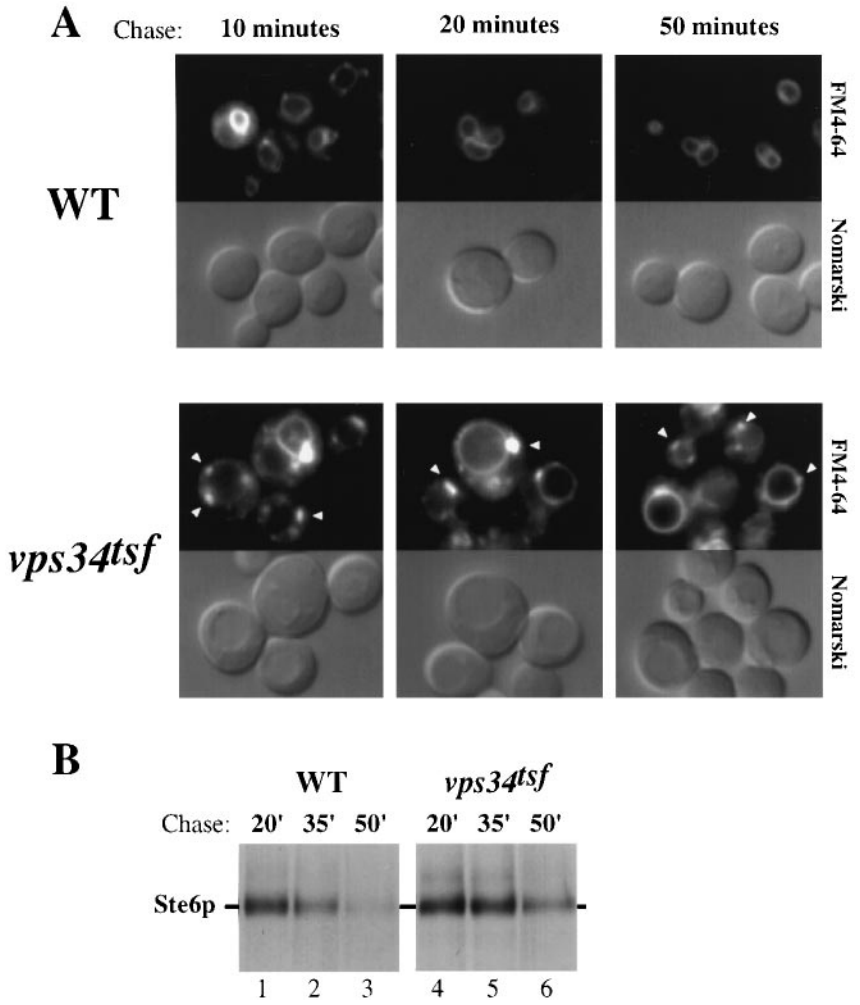

Fig. 7. *vps34*^{tsf} mutant cells exhibit defects in endocytosis at the nonpermissive temperature. Trafficking of FM4-64 and Ste6 protein from the plasma membrane to the vacuole was monitored in wild-type and *vps34*^{tsf} cells. (**A**) Wild-type and *vps34*^{tsf} cells grown at 26°C were shifted to 37°C for 10 min prior to pulse-labeling with FM4-64 for 8 min. The cells were then chased for 10, 20 or 50 min with fresh media after which FM4-64 staining was visualized by fluorescence microscopy. Indentations in the cell surface indicate the position of vacuoles by Nomarski optics. (**B**) Wild-type and *vps34*^{tsf} cells overexpressing Ste6p were grown at 26°C, shifted to 38°C for 10 min and then pulse-labeled with *trans*^{35}S-label for 10 min. After a 20-, 35- or 50-min chase with excess cold methionine/cysteine, 4 OD_{600} units of cells were harvested at each chase point and lysed. Protein extracts derived from these cells were then immunoprecipitated with antiserum specific to the Ste6 protein and analyzed by SDS–PAGE and fluorography.

of FM4-64 adjacent to vacuoles and poor vacuole staining. This defect is indicative of a late block in endocytic transport to the vacuole. Enlarged vacuoles, a documented effect of Vps34p inactivation (Herman and Emr, 1990), were also evident in *vps34*^{tsf} cells at high temperature. At the permissive temperature, the *vps34*^{tsf} strain exhibited no such defects (data not shown). Thus, transient loss of Vps34p function results in a late endocytosis defect characterized by the accumulation of FM4-64 in pre-vacuolar endocytic compartments.

