

Fab1p Is Essential for PtdIns(3)P 5-Kinase Activity and the Maintenance of Vacuolar Size and Membrane Homeostasis

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Abstract. The *Saccharomyces cerevisiae* *FAB1* gene encodes a 257-kD protein that contains a cysteine-rich RING-FYVE domain at its NH₂-terminus and a kinase domain at its COOH terminus. Based on its sequence, Fab1p was initially proposed to function as a phosphatidylinositol 4-phosphate (PtdIns(4)P) 5-kinase (Yamamoto et al., 1995). Additional sequence analysis of the Fab1p kinase domain, reveals that Fab1p defines a subfamily of putative PtdInsP kinases that is distinct from the kinases that synthesize PtdIns(4,5)P₂. Consistent with this, we find that unlike wild-type cells, *fab1Δ*, *fab1^{sf}*, and *fab1* kinase domain point mutants lack detectable levels of PtdIns(3,5)P₂, a phosphoinositide recently identified both in yeast and mammalian cells. PtdIns(4,5)P₂ synthesis, on the other hand, is only moderately affected even in *fab1Δ* mutants. The presence of PtdIns(3)P in *fab1* mutants, combined with previous data, indicate that PtdIns(3,5)P₂ synthesis is a two step process, requiring the production of PtdIns(3)P by the Vps34p PtdIns 3-kinase and the subsequent Fab1p-dependent phosphorylation of PtdIns(3)P yielding PtdIns(3,5)P₂. Although Vps34p-mediated synthesis of PtdIns(3)P is required for the proper sorting of hydro-

lases from the Golgi to the vacuole, the production of PtdIns(3,5)P₂ by Fab1p does not directly affect Golgi to vacuole trafficking, suggesting that PtdIns(3,5)P₂ has a distinct function. The major phenotypes resulting from Fab1p kinase inactivation include temperature-sensitive growth, vacuolar acidification defects, and dramatic increases in vacuolar size. Based on our studies, we hypothesize that whereas Vps34p is essential for anterograde trafficking of membrane and protein cargoes to the vacuole, Fab1p may play an important compensatory role in the recycling/turnover of membranes deposited at the vacuole. Interestingly, deletion of *VAC7* also results in an enlarged vacuole morphology and has no detectable PtdIns(3,5)P₂, suggesting that Vac7p functions as an upstream regulator, perhaps in a complex with Fab1p. We propose that Fab1p and Vac7p are components of a signal transduction pathway which functions to regulate the efflux or turnover of vacuolar membranes through the regulated production of PtdIns(3,5)P₂.

Key words: vacuole • lipid kinase • PtdIns(3,5)P₂ • *VAC7* • *VPS34*

A characteristic feature of eucaryotic cells is the presence of multiple intracellular organelles that are specialized to carry out specific functions. Each organelle is defined by its unique protein and lipid composition that is maintained despite the continuous flow of cargo molecules between compartments. This necessitates having an accurate and efficient sorting system that guarantees the directional and spatial specificity of vesicle-mediated trafficking in the secretory and endocytic pathways

(Palade, 1975; Rothman, 1994). Secretory proteins synthesized at the endoplasmic reticulum are transported to the Golgi complex where proteins destined for the cell surface must be sorted from those trafficking to the lysosome (or yeast vacuole; Kornfeld and Mellman, 1989; Stack et al., 1995). Significant progress has been made in understanding the machinery responsible for the sorting, transport, and docking/fusion of carrier vesicles with the appropriate target organelle (Schekman and Orci, 1996). The current data supports a model where both proteins and lipids function as regulators of these processes. The proteins directly involved in these pathways range from enzymes such as AAA-type ATPases and rab GTPases to polypeptide complexes that serve as membrane vesicle coats or vesicle docking components (Babst et al., 1997; Novick and Ze-

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rial, 1997; Schmid, 1997). Phospholipids, in particular phosphorylated derivatives of phosphatidylinositol (PtdIns),¹ also appear to play a critical role in regulating these transport events (De Camilli et al., 1996; Martin, 1997). Given the numerous modification sites present on the inositol headgroup and the combinatorial manner in which these sites can be modified, phosphoinositides are well suited for the specific regulation of this complex trafficking process. Phosphoinositides also function in a variety of signal transduction pathways ranging from cell growth and differentiation, to apoptosis and cytoskeletal rearrangement (De Camilli et al., 1996; Tokar and Cantley, 1997).

A requirement for phosphoinositides in Golgi to vacuole (lysosome) trafficking in yeast was established by the characterization of the *VPS34* gene product as a PtdIns(3)-kinase, responsible for phosphatidylinositol 3-phosphate (PtdIns(3)P) synthesis (Schu et al., 1993). *VPS34* was identified in a screen for vacuolar protein sorting mutants which missort and secrete proteins that are normally targeted to the yeast vacuole (Banta et al., 1988; Herman and Emr, 1990). Vps34p is recruited from the cytosol to a membrane-bound complex by the protein kinase Vps15p (Stack et al., 1993). Together, Vps15p and Vps34p facilitate protein sorting in both the Golgi to endosome and endosome to vacuole transport pathways through the regulated synthesis of PtdIns(3)P (Munn and Riezman, 1994; Stack et al., 1995). This lipid product appears to be required for the proper localization and/or activation of other proteins essential for Golgi to vacuole transport. Recently, candidate effectors of PtdIns(3)P signaling were identified in yeast as a set of proteins that contain a cysteine-rich RING-FYVE finger domain that binds PtdIns(3)P and not other phosphoinositides (Burd and Emr, 1998). Two of these proteins Vac1p/Vps19p and Vps27p are known to function in the Golgi to endosome and endosome to vacuole transport reactions, respectively (Weisman and Wickner, 1992; Piper et al., 1995; Burd et al., 1997). Similarly, in mammalian cells, wortmannin-induced inhibition of phosphoinositide 3-kinase activities results in lysosomal hydrolase missorting, cathepsin D is secreted into the media (Brown et al., 1995; Davidson, 1995).

Additional polyphosphoinositides have also been implicated in membrane trafficking. PEP1 and PEP3 proteins are required in the Ca⁺⁺-dependent exocytosis of secretory granules from semi-intact PC12 cells (Hay and Martin, 1993; Hay et al., 1995). PEP1 is a PtdIns(4)P 5-kinase, responsible for PtdIns(4,5)P₂ synthesis. PEP3, a PtdIns transfer protein (PITP), exchanges PtdIns and phosphatidylcholine between distinct intracellular membranes, thereby increasing local lipid concentrations. PITPs may also directly present lipid substrates to lipid-modifying enzymes (e.g., PEP1; Liscovitch and Cantley, 1995; Kearns et al., 1998). The activities of these two proteins indicate that the ultimate synthesis of PtdIns(4,5)P₂ plays a direct role in the release of secretory granules, perhaps by recruiting essential regulatory proteins to the proper membrane.

1. *Abbreviations used in this paper:* a.a., amino acid; ALP, alkaline phosphatase; API, aminopeptidase I; CPS, carboxypeptidase S; CPY, carboxypeptidase Y; G6PHD, glucose 6-phosphate dehydrogenase; PITP, PtdIns transfer protein; PtdIns(4)P, phosphatidylinositol 4-phosphate.

Mutation of the yeast PITP, *SECI4*, also results in a block of secretory traffic from the late Golgi (Bankaitis et al., 1990; Kearns et al., 1998).

PtdIns(3)P was the only known D-3 phosphoinositide in yeast, until recently, when PtdIns(3,5)P₂ was identified in yeast, mouse fibroblasts, and plants (Dove et al., 1997; Whiteford et al., 1997). Neither the kinase responsible for PtdIns(3,5)P₂ synthesis nor the biological role of this lipid have been defined. In this report, we provide evidence that yeast Fab1p, a protein that bears homology to a general family of PtdInsP kinases, functions as a PtdIns(3)P 5-kinase. *FAB1* deletion mutants completely lack PtdIns(3,5)P₂ and are viable, but exhibit severe growth defects and have an extremely enlarged vacuole that occupies the majority of the cell (Yamamoto et al., 1995). Proper localization of the vacuolar ATPase (V-ATPase) and alkaline phosphatase (ALP) in *fab1* mutants indicates that Fab1p, and therefore PtdIns(3,5)P₂, is not required for biosynthetic protein and membrane trafficking to the vacuole. A similar enlarged vacuole morphology and severe depletion of PtdIns(3,5)P₂ in *vac7* mutants, indicates that Vac7p functions as a possible regulator or cofactor of the Fab1p kinase. Consistent with the vacuolar phenotypes of *fab1* mutants as well as the vacuolar localization of Vac7p, a large pool of Fab1p cofractionates with the V-ATPase. We propose that Fab1p, together with Vac7p, function downstream of Vps34p to regulate vacuolar membrane recycling/turnover through the production of the polyphosphoinositide PtdIns(3,5)P₂.