Endocytic transport of the plasma membrane **a**-factor transporter, Ste6p, to the vacuole where it is degraded (Kolling and Hollenberg, 1994) was also analyzed in the *vps34*^{tsf} strain. Wild-type and *vps34*^{tsf} cells grown at 26°C, were shifted to 38°C for 10 min prior to pulse-labeling with *trans*^{35}S-label and chasing with excess cold methionine and cysteine for 20-, 35- or 50-min periods. Protein extracts derived from these cells were then incubated with antiserum raised against the Ste6 protein (Browne *et al*., 1996) and the resulting immunoprecipitates subjected to SDS–PAGE and fluorography. At the 20-min chase point, wild-type and *vps34*^{tsf} cells exhibited similar levels of Ste6 protein (Figure 7B, lanes 1 and 4). After a 35-min chase period, Ste6p levels decreased by ~50% in wild-type cells but, in *vps34*^{tsf} cells, Ste6p levels remained constant (lanes 2 and 5). After 50 min of chase, nearly all the Ste6p was degraded in wild-type cells; however, significant levels of Ste6p (30%) were still present in the *vps34*^{tsf} strain (lanes 3 and 6), indicating an impaired delivery of Ste6p to the vacuole.

Since the progression of cargo along the CPY pathway (Stack *et al*., 1995) and the late endocytic pathway are disrupted in the *vps34*^{tsf} strain, it is possible that pre-vacuolar transport intermediates which are discernible by electron microscopy accumulate in this mutant at the nonpermissive temperature. At the permissive temperature (25°C) *vps34*^{tsf} cells did not accumulate aberrant membrane compartments and were wild-type in appearance (Figure 8A). However, after shifting the *vps34*^{tsf} strain to the nonpermissive temperature of 38.5°C for 2 h, we

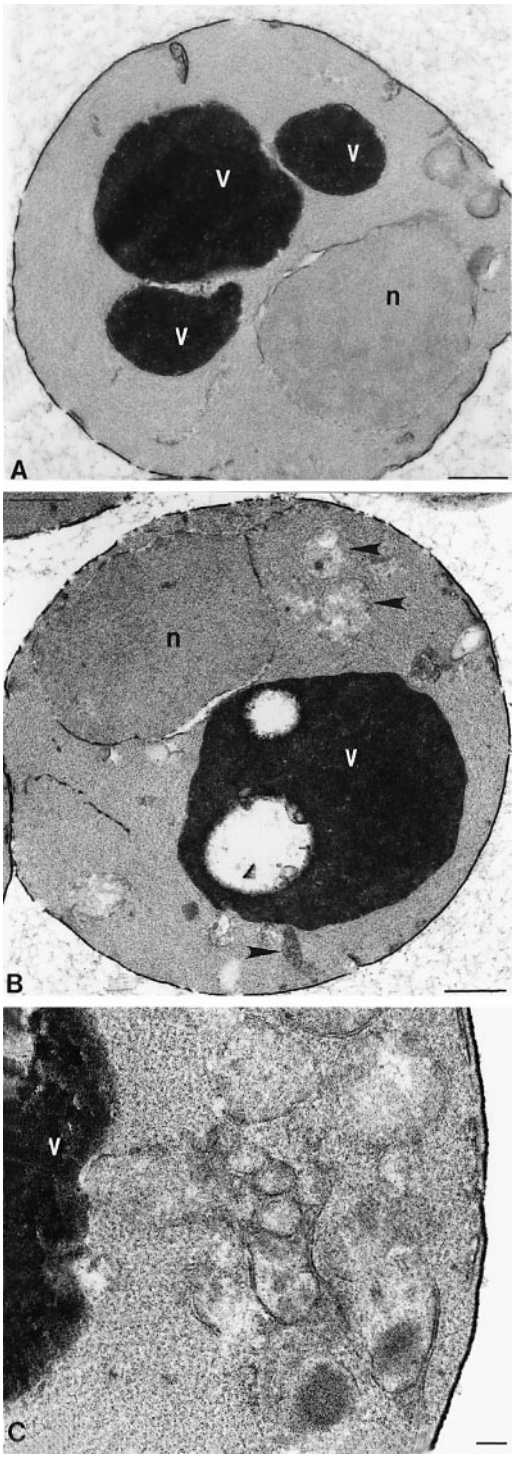

Fig. 8. Electron microscopic analysis of the *vps34^{tsf}* strain. *vps34^{tsf}* cells were grown overnight and split into two aliquots. One aliquot was maintained at the permissive temperature while the other was shifted to the nonpermissive temperature to inactivate Vps34p. (**A**) *vps34^{tsf}* strain maintained at the permissive temperature, 25°C. Bar = 0.5 μm. (**B**) *vps34^{tsf}* strain after a 2 h shift to 38.5°C. Several prevacuolar compartments are indicated by arrowheads. Bar = 0.5 μm. (**C**) High magnification image of membrane structures present in the *vps34^{tsf}* after 2 h at 38.5°C. Bar = 0.1 μm. V, vacuole; N, nucleus.

observed the accumulation of a variety of compartments peripheral to the vacuole (Figure 8B and C). Similar structures were also evident in the *vps34^{tsf}* strain shifted to the nonpermissive temperature for 30 min (data not shown). Collectively, our data suggest that Vps34p function is required for the transport/fusion of sorting intermediates with the vacuole but, apparently, is not required for their formation.

Discussion

Previous studies have shown that Vps15p, a protein kinase localized to the late Golgi and/or endosomal membranes, recruits Vps34p to the membrane fraction and activates its PtdIns 3-kinase activity (Herman *et al*., 1991; Stack *et al*., 1993, 1995). It is likely that PtdIns(3)P is an essential second messenger in vacuolar protein transport which functions to modulate the activity and/or localization of Vps proteins. However, insights into the possible site(s) of PtdIns(3)P function in vacuolar protein sorting have, to date, remained elusive. Here, we localize the site of PtdIns(3)P turnover to the vacuole/lysosome. While findings in mammalian systems suggest that the turnover of some 3-phosphoinositides (i.e. phosphatidylinositol 3,4,5-trisphosphate) may be mediated by cytoplasmic phosphatases (Woscholski and Parker, 1997), these data indicate that the majority of PtdIns(3)P turnover in yeast and possibly mammalian cells is dependent upon the activity of vacuolar/lysosomal degradative enzymes. An intact CPY transport pathway is also required to deliver PtdIns(3)P to the vacuole, revealing that PtdIns(3)P is a cargo of membrane transport. An implication of these results is that PtdIns(3)P transits through a prevacuolar endosome as it progresses to the vacuole, suggesting that PtdIns(3)P may also regulate endosome function. Consistent with this, we have found that temperature inactivation of the *Vps34^{tsf}* led to defects in the transport of endocytic cargo to the vacuole.

Turnover of PtdIns(3)P requires vacuolar hydrolase activities

Deletion of *PRC1* (CPY) or *CPS1* (CPS), which are not known to activate vacuolar zymogens (Klionsky *et al*., 1990; van den Hazel *et al*., 1996), had no effect on PtdIns(3)P turnover. On the other hand, deletion of *PEP4* (PrA) or *PRB1* (PrB), which function to process proteolytically many vacuolar proenzymes to their mature/active forms (Hirsch *et al*., 1992; van den Hazel *et al*., 1992, 1996), led to several-fold increases in PtdIns(3)P (Figure 3). These data suggest that PrA and PrB play an important part in converting a vacuolar enzyme(s) involved in some aspect of PtdIns(3)P turnover from its precursor form to its mature form. One enzyme that could participate in PtdIns(3)P turnover is a phosphatase. Deletion of ALP, a vacuolar phosphatase which is proteolytically matured in a PrA-dependent manner (Klionsky and Emr, 1989), did not result in increases in cellular PtdIns(3)P, suggesting that ALP is not the relevant target of PrA (unpublished observations). Alternatively, a lipase activated by PrA and/or PrB may carry out PtdIns(3)P turnover. Vacuolar/lysosomal lipases have been previously implicated in the degradation of cellular lipids. Fluorescent analogues of phosphatidylcholine have been found to be internalized from the plasma membrane and trafficked via endocytic routes to the vacuole in yeast (Kean *et al*., 1993). Several distinct breakdown products of this lipid were observed, suggestive of lipase-mediated turnover, and the turnover

rate was slowed 5-fold in a PrA- and PrB-deficient strain (Kean *et al*., 1993). Glycosphingolipids are also known to be endocytosed and turned over within the lysosome in a sphingomyelinase-dependent manner (Sandhoff and Kolter, 1996). Patients deficient in sphingomyelinase activity are afflicted with Niemann–Pick disease, a lipid storage disorder (Sandhoff and Kolter, 1996). Database searches of the yeast genome indicate that there are many potential lipases that are, as yet, uncharacterized and we are in the process of testing whether any of these play a role in PtdIns(3)P turnover.