Materials and Methods

Strains and Media

The *Escherichia coli* strain XL1-Blue (*supE44 thi-1 lac endA1 gyrA96 hsdR17 relA1 F' proAB Laq1q ZΔM15*) was grown with standard LB media. Unless otherwise specified, the *Saccharomyces cerevisiae* strains used in this study were grown in standard YPD and SD minimal media with the necessary auxotrophic supplements. *S. cerevisiae* strains used in this study were as follows: (a) SEY6210 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*; Robinson et al., 1988). (b) *fab1Δ1* (SEY6210 *fab1Δ::LEU2*, this study). (c) *fab1Δ2* (SEY6210 *fab1Δ::HIS3*, this study). (d) LWY1481 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pep4-Δ1137 vac7Δ::HIS3*; Bonangelino et al., 1997). (e) LWY2365 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pep4-Δ1137 vac14-1*; Bonangelino et al., 1997). (f) EMY119 (*MATα leu2-3,112 ura3-52 fab1-2*; lab strain).

Genetic and DNA Manipulations

All restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). T4 DNA ligase was received from GIBCO BRL (Gaithersburg, MD). PCR was performed using synthetic oligonucleotide primers and dNTPs purchased from GIBCO BRL. All PCR reactions used a 2:1 mixture of KlenTaq polymerase (Ab Peptides, Inc., St. Louis, MO) and Pfu polymerase (Stratagene, La Jolla, CA) as described in (Barnes, 1994). Otherwise standard molecular biology techniques were used (Maniatis et al., 1982). Yeast transformations were performed using the lithium acetate method (Ito et al., 1983) and genomic DNA was isolated using a modified version of the procedure described by Hoffman and Winston (1987).

Disruption of *FAB1*. The entire open reading frame of *FAB1* was deleted in SEY6210 through transformation with a 1.1-kbp PCR fragment containing the *HIS3* marker flanked by 52 nucleotides upstream and downstream of the *FAB1* sequence. Histidine prototrophs were selected on histidine-free minimal media plates and confirmation of the disruption was done by PCR using two different sets of primers.

fab1-2 Construction. A 6,350 bp AatII-NruI fragment of the wild-type

FAB1 gene was excised from pAY60 (*pRS416-FAB1*; Yamamoto et al., 1995). The resulting linearized 9,100-bp pRS416 vector containing ~2,000 bp of flanking genomic sequence 5' of the AatII site and 3' of the NruI site was then transformed into EMY119, a strain carrying the *fab1-2* allele (Yamamoto et al., 1995) and selected on uracil-free plates. Through homologous recombination and the subsequent purification of the gap-repaired plasmid, the *fab1-2* allele was rescued from the genome.

Generation of *Fab1p* Kinase Domain Point Mutations. The G2042/2045V point mutant construct was made using the gene SOE (splicing by overlap extension) and plasmid gap repair techniques (Muhlrad et al., 1992; Horton et al., 1993). For the gene SOE, two successive rounds of PCR were used to generate the final 1.9-kbp fragment containing the desired mutations. In the first round, the 5' and 3'-PCR fragments were synthesized in two separate PCR reactions. For the second round of PCR, the products from the 5' and 3' reactions were diluted 100-fold and amplified in the presence of both outside primers to create the full-length fragment containing the desired mutations. pAY60 (Yamamoto et al., 1995) was then digested with NheI and NruI and the gapped vector was purified and cotransformed into the *fab1Δ2* strain with the full-length gene SOE fragment. Plasmid DNA was rescued from yeast cells that had become uracil prototrophs, indicating successful gap repair of the pRS416-based plasmid. The gene SOE fragment containing the D2134R point mutation was made in a similar fashion. However, after synthesis of the full-length gene SOE fragment, it was digested with NheI and NruI and introduced into the gapped vector by direct ligation. Once transformed into *E. coli* the plasmid constructs for both point mutations were sequenced to confirm the presence of only the desired mutation. DNA sequencing was done at the UCSD CFAR core facility using the ABI Prism BigDye chemistry with Amplitaq DNA Polymerase FS. Gels were analyzed with a 373XL DNA Sequencer (PE Applied Biosystems, Foster City, CA). Correct constructs were then retransformed into *fab1Δ2* for phenotypic analysis.

Construction of HA-tagged *Fab1p*. Primers specific to *FAB1* were used to separately amplify a 350-bp and a 1,300-bp region upstream and downstream of codon 8, while also engineering a BglII site at codon 8. The 350-bp fragment was cut with SnaBI/BglII while the 1,300-bp *FAB1* fragment was cut with BglII/Bsu36I. These fragments were then simultaneously ligated into pAY60 (Yamamoto et al., 1995) and pEMY105 (*pRS426-FAB1*) digested with SnaBI/Bsu36I. The resulting *pRS416-FAB1*-BglII and *pRS426-FAB1*-BglII constructs contain the full-length *FAB1* gene with a unique BglII site engineered at codon 8. A triple-HA epitope repeat was also amplified using PCR primers that introduced BglII sites into both ends of the PCR product to make the triple-HA repeat in frame with the BglII site engineered into *FAB1*. This HA insert was ligated into *pRS416-FAB1*-BglII and *pRS426-FAB1*-BglII via the BglII sites.

In Vivo Labeling and Immunoprecipitation of *Fab1p*

Approximately 6 OD₆₀₀ units of yeast were harvested by centrifugation and resuspended in 2 ml of minimal media and allowed to recover at 24°C for 10 min. Express [³⁵S]-protein labeling mix (DuPont NEN, Boston, MA) was then added to a final concentration of 5 μCi/OD₆₀₀ unit. After a 20-min incubation at 24°C, cells were chased for 45 min at either 24 or 38°C with 5 mM methionine, 1 mM cysteine, 0.4% yeast extract and 2% glucose. The chase was ended with the addition trichloroacetic acid was added to a final concentration of 10%. Trichloroacetic acid precipitates were subjected to glass bead lysis and *Fab1p* was then immunoprecipitated as previously described (Gaynor et al., 1994) with antisera raised against a TrpE fusion of the *Fab1* protein.

Subcellular Fractionation

Spheroplasted wild-type cells were subjected to gentle osmotic lysis as previously described (Gaynor et al., 1994). The resulting cellular extract was then sequentially centrifuged at 325 g for 5 min to remove unbroken cells, 13,000 g for 10 min to generate pelletable, P13 and soluble S13 fractions and 100,000 g for 30 min to yield P100 and S100 fractions. Protein present in all fractions was then precipitated using TCA to a final concentration of 5%. In the case of *Fab1p* and glucose 6-phosphate dehydrogenase, the extracts were derived from wild-type cells pulse-labeled with Express [³⁵S]-protein labeling mix for 20 min and chased with 5 mM methionine, 1 mM cysteine, 0.4% yeast extract for 40 min. Fractions were then immunoprecipitated for *Fab1p* and glucose 6-phosphate dehydrogenase using antiserum raised against these proteins as described (Gaynor et al., 1994). The presence of Kex2p and the 100-kD subunit of the vacuolar ATPase in specific fractions were determined using antisera specific to

these proteins for immunoblotting and ECL as described in (Babst et al., 1997).

In Vivo Analysis of Phosphoinositides

To label strains with myo-[2-³H]inositol (Nycomed Amersham Inc., Princeton, NJ), cells were grown for 24 h in YPD or SD minimal media (if selecting for a plasmid). 0.2–0.5 OD₆₀₀ units of cells were harvested, washed with 1 ml of synthetic media lacking inositol and used to inoculate 5 ml of inositol-free media containing 75 μCi myo-[2-³H]inositol. Cells were labeled 12 h at 22°C, harvested by centrifugation, washed with fresh synthetic media and then resuspended in 100 to 200 μL of synthetic media. An equal volume of 1.2 M NaCl was then added to the cells which were then incubated for a 10-min period to osmotically shock cells. This was carried out at 22°C unless otherwise indicated. Following this treatment, labeled cells were lysed in 1.5 ml of 1 M HCl/chloroform/methanol (1:1:1) by vortexing 10 × 30 s periods in the presence of 0.5 g of 0.25-mm glass beads. As previously described, cellular lipids were extracted, deacylated and analyzed by HPLC (Schu et al., 1993; Stack et al., 1995). Fractions eluting from an HPLC column (catalog no. 4611-1505; Whatman Inc., Clifton, NJ) were collected every 0.66 min. [³H]-PtdIns(3,5)P₂ standards were used to determine the elution point of PtdIns(3,5)P₂ from our column were a generous gift of Robert H. Michell (University of Birmingham, AL). The data presented are representative of multiple experiments. A variation of 10% was observed between experiments.