How does PtdIns(3)P gain access to vacuolar hydrolase activities? Protease protection and cellular fractionation experiments have shown that Vps15p and Vps34p are localized to the cytoplasmic surface of the membrane which restricts the synthesis of PtdIns(3)P to the cytoplasmic membrane leaflet (Herman *et al*., 1991; Stack *et al*., 1993). The fact that PrA and PrB function within the lumen of the vacuole suggests that a mechanism must exist to overcome this topological difference and enable PtdIns(3)P exposure to vacuolar hydrolase activities. There are several potential mechanisms by which this could occur. PtdIns(3)P turnover could be carried out by a membrane-spanning lipase or phosphatase that contains a cytoplasmic catalytic domain, a transmembrane domain and a tail domain within the lumen of the vacuole. The conversion of the precursor form of such an enzyme to its mature/active state would require PrA- and/or PrB-dependent proteolytic cleavage of the lumenal tail domain. This event would enable PtdIns(3)P to be processed in a PrA/PrB-dependent manner in the cytoplasmic membrane leaflet of the vacuole. A vacuolar hydrolase with this topology has yet to be documented. Alternatively, PtdIns(3)P may be transferred by a flipase to the inner-membrane leaflet of the vacuole making it accessible to vacuolar enzymes. Lipid transfer between membrane leaflets has been reported (van Helvoort *et al*., 1996); however, a flipase specific for polyphosphoinositides has not been identified. In this paper we present evidence that a third possibility exists in yeast. *vma4* null mutants not only exhibit 2.5-fold increases in PtdIns(3)P, but also accumulate a pool of intravacuolar vesicles (Figures 3 and 4). PtdIns(3)P present in the cytoplasmic leaflet of the endosome (or vacuole) could be sorted into vesicles that invaginate into the compartment. The PtdIns(3)P would be present in the inner-leaflet of such vesicles, and upon delivery to the vacuole these vesicles would be exposed to lumenal hydrolase (lipase) activities and degraded (Figure 9). Further experimentation is necessary to distinguish between these possibilities.

The vacuole is perceived to function in the bulk degradation of macromolecules, metabolite storage and ion homeostasis (Klionsky *et al*., 1990). We have presented evidence that the vacuole also functions to downregulate signals mediated by a PtdIns 3-kinase. Among phosphoinositide-signaling molecules in yeast, this degradation pathway may be unique to PtdIns(3)P as neither PtdIns(4)P or PtdIns(4,5)P_2 levels increased in cells lacking PrB. Therefore, the predominant pools of PtdIns(4,5)P_2 and PtdIns(4)P are likely to be turned over by alternate mechanisms at other membrane sites in the cell (Rhee, 1991; McPherson *et al*., 1996). Recently, it has been found that a pool of PtdIns(3)P can be converted to phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] (Dove *et al*., 1997). Under normal growth conditions, steady-state PtdIns(3,5)P_2 is much less abundant than PtdIns(3)P (Dove *et al*., 1997), suggesting that only a small pool of PtdIns(3)P is phosphorylated to PtdIns(3,5)P_2. Deletion of *PRB1* (and *VAM3*) did not influence PtdIns(3,5)P_2 levels (unpublished observations), demonstrating both that vacuolar hydrolase activity is not required for PtdIns(3,5)P_2 turnover and that *PRB1* deletion does not bring about increases in PtdIns(3)P by reducing steady-state PtdIns(3,5)P_2 levels.

PtdIns(3)P is a lipid cargo of transport intermediates destined for the vacuole

As PtdIns(3)P synthesis is thought to occur at either the late Golgi or endosome (Herman *et al*., 1991), transport of PtdIns(3)P to the vacuole is also required in order for it to be turned over by vacuolar hydrolases. Deletion of *VAM3*, a vacuolar t-SNARE (Wada *et al*., 1997), or *YPT7*, the yeast rab7 homologue (Wichmann *et al*., 1992), caused >4-fold increases in PtdIns(3)P. Loss of Vam3p and Ypt7p function did not induce increases in Vps34p PtdIns 3-kinase activity. Transient interruption of endosome-to-vacuole trafficking through the inactivation of the *vam3^{tsf}* and *vps33^{tsf}* led to 2-fold increases in PtdIns(3)P. *vam3* null mutants cause steady-state blocks in endosome-to-vacuole traffic and therefore block more rounds of transport than does the 20-min inactivation of the *vam3^{tsf}*, perhaps explaining why PtdIns(3)P is significantly more stable in *vam3*Δ mutants than the *vam3^{tsf}*. Moreover, simultaneous inactivation of both Vps34 PtdIns 3-kinase and Vam3p-dependent transport of PtdIns(3)P to the vacuole resulted in the stabilization of PtdIns(3)P (Figure 5). This indicates that a large percentage of the total pool of PtdIns(3)P synthesized by yeast is delivered to and turned over at the vacuole.

Vam3p activity is required for multiple transport pathways to the vacuole, including the ALP, CPY and AP1 pathways (Darsow *et al*., 1997). Inactivation of *VAM3* causes the immature precursor forms of these hydrolases to accumulate in distinct sorting intermediates which fail to fuse with the vacuole (Darsow *et al*., 1997). In *vam3* mutants, it is likely that PtdIns(3)P also accumulates at one or multiple prevacuolar compartments, sequestered away from active vacuolar hydrolases. Because PtdIns(3)P is rapidly degraded following cell lysis, our attempts to biochemically localize PtdIns(3)P to specific sorting intermediates have been unsuccessful (unpublished observations). As an alternative approach to address which sorting compartments mediate PtdIns(3)P transport to the vacuole, we monitored PtdIns(3)P levels in mutants which differentially affect the fusion of specific sorting intermediates with the vacuole. We cannot yet rule out that AP1-containing autophagosomes are partly responsible for delivery of PtdIns(3)P to the vacuole. However, analysis of PtdIns(3)P levels in *apm3*, *sec1* and *end4* mutants indicated that PtdIns(3)P does not rely on the ALP, late secretory or early endocytic pathways for vacuolar transport. Vps4p encodes a AAA-type ATPase that is required for transport along the CPY pathway (Babst *et al*., 1997). Inactivation of the *Vps4^{ts}* leads to an accumulation of endosomal intermediates which contain inactive CPY precursor protein (Babst *et al*., 1997).

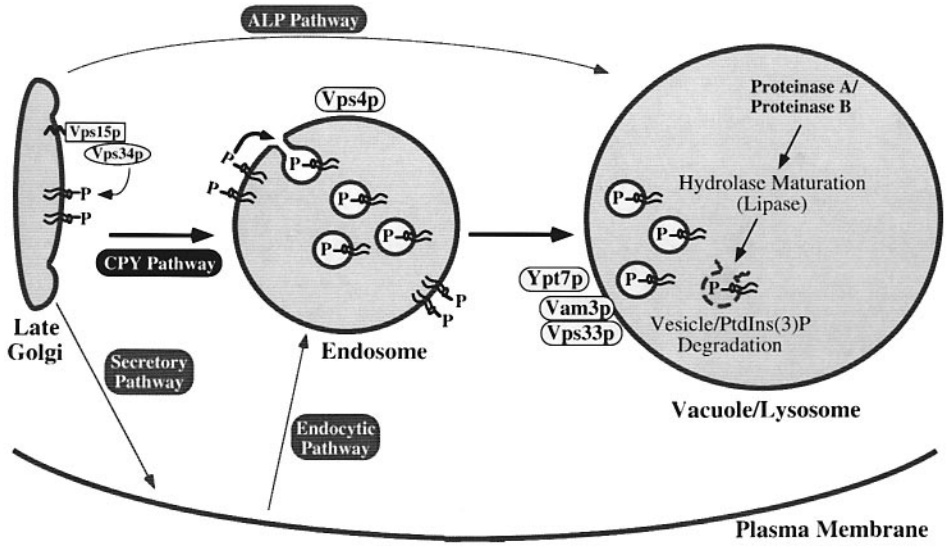

Fig. 9. Model for the uptake and turnover of PtdIns(3)P. Following Vps15p-/Vps34p-mediated synthesis of PtdIns(3)P in the cytoplasmic leaflet of the late Golgi or endosome, our data suggests that PtdIns(3)P is delivered to the hydrolytically active interior of the vacuole. One mechanism by which this process may occur is through the formation of endosomal or vacuolar invaginations that pinch off into uniformly-sized vesicles that contain PtdIns(3)P in their inner-leaflet. Subsequent delivery to the vacuole results in hydrolase-dependent degradation of the vesicles together with PtdIns(3)P in the lumen of the vacuole. Turnover of PtdIns(3)P is, thus, a multistep process which depends upon Vps4p, Vps33p, Vam3p and Ypt7p to transport PtdIns(3)P to the vacuole and proteinase A and B to activate lipases and/or phosphatases involved in its turnover.