FM4-64 Labeling of Yeast Vacuoles

Approximately 1 OD₆₀₀ of yeast were harvested at an OD₆₀₀ of 0.6–0.8, and then labeled with the vital vacuolar dye FM4-64 as previously described (Vida and Emr, 1995). FM4-64 [N-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl) pyridinium dibromide] was obtained from Molecular Probes Inc. (Eugene, OR). For temperature-shift experiments, cells were labeled at the permissive temperature and then shifted to the elevated temperature for the remainder of the incubation in the absence of the fluorophore.

Vacuolar Protein Sorting Assays

For analysis of CPS, CPY, ALP, and API processing, whole cells were labeled as previously described (Gaynor et al., 1994). Express [³⁵S]-protein labeling mix was added to a final concentration of 10 μCi/μl and cells were labeled during a 10-min pulse at 24°C. For temperature-shift experiments, cells were preshifted to 38°C for 30 min before labeling. The cultures were kept at the identical temperatures and chased as described above. After the chase, cells were harvested and lysates generated as previously described (Gaynor et al., 1994). Extracts were immunoprecipitated with antisera against CPY, ALP, CPS, and API, that have been previously characterized (Klionsky and Emr, 1989; Cowles et al., 1997b; antibody to API was a generous gift from Dan Klionsky, University of California, Davis, CA; Klionsky et al., 1992). Radioactive immunoprecipitates of CPS were treated with endoglycosidase H (DuPont NEN) as previously described (Gaynor et al., 1994). SDS-PAGE was done as described above however, gels were run at 25 mA constant current.

Microscopy

For observing the indirect immunofluorescence of the 60-kD subunit of the vacuolar ATPase, the method of (Redding et al., 1997) was used. In brief, exponentially growing cells were fixed in 4% formaldehyde and 0.1 M potassium phosphate, pH 6.5 for 16 h. The cell wall was removed by incubating cells with 45 μg/ml Zymolyase 100T (Seikagaku Kogyo, Tokyo, Japan) and cells were then treated with 1% SDS for 10 min. The fixed spheroplasts were first incubated in a 1:40 dilution of monoclonal antibody specific for the 60-kD subunit of the vacuolar ATPase (Molecular Probes Inc.), followed by successive incubations in goat anti-mouse IgG (1:3,000), and donkey anti-goat (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). GFP-ALP was transformed into the *fab1Δ2* strain and observed as previously described (Cowles et al., 1997a). 50 OD₆₀₀ units of cells grown to mid-log phase were harvested for each sample analyzed by electron microscopy. All cells were grown in YNB supplemented with the required amino acids and 2% dextrose. Cells were then fixed and processed for electron microscopy as described (Rieder et al., 1996).

Immunofluorescence localization of an HA-tagged version of *Fab1p* was performed as previously described (Bonangelino et al., 1997). In

brief, cells overexpressing Fab1p-HA were resuspended in 0.1 M sodium phosphate buffer (pH 6.5) and fixed in 4.4% formaldehyde for 40 min at 30°C. Fixed cells were converted to spheroplasts by incubation in 1.2 M sorbitol in 0.1 M phosphate buffer (pH 6.5) with 1% mercaptoethanol and 150 g of oxalyticase (Enzogenetics, Eugene, OR) per ml for 15 min at 30°C. After adhering the fixed spheroplasts to 1% polyethyleneimine-coated wells, cells were incubated with monoclonal anti-HA (MMSR101; Berkeley Antibody Co., Inc., Richmond, CA) at a 1:200 dilution overnight (16 h), followed by affinity-purified rabbit anti-Vac8p (Wang et al., 1998) at a 1:50 dilution for 1 h. The primary mouse antibodies were detected by using a 1:200 dilution of Oregon-Green-488 conjugated goat anti-mouse (Molecular Probes) and the rabbit antibodies were detected by a 1:200 dilution of Rhodamine red-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories Inc.). Incubations with the fluorophore-conjugated secondary antibodies were performed in a dark humidity chamber for 1 h. Images were collected using an MRC 1024 Scanning Confocal head mounted on a Nikon Optiphot equipped with a 100× oil immersion lens.

Results

Fab1p Is a Member of a Distinct Family of PtdInsP Kinases

Many of the phosphoinositides and phosphoinositide kinases (Fig. 1 A) present in mammalian cells are also found in the yeast *S. cerevisiae*. Homologs of the PtdIns 4-kinase (Stt4p and Pik1p; Flanagan et al., 1993; Yoshida et al.,

1994), PtdIns 3-kinase (Vps34p; Schu et al., 1993), and PtdIns(4)P 5-kinase (Mss4p; Desrivieres et al., 1998; Homma et al., 1998) have been identified in yeast. Two groups recently have identified a novel lipid, PtdIns(3,5)P₂, in yeast and mammalian cells, that is produced by the sequential phosphorylation of PtdIns at the 3' and 5'-positions (Dove et al., 1997; Whiteford et al., 1997). The PtdIns(3)P 5-kinase required for the synthesis of this previously undocumented lipid has not yet been identified.

Based on initial sequence analyses, Fab1p from *S. cerevisiae*, as well as its homologs from *S. pombe* and *C. elegans*, were proposed to be PtdIns(4)P 5-kinases (Yamamoto et al., 1995; Boronenkov and Anderson, 1995). A BLASTP search (NCBI; Altschul et al., 1997) with a COOH-terminal region of Fab1p (a.a. 2,000–2,278), containing the lipid kinase domain, reveals that the most closely related sequences with known enzymatic activities belong to a subset of PtdInsP kinases which include the Type I and Type II mammalian PtdIns(4)P 5-kinases. The Fab1p kinase domain therefore, is more closely related to the kinase domains of PtdInsP kinases (Fig. 1 B) than PtdIns kinases such as the 3-kinase Vps34p, the 4-kinases Stt4p and Pik1p, and the PtdIns kinase homologs Tor1p and Tor2p (Kunz et al., 1993).

Recent evidence supports the reclassification of the Type II PtdIns(4)P 5-kinases as PtdIns(5)P 4-kinases (Rameh et al., 1997) and this biochemical distinction of enzymatic activity is also borne out in sequence comparisons. Construction of a phylogenetic tree based on the pairwise alignment of the kinase domains of these groups, separates the Type I and Type II kinase domains into two classes (Fig. 1 B; Loijens and Anderson, 1996). Interestingly, Fab1p belongs to a third, distinct class of PtdInsP kinases when included in this analysis (Fig. 1 B; Loijens and Anderson, 1996). Consistent with this phylogenetic analysis, the yeast Mss4 protein has recently been identified as a PtdIns(4)P 5-kinase (Desrivieres et al., 1998; Homma et al., 1998; Fig. 1 B), indicating that this additional class does not appear to have been established simply on the basis of comparing lipid kinases from different species. These results suggest that the lipid kinase domain of Fab1p is distinct from analogous domains of known PtdIns(4)P 5-kinases and PtdIns(5)P 4-kinases, raising the possibility that Fab1p phosphorylates a PtdInsP substrate distinct from either PtdIns(4)P or PtdIns(5)P, perhaps PtdIns(3)P.