PtdIns(3)P was stabilized at the nonpermissive temperature in this mutant, suggesting that at least a portion of the total pool of PtdIns(3)P traffics in a Vps4p-dependent manner via a CPY-containing endosomal compartment to the vacuole. Loss of Vps4p or Vam3p function results in an accumulation of prevacuolar intermediates including multivesicular bodies (MVBs) (Babst *et al*., 1997; Darsow *et al*., 1997). PtdIns(3)P presumably also accumulates in these membranes, sequestered away from the hydrolases that degrade it (Figure 9).

PtdIns(3)P as a regulator of endosome function

Vps15p and Vps34p have been localized to the late Golgi and/or prevacuolar endosome (Herman *et al*., 1991). This pattern of localization is consistent with the rapid sorting defects observed in the *vps34^{tsf}* mutant (Stack *et al*., 1995). It is likely that PtdIns(3)P activates/recruits other class D *VPS* gene products that are essential for transport between the Golgi and endosome (Stack *et al*., 1993, 1995). Vac1p and the Vps21p rab GTPase are two such class D candidates (Horazdovsky *et al*., 1994; Singer-Kruger *et al*., 1994; Burd *et al*., 1997). EEA1, a mammalian homologue of Vac1p, binds PtdIns(3)P (Patki *et al*., 1997). In addition, wortmannin, a potent inhibitor of PtdIns 3-kinases, blocks the activation of the mammalian Vps21p homologue, rab5 (Li *et al*., 1995), and the sorting of cathepsin D to the lysosome (Brown *et al*., 1995; Davidson, 1995). Therefore, PtdIns(3)P may act as a critical signal during rab-activated docking/fusion of Golgi-derived vesicles at the endosome (Stack *et al*., 1993). Our data suggest that a pool of PtdIns(3)P is likely to traffic from a prevacuolar endosome to the vacuole and that PtdIns(3)P is likely to be a component of endosomal membranes. Thus, PtdIns(3)P may have additional roles in transport events which occur late in the pathway.

An endosomal role for PtdIns(3)P is evident from the fact that Vps34p PtdIns 3-kinase activity is required at a late endocytic step. Transiently-induced reductions in the levels of PtdIns(3)P caused by shifting the *vps34^{tsf}* strain to the nonpermissive temperature impedes the transport of endocytosed fluorescent dye (FM4-64) to the vacuole. Instead, FM4-64 builds up in small compartments adjacent to the vacuole. Some vacuoles did appear to be partially stained with FM4-64, suggesting that FM4-64 can partially bypass Vps34p-dependent sorting steps to the vacuole. Indeed, more than one endocytic pathway to the vacuole may exist (Kolling and Hollenberg, 1994), raising the possibility that inactivation of Vps34p may impair one endocytic route while leaving another intact. Endocytosed plasma membrane proteins destined for degradation in the vacuole (Kolling and Hollenberg, 1994) may also accumulate in prevacuolar endosomal compartments since Ste6p degradation was delayed in the *vps34^{tsf}* strain at the nonpermissive temperature. Even after loss of Vps34p function, some turnover of Ste6p was observed, suggesting that a pool of this protein may follow a Vps34p-independent route to the vacuole or that kinetically-delayed turnover of Ste6p can occur within a prevacuolar compartment(s). Overall, these results are consistent with previous work which suggested that the turnover of endocytosed α-factor was impaired in a *vps34* null mutant (Munn and Riezman, 1994). However, interpretation of results obtained with the null mutant is difficult because of the loss of vacuolar hydrolase activity in null *vps34* strains (Herman and Emr, 1990).

How might Vps34p PtdIns 3-kinase activity be required to regulate class D Vps protein function and the fusion of endocytic cargo-containing intermediates with the vacuole? PtdIns(3)P produced at the late Golgi or endosome may initially control the activities of class D proteins functioning at an early step of CPY sorting. After regulating this stage of the CPY pathway, PtdIns(3)P could participate in interactions with other, later-acting effectors. For example, PtdIns(3)P may modulate the functions of certain class E Vps proteins which control the sorting activity of endosome compartments. Like Vps34p, class

E proteins are required for both CPY sorting and a late step of endocytic traffic to the vacuole (Munn and Riezman, 1994; Stack *et al*., 1995; Babst *et al*., 1997).

Our conclusions are consistent with morphological studies in mammalian cells. In these studies, the MVB has been characterized as a late-sorting compartment in the endosomal membrane system (Gruenberg and Maxfield, 1995). It has been shown that the downregulation and degradation of ligand-bound epidermal growth factor receptor requires its internalization into MVBs and that failure to do so results in the recycling of the receptor from internal membranes back to the cell surface (Felder *et al*., 1990). Conversely, titration of a specific lipid component of MVBs (lysobisphosphatidic acid) results in the sequestration of the mannose-6-phosphate receptor (but not lgp120) in lumenal vesicles and tubules of MVBs instead of its recycling from endosomal to Golgi membranes (Kobayashi *et al*., 1998). Thus, the MVB selectively recruits proteins and possibly specific lipids to invaginating vesicles. Yeast should provide a useful model system to address the molecular mechanisms by which specific proteins and lipids sort into the newly forming vesicles contained within the MVB and, possibly, the vacuole.

Materials and methods

Plasmids and DNA methods

DNA ligations and bacterial minipreps were performed by standard methods (Maniatis *et al*., 1982). Restriction and modifying enzymes were purchased from Boehringer Mannheim, Indianapolis, IN. DNA fragments were purified using Gene Clean (BIO 101, La Jolla, CA). Bacterial transformations were carried out as previously described (Hanahan, 1983). *pVPS33-8.416*, *pVAM3-6.416* (Darsow *et al*., 1997), *pMB59* (Babst *et al*., 1997), 2μ plasmids containing *VPS15* or *VPS34* and *pvps34^{tsf}* (Stack *et al*., 1995) plasmids have been described. *pvps34^{tsf}-2* was made by ligating *Cla*I–*Sma*I digested pRS413 (Sikorski and Hieter, 1989) with the 4.1 kDa gel-purified *vps34^{tsf}* gene treated sequentially with *Kpn*I, T4 Polymerase and *Cla*I. pBHY7-1, *ypt7*Δ, was constructed by replacing a *Hin*cII–*Sph*I fragment of the *YPT7* gene (1.37 kDa *Stu*I–*Pac*I fragment cloned into pBlueScript, Stratagene, La Jolla, CA) with the *HIS3* gene.

Strains

SEY6210 [MATα leu2-3,112 ura3-52 his3-Δ 200 trp1-Δ 901 lys2-801 suc2-Δ 9; (Robinson *et al*., 1988)]. (i) TDY2 [SEY6210 vam3Δ::LEU2; (Darsow *et al*., 1997)]. (ii) WSY99 (SEY6210 ypt7Δ::HIS3, this study). (iii) LBY317 + pVPS33-8.416 [SEY6210 vps33Δ::HIS3, pVPS33-8.416, (Darsow *et al*., 1997)]. (iv) TDY2 + pVAM3-6.416 [TDY2, pVAM3-6.416, (Darsow *et al*., 1997)]. (v) TVY614 (SEY6210 prc1Δ::HIS3, pep4Δ::LEU2 prb1Δ::HISG, laboratory strain). (vi) TVY1 (SEY6210 pep4Δ::LEU2, laboratory strain). (vii) TVY2 (SEY6210 prb1Δ::LEU2, laboratory strain). (viii) TVY6 (SEY6210 prc1Δ::HIS3, laboratory strain). (ix) CCY241 [SEY6210 cps1Δ::URA3, (Cowles *et al*., 1997a)]. (x) KMY1004α [SEY6210 vma4Δ::URA3 (Morano and Klionsky, 1994)]. (xi) vps34^{tsf} (SEY6210 vps34Δ1::TRP1, pvps34^{tsf}-2, this study). (xii) AWY1 (SEY6210 vps34Δ1::TRP1, vam3Δ::LEU2, pvps34^{tsf}-2, pVAM3-6.416, this study). (xiii) vps4^{ts} [SEY6210 vps4Δ::TRP1, pMB59 (Babst *et al*., 1997)]. (xiv) RH268-1C (MATa ura3 his4 leu2 trp1::URA3 bar1-1 end4, laboratory strain). (xv) GOY3 [SEY6210 apm3Δ1::HIS3 (Cowles *et al*., 1997a)]. (xvi) sec1^{ts} (MATa leu2-3, 112 ura3-52 suc2Δ9 sec1-1, laboratory strain). Strains were made using the lithium acetate transformation method (Ito *et al*., 1983).