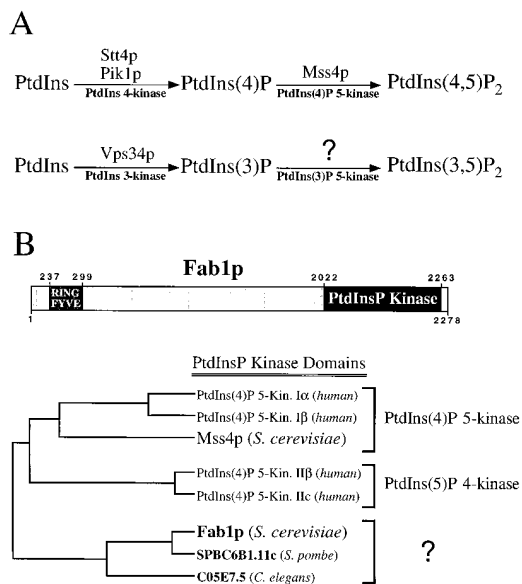


Figure 1. Phosphatidylinositol derivatives and their kinases in yeast. (A) A diagram of the D-3 and D-4 phosphorylated phosphoinositides present in the yeast *S. cerevisiae*. Above the arrows, the specific kinases determined to be involved in their biosynthesis are listed. (B) A phylogenetic comparison of the Fab1p lipid kinase domain to the analogous domain of other PtdInsP kinases. Using the DNASTar program MegAlign, the *S. cerevisiae* Fab1p kinase domain (a.a. 2022–2263) and its homologues SPBC6B1.11c (AL021838; a.a. 358–595) and C05E7.5 (Z67879; a.a. 924–1170) from *S. pombe* and *C. elegans*, respectively, were aligned and a phylogenetic tree was constructed with the corresponding kinase domains of human PtdIns(4)P 5-kinases Type Ia (U78575; a.a. 125–449), Type Ib (U52376; a.a. 1–288), Type IIβ (U85245; a.a. 100–416), Type IIc (P53807; a.a. 110–406), and *S. cerevisiae* Mss4p (D13716; a.a. 439–765). The multiple sequence alignment was performed using the Jotun-Hein method with default program parameters, using the PAM250 residue weight table.

Inactivation of Fab1p Results in Undetectable Levels of PtdIns(3,5)P₂

To determine if Fab1p is required for the production of specific phosphatidylinositol derivatives in vivo, we analyzed phosphoinositide levels in wild-type yeast cells and in a strain deleted for *FAB1*. SEY6210 (wild-type) and *fab1Δ1* cells were labeled with myo-[2-³H]inositol for 12 h. At the end of this labeling period, cells were osmotically shocked with 0.9 M NaCl for 10 min. Hyperosmotic shock facilitates the analysis of PtdIns(3,5)P₂, which is difficult to detect in the absence of this treatment, without dramatically affecting the levels of other observable lipids (Dove et al., 1997). The labeled cells were then lysed in acidified chloroform/methanol and the cellular lipids were deacylated and identified by HPLC analysis (see Materials and

Methods). This method enables the resolution and quantification of cellular PtdIns(3)P, PtdIns(4)P, PtdIns(3,5)P₂ and PtdIns(4,5)P₂ (Auger et al., 1989; Schu et al., 1993; Dove et al., 1997).

When this analysis is carried out using wild-type yeast cells, four distinct glycerophosphoinositides corresponding to PtdIns(3)P (13,700 cpm, fraction 32-33), PtdIns(4)P (13,700 cpm, fraction 35-36), PtdIns(3,5)P₂ (2,600 cpm, fraction 66-67) and PtdIns(4,5)P₂ (4,100 cpm, fraction 73-74) are clearly separated (Fig. 2 A). The identities of the deacylated lipid products were confirmed using tritiated standards (data not shown). When an equal quantity of labeled cellular lipids isolated from *fab1Δ1* cells are assayed in the same manner, the most dramatic effects are observed on PtdIns(3,5)P₂ levels, which are undetectable (<50 cpm; Fig. 2 A). Relative to wild-type cells, other phosphoinositide levels are also influenced in the *fab1Δ1* mutant cells: PtdIns(3)P increases ~100% (25,000 cpm), PtdIns(4)P decreases ~50% (7,300 cpm) whereas PtdIns(4,5)P₂ decreases 60% (1,600 cpm).

To determine if the dramatic changes in PtdIns(3,5)P₂ levels seen in the *fab1Δ1* strain resulted directly from a

loss of Fab1p activity, we assayed PtdIns(3,5)P₂ in a *fab1Δ2 + pfab1-2 (fab1^{tsf})* strain briefly shifted to nonpermissive temperature (Yamamoto et al., 1995; see Materials and Methods). At the permissive temperature, *fab1^{tsf}* cells have normal growth characteristics and wild-type vacuole morphology. However, shifting these cells to 37°C results in growth defects and aberrant vacuole morphologies (Bonangelino et al., 1997; Yamamoto et al., 1995). Wild-type and *fab1^{tsf}* cells were labeled for 12 h with myo-[2-³H]inositol at 22°C, as before. At the end of this labeling period, half of the wild-type and *fab1^{tsf}* cells were incubated with 0.9 M NaCl for 10 min at permissive temperature. The remaining cells were exposed to 0.9 M NaCl for 5 min at 22°C and shifted to the nonpermissive temperature of 38°C for the final 5 min of osmotic shock. Even at the permissive temperature (22°C) PtdIns(3,5)P₂ levels are reduced sevenfold (800 cpm) in the *fab1^{tsf}* cells compared to the wild-type control (Fig. 2), whereas the levels of other phosphoinositides remain only moderately affected (data not shown). In the *fab1^{tsf}* strain at the nonpermissive temperature, PtdIns(3,5)P₂ synthesis is dramatically affected, reaching undetectable levels (<50 cpm) after a 5-min shift. Compared to the wild-type control, the high temperature incubation results in no significant changes in the other lipids. Given the homology of Fab1p to the family of PtdInsP kinases and the fact that in yeast Vps34p is the only source of PtdIns(3)P, the rapid depletion of PtdIns(3,5)P₂ observed in the *fab1* mutant cells suggests that Fab1p encodes a PtdIns(3)P 5-kinase.

Mutations within the Kinase Domain of Fab1p Cause a Dramatic Decrease in Cellular PtdIns(3,5)P₂ Levels

The results demonstrate that Fab1p is required for normal PtdIns(3,5)P₂ levels. To address the role of the putative kinase domain of Fab1p in phosphatidylinositol metabolism, we used site-directed mutagenesis to make substitutions in highly conserved amino acid positions of the kinase domain. Specifically, Gly2042 and Gly2045 were simultaneously changed to valine residues (G2042/2045V) and Asp2134 was replaced with an arginine residue (D2134R; Fig. 3 A; see Materials and Methods). The GGxxG glycine triad motif containing Gly2042 and Gly2045 of Fab1p is reminiscent of the highly conserved GxGxxG motif present in the nucleotide binding site of numerous protein kinases (Bossemeyer et al., 1993; Hemmer et al., 1997; Sicheri et al., 1997; Xu et al., 1997). In the protein kinases, these glycine residues are included in the conserved glycine-rich loop and mutation of these residues to alanine or serine residues results in decreases in enzymatic activity (Hemmer et al., 1997; Grant et al., 1998). This comparison predicts that the substitution of these glycine residues to valine residues in Fab1p would result in commensurate impairment of lipid kinase activity. By similar sequence comparisons, Asp2134 of Fab1p appears to be analogous to the catalytically important Asp386 of the c-Src protein kinase (Xu et al., 1997) and mutation of this aspartate residue would be expected to severely affect phosphotransfer (Bossemeyer et al., 1993). An analogous mutation of this aspartate residue to an alanine residue in the Vps34p lipid kinase also lacked detectable amounts of PtdIns(3)P synthesis (Schu et al., 1993). Because of the apparent impor-