Ste6p labeling and immunoprecipitation

Cells to be metabolically labeled with *trans*^{35}S-label were grown at 26°C to an OD_{600} of 0.7–0.8 in synthetic medium (YNB) supplemented with 2% dextrose and the required amino acids. Four OD_{600} units per immunoprecipitation were harvested and resuspended in 1 ml of YNB, the necessary amino acids and 2% dextrose, and preincubated at 38°C for 10 min. Cells maintained at 38°C, were labeled with 80 μCi *trans*^{35}S-label per immunoprecipitation for 10 min and chased for 20–50 min with 5 mM methionine, 1 mM cysteine and 0.2% yeast extract. Protein was precipitated by adding trichloroacetic acid to 10% to whole cells. Trichloroacetic acid pellets (13 000 *g*) were resuspended, lysed with glass beads and subjected to immunoprecipitation as described (Rieder *et al*., 1996) with antiserum specific to Ste6p (Browne *et al*., 1996).

In vivo phosphoinositide analysis

Cells to be labeled with myo-[2-^{3}H]inositol (Amersham, Arlington Heights, IL) were grown for 24 h at 26°C in YNB, the required amino acids and 2% dextrose if selecting for a plasmid, otherwise in yeast extract/peptone/dextrose (YPD) to an OD_{600} per ml of 0.8–1.0. *vma4*Δ cells were always grown in media supplemented with 50 mM MES (pH 5.5). 0.2 OD_{600} units of these cells were harvested, washed with 1 ml of synthetic media lacking inositol and, subsequently, used to inoculate 5 ml of inositol-free media containing 50 μCi myo-[2-^{3}H]inositol. Cells were grown for either 12 h at 26°C (Figures 1, 3, 5 and 6) or 11.84–11.33 h at 26°C and shifted for 10–40 min, as indicated, to 38°C (Figures 1B, 5 and 6). After this growth period, cells were typically at a density of 0.8–1.0 OD_{600} units per ml. Cells were harvested by centrifugation, washed with water prewarmed to either 26 or 38°C, and subsequently lysed in 2 ml of 1 M HCl, chloroform, methanol, 0.25 mm glass beads (1:1:1:1) by vortexing for ten 30 s periods. Total cell lipids were extracted, deacylated and analyzed by HPLC as described (Schu *et al*., 1993; Stack *et al*., 1995). Instead of collecting 0.33 min fractions, 0.66 min fractions were isolated using a Whatman HPLC column, Cat. # 4611–1505 (Whatman Inc. Clifton, NJ). The precise elution of PtdIns(4,5)P_2 from our column was determined using tritiated PtdIns(4,5)P_2 standard (Amersham, Arlington Heights, IL). The HPLC data presented are representative of multiple experiments. Absolute levels of phosphoinositides varied 10% from experiment to experiment.

PtdIns 3-kinase assays

Spheroplasts were lysed and separated into P100 and S100 fractions by a 30-min 100 000 *g* spin as previously described (Schu *et al*., 1993; Stack *et al*., 1995). Because active Vps34p resides in the P100 fraction (Schu *et al*., 1993), the activity found in this fraction was assayed. PtdIns 3-kinase reactions were carried out by incubating 2 μg P100 extract in 50 μl 20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 0.2 mg/ml sonicated PtdIns, 60 μM ATP and 0.2 mCi/ml [γ-^{32}P]ATP at 25°C for 5 min. Reactions were stopped with the addition of 240 μl 1 M HCl/chloroform/methanol (1:1:1) followed by vortexing. The organic phase was analyzed by TLC as described (Schu *et al*., 1993; Stack *et al*., 1995).

Microscopy

FM4-64 labeling was performed as previously described (Vida and Emr, 1995). Wild-type and *vps34^{tsf}* cells were grown in YNB, selective amino acids and 2% dextrose at 26°C and shifted to 37°C for 10 min prior to labeling cells with FM4-64 (Molecular Probes, Inc.). One OD_{600} unit of cells was labeled in 50 μl of prewarmed YPD containing 32 μM FM4-64 for 8 min at 37°C. Cells were then chased in 1 ml of fresh YPD media (prewarmed to 37°C) for 10–50 min, as indicated. Cells were then viewed by both fluorescence microscopy and Nomarski optics. For electron microscopy, 50 OD_{600} units of *vma4*Δ or *vps34^{tsf}* cells were harvested after being grown to mid-log phase. *vma4*Δ cells were grown in YPD supplemented with 50 mM MES, pH 5.5 at 26°C while *vps34^{tsf}* cells were grown at 26°C in YNB, the required amino acids and 2% dextrose. For the final 2 h of growth, *vps34^{tsf}* cells were shifted to 38.5°C. Cells were fixed and processed for electron microscopy as previously described (Rieder *et al*., 1996).

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