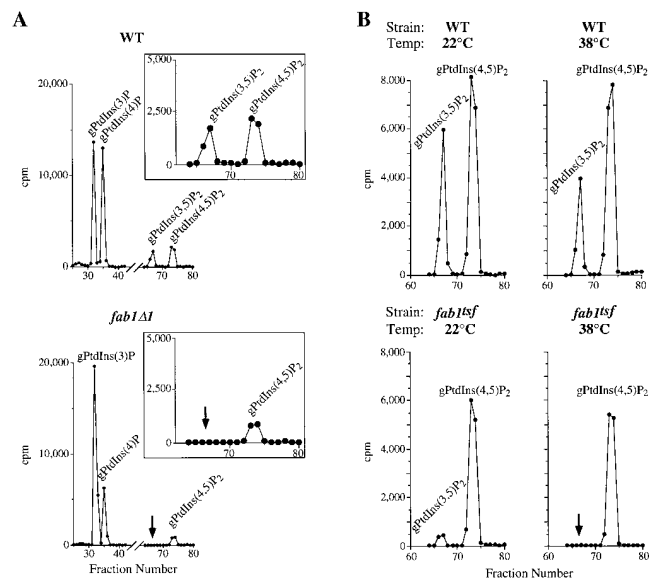


Figure 2. PtdIns(3,5)P₂ is undetectable in *fab1* mutant cells. (A) Wild-type (SEY6210) and *fab1Δ1* cells were labeled with myo-[2-³H]inositol for 12 h at 22°C and hyperosmotically shocked with 0.9 M NaCl for 10 min. Cells were then glass bead lysed and the cellular lipids extracted, deacylated and separated by HPLC (see Materials and Methods). Fractions (0.67 min) were collected and counted for [³H] radioactivity. Deacylated (glycero-) PtdIns(3)P, PtdIns(4)P, PtdIns(3,5)P₂, and PtdIns(4,5)P₂ are indicated. Arrows indicate the expected position of gPtdIns(3,5)P₂. (B) Wild-type and *fab1^{tsf}* cells were labeled for 12 h with myo-[2-³H]inositol at 22°C, after which both cultures were separated into two equal aliquots. One aliquot of each strain was incubated with 0.9 M NaCl for 10 min at 22°C. The remaining wild-type and *fab1^{tsf}* cells were incubated with 0.9 M NaCl for 5 min at 22°C and, for the final 5 min of hyperosmotic shock, shifted to the nonpermissive temperature of 38°C. The peaks representing PtdIns(3,5)P₂ and PtdIns(4,5)P₂ are indicated. The arrow highlights the elution point of the gPtdIns(3,5)P₂ standard. The data presented are representative of multiple experiments.

Table I. Cellular Distribution of Fab1 Protein

	P13	P100	S100
Fab1p	30%	40%	30%
V-ATPase	70%	25%	<5%
Kex2p	10%	90%	<5%
G6PDH	5%	5%	90%

100,000 g yielding soluble fractions (S13 and S100) and pelletable fractions (P13 and P100; see Materials and Methods). All fractions were assayed for the presence of Fab1p, the 100-kD vacuolar ATPase subunit, the Golgi/endosome localized protease, Kex2p and the cytosolic marker glucose 6-phosphate dehydrogenase (G6PDH) using antibodies specific to these proteins. The majority of Fab1p is detected in the pellet fractions (30% P13, 40% P100) and the remainder (30%) is found in the S100 fraction (Table I). In this experiment, vacuolar membranes, identified by the presence of the vacuolar ATPase 100-kD subunit, are found predominantly in the P13 fraction (75%) and also in the P100 (25%). Golgi and endosomal compartments containing Kex2p are enriched in the P100 fraction whereas the cytosolic fraction (G6PDH) is predominantly S100. Fab1p, therefore, appears to be enriched in fractions that contain endosomal and vacuolar membranes, suggesting that Fab1p functions at one (or more) of these compartments.

To refine the localization of native Fab1p, CEN and 2 μ versions of HA-tagged Fab1p (Fab1p-HA) have been constructed (see Materials and Methods). These constructs complement the growth and vacuolar morphology defects of the *fab1* deletion strain (data not shown). Indirect immunofluorescence using the CEN construct did not produce a discernible signal above background (data not shown). However, at multicopy levels (8–10-fold overexpression; data not shown), we find that a pool of Fab1p-HA colocalizes with Vac8p (Fig. 5), a protein associated with the vacuolar membrane (Wang et al., 1998). Cytoplasmic accumulations of the Fab1p-HA protein are also evident. In some cells, no signal could be detected above background, presumably due to variable levels of Fab1p-HA overexpression within the cell population. Consistent with the subcellular fractionation results, which suggest that Fab1p is distributed among multiple membranes (Table I), this data indicates that Fab1p is associated with vacuolar and prevacuolar compartments.

Point Mutations within the Fab1p Kinase Domain Result in Abnormal Vacuolar Morphology

Transformation of the *fab1* null background with either of the Fab1p point mutant constructs results in a differential suppression of the *fab1* phenotypes. As shown previously (Yamamoto et al., 1995), these defects include: (a) temperature-sensitive growth, (b) vacuole acidification defects and, (c) a 2.5-fold increase in vacuolar surface area, which leads to inappropriate nuclear segregation during mitosis. The temperature-sensitive growth of the *fab1* $\Delta 2$ at 30°C is complemented in cells expressing either of the Fab1p point mutant constructs. However, although the *fab1*^{G2042/2045V} strain grows even at 38°C, the *fab1*^{D2134R} strain does not. Similarly, rescue of the vacuole acidification defect as

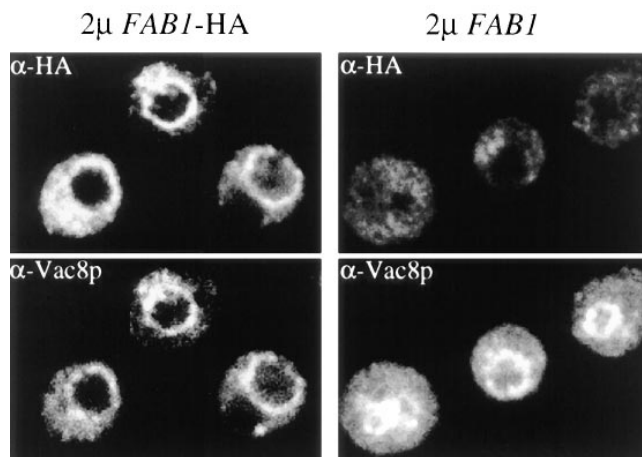


Figure 5. Localization of HA-tagged Fab1p by indirect immunofluorescence. Cells overexpressing HA-tagged Fab1 or wild-type Fab1 protein from multi-copy plasmids were fixed, converted to spheroplasts and then incubated with monoclonal anti-HA or anti-Vac8p antibodies as described in the Materials and Methods. The localization of Fab1p-HA and Vac8p were determined by indirect immunofluorescence. HA-tagged Fab1p and Vac8p signals are indicated.

measured by quinacrine staining of the vacuoles in the *fab1* mutants also varied. Vacuolar quinacrine fluorescence in both the *fab1* $\Delta 2$ and the *fab1*^{D2134R} strains is extremely weak, indicating a severe acidification defect. However, the *fab1*^{G2042/2045V} vacuole appears to have the same fluorescent intensity as the wild-type control (data not shown). By FM4-64 staining of the vacuolar membrane and Nomarski optics, the yeast vacuole appears as a

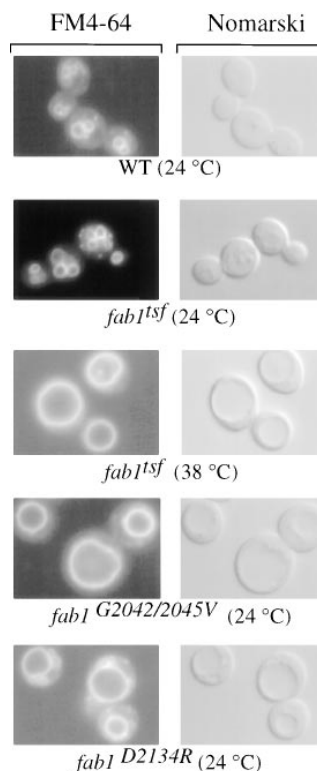


Figure 6. *fab1* mutant cells have an abnormal vacuole morphology. In the right panels, vacuoles were observed by Nomarski optics. On the left, the same fields are shown under fluorescent illumination (rhodamine channel). Cells were grown to mid-log phase at 24°C and the vacuoles labeled with the vital stain FM4-64 for 15 min (see Materials and Methods). After labeling, all of the strains were chased in the absence of dye for 1 h at 24°C except a duplicate of the *fab1*^{tsf} strain that was chased at the nonpermissive temperature of 38°C.

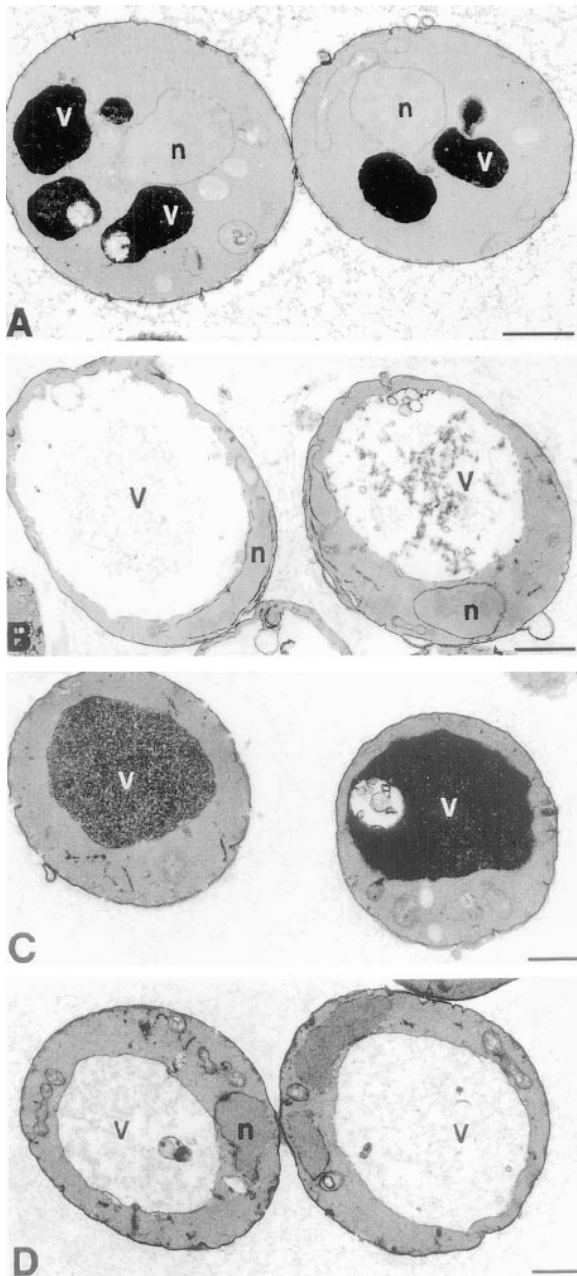


Figure 7. Electron microscopy of *fab1* mutants. Cells were grown to mid-log phase and fixed with 3% glutaraldehyde. Cells were then converted to spheroplasts and visualized by electron microscopy (see Materials and Methods). (A) Wild-type (SEY6210) cells. (B) *fab1Δ1* cells. (C) *fab1^{G2042/2045V}* cells. (D) *fab1^{D2134R}* cells. Bar, 0.5 μm.

multilobed structure. However, the vacuolar phenotype of the *fab1^{G2042/2045V}* and *fab1^{D2134R}* strains are similar to that of the *fab1* null strain (Yamamoto et al., 1995) or the *fab1^{tsf}* after a 30-min shift to the nonpermissive temperature (Fig. 6). The vacuole in these mutants appears as a single grossly enlarged organelle that comprises the majority of the cell volume. Smaller compartments were also revealed immediately adjacent to the vacuole in the *fab1^{D2134R}* strain (Fig. 6) At the permissive temperature

however, the vacuole morphology of the *fab1^{tsf}* strain is indistinguishable from the wild-type control (Fig. 6).

Electron microscopic analysis not only highlights the enlarged vacuolar phenotype resulting from decreases in PtdIns(3,5)P₂ synthesis, but also shows the differential effects of Fab1p kinase domain point mutations on vacuole morphology. The electron-dense vacuole of the wild-type and *fab1^{G2042/2045V}* strains contrasts greatly with the electron-transparent nature of the vacuole in the *fab1Δ1* and *fab1^{D2134R}* strains (Fig. 7). The opacity of the vacuole is likely to be a measure of vacuole acidification, as inactivation of the vacuolar ATPase results in electron-transparent vacuoles (Wurmser and Emr, 1998). Thus, the relatively severe acidification defect in the *fab1^{D2134R}* mutant compared to the *fab1^{G2042/2045V}* mutant strain correlates with the levels of PtdIns(3,5)P₂ synthesis. Fab1p kinase activity is therefore not only essential for maintenance of vacuolar size and acidification, but may also influence temperature-sensitive growth.

***Fab1p* Kinase Activity Is Not Required for Proper Vacuolar Hydrolase Sorting**

The Vps34p PtdIns 3-kinase is crucial in Golgi to vacuole transport of hydrolases, and recent evidence suggests that one function of PtdIns(3)P is to regulate RING-FYVE-containing proteins (i.e., Vac1p and Vps27p) that are essential for hydrolase transport from the Golgi to the vacuole (Weisman and Wickner, 1992; Piper et al., 1995; Burd et al., 1997). The finding that PtdIns(3)P can serve as a substrate for a PtdIns(3)P 5-kinase raises the possibility that the requirement for Vps34p in vacuolar protein transport arises through its role in the production of PtdIns(3,5)P₂ (Dove et al., 1997). Our findings that PtdIns(3,5)P₂ levels are specifically and dramatically decreased in *fab1* mutant strains provides a direct means to assess this hypothesis.

Several trafficking routes from the Golgi to the yeast vacuole have been characterized. Soluble CPY and the type II integral membrane protein carboxypeptidase S, CPS, transit to the vacuole via an endosomal-dependent trafficking route, the CPY pathway (Banta et al., 1988). Alkaline phosphatase (ALP) however, arrives at the vacuole by a distinct pathway that bypasses the endosome, the ALP pathway (Cowles et al., 1997a,b). A third route for protein delivery to the vacuole delivers the protease aminopeptidase I (API) from the cytoplasm to the vacuole through autophagy (Scott et al., 1997). To address the effects that the loss of PtdIns(3,5)P₂ synthesis may have on each pathway, we assayed the proteolytic maturation of representative hydrolases from the three known pathways in the *fab1* mutant strains. The conversion of the precursor hydrolase to the mature form occurs upon delivery of the hydrolase to a proteolytically competent vacuole.

Data from the original *fab1-2* temperature sensitive allele supports the idea that PtdIns(3,5)P₂ is not required for anterograde flow of traffic to the vacuole via the CPY pathway. After a 60-min preshift at the nonpermissive temperature, CPY was properly sorted and processed (Yamamoto et al., 1995). Our data with the *fab1^{tsf}* strain also indicate that the other transport pathways to the vacuole do not depend on Fab1p function. As shown for CPY,

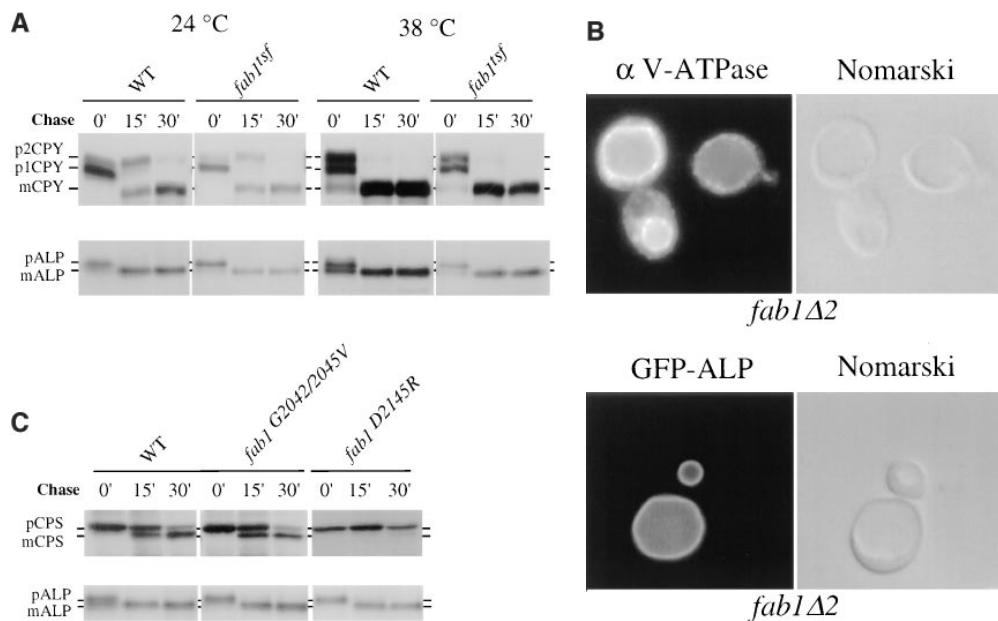


Figure 8. Intracellular sorting of vacuolar hydrolases in *fab1* mutant cells. Yeast cells were metabolically labeled with Express [³⁵S]-protein labeling mix during a 10-min pulse and then chased in the presence of an excess of non-radiolabeled methionine and cysteine for 0, 15, or 30 min (see Materials and Methods). Each of the vacuolar enzymes was immunoprecipitated, followed by SDS-PAGE and autoradiography. The migration positions of the precursor and mature forms of the hydrolases are indicated on the left side of the figure panels. (A) The maturation of CPY and ALP was examined in the *fab1^{tsf}* strain compared with wild-type, as described in Materi-

als and Methods. However, for the nonpermissive temperature, cells were preshifted to 38°C for 30 min before labeling and maintained at the elevated temperature throughout the chase period. (B) The upper panel, on the left, shows indirect immunofluorescence localization of the 60-kD subunit of the vacuolar ATPase using a monoclonal antibody in the *fab1Δ2* strain. For the immunofluorescence, cells were fixed overnight, spheroplasted, and then incubated with antibody as described in Materials and Methods. The lower panel, on the left, shows GFP-ALP localization in a *fab1Δ2* strain, observed by fluorescence microscopy (see Materials and Methods). On the right side for both panels are the identical fields observed under Nomarski optics. (C) The maturation of the vacuolar hydrolases CPS and ALP in the two point mutant strains (*fab1^{G2042/2045V}* and *fab1^{D2134R}*) and the wild-type strain SEY6210. The labeling and chase were done as in A however, the cells were maintained at 24°C.

ALP is also properly matured (Fig. 8 A) after a 30-min preshift to the nonpermissive temperature. The processing of two additional vacuolar hydrolases, CPS and API, was also found to proceed as in wild-type cells (data not shown). This sorting data from the *fab1^{tsf}* strain (Fig. 8 A) at the nonpermissive temperature combined with the PtdIns(3,5)P₂ quantitation (Fig. 2 B) from the same strain under similar temperature shift conditions indicates that Fab1p and PtdIns(3,5)P₂ are not directly involved in the CPY, ALP, or API sorting pathways. In contrast, after only a 5-min preshift to the nonpermissive temperature, the *vps34^{tsf}* missorts and secretes >95% of the pulse-labeled CPY (Stack et al., 1995).

As an additional test for protein trafficking to the vacuole, we used indirect immunofluorescence in *fab1Δ2* cells with monoclonal antibody against the 60-kD vacuolar ATPase subunit (V₁ complex). This subunit is found distributed on the vacuolar membrane in wild-type cells and arrives at the vacuole via the CPY pathway (Rieder et al., 1996; Piper et al., 1997). In *fab1Δ2* cells, the fluorescence for the 60-kD vacuolar ATPase subunit is coincident with the vacuolar membrane observed by Nomarski optics (Fig. 8 B). The ATPase subunit is similarly localized in the original *fab1-2* temperature-sensitive strain after incubation at the nonpermissive temperature for 2 h (Bonangelino et al., 1997). Similarly, we used a GFP-ALP construct to localize ALP in the *fab1Δ2* mutants. GFP-ALP also was properly localized to the vacuolar membrane in the *fab1Δ2* cells (Fig. 8 B). Therefore, Fab1p function, and PtdIns(3,5)P₂ synthesis, is not essential for delivery of biosynthetic cargo

to the vacuole via either the CPY or ALP transport pathways.

In the *fab1^{G2042/2045V}* and *fab1^{D2134R}* strains, with reduced and undetectable steady-state levels of PtdIns(3,5)P₂, respectively, we assessed the proteolytic processing of both CPS and ALP (Fig. 8 C). In both mutant strains, ALP maturation occurs at a rate indistinguishable from wild-type, even at the 15-min chase point (Fig. 8 C). Compared to the control, CPS processing also occurs with wild-type kinetics in *fab1^{G2042/2045V}* mutant cells (Fig. 8 C). However, there is a significant kinetic delay in CPS maturation in *fab1^{D2134R}* cells (Fig. 8 C). Based on chase times extending to 120 min (data not shown), the half-time for processing CPS to the mature form in wild-type and *fab1^{G2042/2045V}* cells is approximately 20 min at 24°C, whereas in the *fab1^{D2134R}* strain it is >120 min. Mature API is also detectable in both of these mutants, however with the *fab1^{G2042/2045V}* mutant, there is a slight kinetic delay in maturation that is more severe in the *fab1^{D2134R}* mutant (data not shown). These data indicate that all three vacuolar transport pathways are generally intact, as a significant pool of hydrolases delivered to the vacuole by these routes are matured independent of Fab1p kinase activity.

The kinetic delay in hydrolase maturation observed in the *fab1Δ* (Yamamoto et al., 1995) or point mutant strains (Fig. 8 C) may be due to vacuolar acidification defects that result from the loss of Fab1p kinase activity. Loss of vacuolar acidification is known to reduce the activity of proteinase A (Sorensen et al., 1994), a vacuolar protease that plays a key role in the maturation of many vacuolar hydro-

lases (Morano and Klionsky, 1994; Van Den Hazel et al., 1996). As a result, cells lacking vacuolar ATPase function exhibit kinetic delays in the conversion of many hydrolases to their mature forms (Morano and Klionsky, 1994). As assessed by quinacrine staining, the lipid kinase activity of Fab1p is also required for proper vacuole acidification. The normal quinacrine fluorescence in the *fab1*^{G2042/2045V} strain (data not shown), indicates that even low levels of PtdIns(3,5)P₂ are sufficient to maintain vacuolar pH. However, the *fab1*^{D2134R} mutant strain shows a severe vacuole acidification defect (data not shown). The processing delays observed in the *fab1* point mutants are likely to be directly due to the slow maturation of certain hydrolases in a compromised vacuole. Consistent with this hypothesis, the degree of vacuole acidification in the two point mutants correlates well with the hydrolase maturation delays observed in these strains.

Vac7p Is Required for PtdIns(3,5)P₂ Synthesis

A number of observations suggest that an upstream regulatory factor(s) may be required for Fab1p activity. First, overexpression of *FAB1* from a multicopy vector fails to cause elevations in endogenous PtdIns(3,5)P₂ (data not shown), suggesting that the concentration of Fab1-mediated synthesis of PtdIns(3,5)P₂ requires a limiting cofactor. Furthermore, immunoprecipitations of Fab1p from yeast protein extracts did not contain detectable PtdIns(3)P 5-kinase activity (data not shown), possibly due to the dissociation of Fab1p from an activator protein(s) during immunopurification. Finally, several of the recently described *vac* mutants exhibit phenotypes which are strikingly similar to the *fab1* mutants, *VAC7* is the best characterized. Like *fab1*, *vac7* mutants exhibit acidification defects and an enlarged vacuolar phenotype (Bonangelino et al., 1997). This raises the possibility that Fab1p and Vac7p function together to regulate a common cell function (i.e., vacuole membrane homeostasis). To further assess whether Vac7p functions in the Fab1p pathway, we examined the morphology of *vac7Δ* cells by electron microscopy and assayed a *vac7* mutant for changes in PtdIns(3,5)P₂ levels.

Relative to wild-type cells, which exhibited two to three electron-dense staining vacuoles when examined by electron microscopy, the *vac7Δ* (LWY1481) strain has a single enlarged vacuole that appears less electron dense (Fig. 7 and 9 A). Heterogeneous intravacuolar structures appear more prominently in *vac7Δ* cells than in *fab1Δ1* cells (Fig. 7 B and 9 A). It is also important to note that aberrant membranes (vesicles and endosomal intermediates) do not appear to accumulate in the cytoplasm of the *fab1Δ1* or *vac7Δ* cells, a hallmark of many mutants which have Golgi to vacuole transport defects (Cowles et al., 1994; Babst et al., 1997).

As the similar morphological defects of *fab1Δ1* and *vac7Δ* cells suggest, if Fab1p and Vac7p function in the same pathway, then the mutation of *VAC7* might also have effects on PtdIns(3,5)P₂ levels. Therefore, we analyzed PtdIns(3,5)P₂ in a *vac7* mutant strain. Wild-type and *vac7Δ* cells were labeled 12 h with myo-[2-³H]inositol, osmotically shocked for 10 min with 0.9 M NaCl, lysed and analyzed by HPLC as described earlier. PtdIns(3,5)P₂

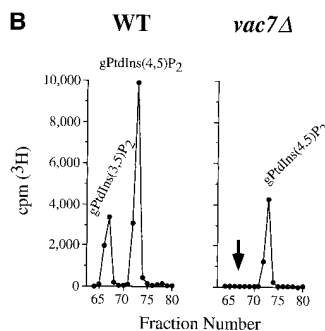
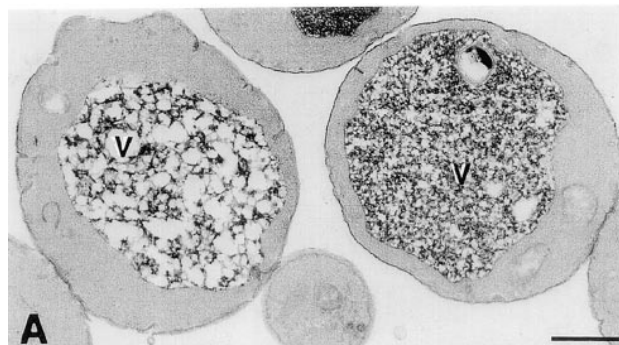


Figure 9. The *vac7Δ* strain displays phenotypes similar to the *fab1Δ1* strain. (A) Electron microscopy of *vac7Δ* cells prepared as in Fig. 6. (B) Wild-type and *vac7Δ* cells were labeled 12 h with myo-[2-³H]inositol and osmotically shocked with 0.9 M NaCl for 10 min at 22°C. Each strain was then lysed and the extracted cellular lipids deacylated and separated by HPLC as in Fig. 2. Deacylated products representing PtdIns(3,5)P₂ and PtdIns(4,5)P₂ are indicated. The arrow indicates the expected position of PtdIns(3,5)P₂. The data are representative of several experiments. Bar, 0.5 μm.

(5,300 cpm total) was observed in wild-type cells, however, PtdIns(3,5)P₂ was undetectable in *vac7Δ* cells (<50 cpm; Fig. 9 B). As seen in *fab1Δ1* cells (Fig. 2 A), the *vac7Δ* mutant also exhibits a ~50% decrease PtdIns(4,5)P₂ (Fig. 9 B), 100% increase in PtdIns(3)P and a 50% decrease in PtdIns(4)P (data not shown). Vac7p is not homologous to any other known protein and does not contain kinase motifs (Bonangelino et al., 1997). These data suggest that Vac7p, possibly in association with Fab1p, is required for PtdIns(3,5)P₂ synthesis.

Discussion

Fab1p Kinase Activity Is Required for PtdIns(3,5)P₂ Synthesis in *S. cerevisiae*

Sequence comparisons of the putative lipid kinase domain of Fab1p (a.a. 2,022–2,263) with other kinases of known enzymatic activities categorized Fab1p into a unique subclass of general PtdInsP kinases (Fig. 1 B). Two distinct doubly phosphorylated derivatives of PtdIns have been identified in yeast, PtdIns(4,5)P₂ and PtdIns(3,5)P₂, and the enzyme activities involved in their stepwise synthesis already have been suggested (Fig. 1 A). Although its identity was not known, the existence of a specific PtdIns(3)P 5-kinase is supported by the sequential synthesis of PtdIns(3,5)P₂ in murine Ph cells from PtdIns(3)P, as well as the requirement for Vps34p activity in yeast for PtdIns(3,5)P₂ production (Dove et al., 1997; Whiteford et al., 1997). Given that the sequence of Fab1p kinase domain

distinguishes it from the Type I and Type II PtdIns(4)P 5-kinases, Fab1p is the likely PtdIns(3)P 5-kinase in yeast.

Consistent with this hypothesis, our data show that *fab1Δ* cells have undetectable levels of PtdIns(3,5)P₂, whereas the amounts of other phosphoinositides are only moderately affected (Fig. 2 A). To determine if this decrease in PtdIns(3,5)P₂ is the direct result of Fab1p inactivation, we assessed the phosphoinositide levels in a strain harboring a temperature-sensitive allele of *FAB1*. Despite the fact that this mutant protein is stable during the shift to the nonpermissive temperature, cellular PtdIns(3,5)P₂ levels show a rapid decrease, reaching undetectable levels within 5 min at 38°C, whereas the amounts of other phosphoinositides do not change relative to wild-type cells treated in the same manner (Fig. 2 B). The differential loss of PtdIns(3,5)P₂ in cells expressing either of the two kinase domain point mutant constructs also provides evidence that Fab1p is the PtdIns(3)P 5-kinase (Fig. 4). By comparison with known structures of protein kinases, the glycine residues replaced in the Fab1p G2042/2045V mutant are located in a conserved glycine-rich loop region between two β-strands that form the ATP-positioning motif (Sicheri et al., 1997; Xu et al., 1997). Mutagenesis of these residues in cAPK and the insulin receptor results in reduced kinase activity (Hemmer et al., 1997; Odawara et al., 1989). Therefore, the residual PtdIns(3,5)P₂ synthesis observed in the *fab1*^{G2042/2045V} strain is consistent with Fab1p directly catalyzing the synthesis of PtdIns(3,5)P₂ from PtdIns(3)P. Additionally, mutation of the aspartate residue at position 2,134 eliminates the production of PtdIns(3,5)P₂. This aspartate is analogous to the catalytically critical Asp166 of cAPK (Bossemeyer et al., 1993). Mutation of the comparable residue in the v-fps and c-kit protein kinases as well as the yeast lipid kinase Vps34p results in the loss of kinase activity (Moran et al., 1988; Tan et al., 1990; Schu et al., 1993).

Although the *in vivo* lipid quantitation data from the *fab1* mutants provide strong evidence that Fab1p is the functionally relevant PtdIns(3)P 5-kinase *in vivo*, it has not yet been possible to demonstrate the activity *in vitro*. Immunopurification of Fab1p from native yeast extracts did not contain detectable PtdIns(3)P 5-kinase activity (data not shown), nor were we able to demonstrate the production of PtdIns(3,5)P₂ in crude yeast lysates (data not shown). We suggest that these negative *in vitro* results may be due to the instability or lack of necessary cofactors required for Fab1p 5-kinase activity (see below) or the relative inefficiency of the enzyme under our *in vitro* conditions.

Vac7p as an Upstream Regulator of Fab1p

The catalytic activity and localization of most if not all phosphoinositide kinases are controlled by interactions with regulatory proteins *in vivo*. The p85/p110 phosphoinositide 3-kinase translocates to the plasma membrane through its interaction with the cytosolic tail of ligand-bound tyrosine kinase receptors (Hu et al., 1992), whereas the p110 phosphoinositide 3-kinase γ-isoform is activated in response to heterotrimeric G-protein-coupled receptor stimulation (Stoyanov et al., 1995; Stephens et al., 1997). Similarly, the Vps34p PtdIns 3-kinase binds a membrane

associated serine/threonine protein kinase, Vps15p (Stack et al., 1993, 1995). This interaction localizes Vps34p to an intracellular membrane compartment and activates its PtdIns 3-kinase activity (Schu et al., 1993; Stack et al., 1995). We have made several observations which point to the existence of a protein cofactor(s) that is required for Fab1p activity. First, cells overexpressing Fab1p failed to exhibit increases in PtdIns(3,5)P₂ levels, suggesting that a protein critical for Fab1p activation may be limiting. Similarly, overexpression of Vps34p does not elevate cellular PtdIns(3)P levels (data not shown), presumably due to the limiting pool of activated Vps15p available to properly recruit and regulate Vps34p activity. Second, the lack of detectable PtdIns(3)P 5-kinase activity in Fab1p *in vitro* experiments also suggests that stimulatory signals from an upstream regulator may be absent from immunoprecipitated Fab1p or unstable in crude lysates.

The identification of a possible upstream activator for Fab1p came from a genetic screen in yeast for mutants exhibiting aberrant vacuole segregation structures during cytokinesis (Wang et al., 1996). This screen revealed several complementation groups displaying the enlarged vacuolar phenotypes of *fab1* cells. Indeed *FAB1* was isolated numerous times as well as a previously uncharacterized gene, *VAC7* (Bonangelino et al., 1997). To test the possibility that *Vac7p* and Fab1p function in the same pathway, we assayed phosphoinositide levels in *vac7Δ* cells and found that PtdIns(3,5)P₂ was undetectable in this mutant strain (Fig. 9 B). Thus, *Vac7p* activity, like Fab1p, is required for PtdIns(3,5)P₂ production, suggesting that *Vac7p* functions upstream of Fab1p. *Vac7p* encodes a 1,165 a.a. protein that is enriched in vacuole membranes and bears no homology to lipid kinases (Bonangelino et al., 1997). It contains a potential transmembrane domain (a.a. 919–943) and is not stripped from the membrane fraction with high NaCl, urea, or Na₂CO₃ treatment indicating that *Vac7p* is an integral membrane protein. *Vac7p* however, is unlikely to be critical for localizing Fab1p to the membrane as its deletion failed to increase the soluble pool of Fab1p (unpublished observation). This suggests that *Vac7p* may function to stimulate Fab1p PtdIns(3)P 5-kinase activity rather than recruit Fab1p to the membrane.

Other members of the *FAB1/VAC7* gene family are likely to exist. In fact, a complementation group distinct from *fab1* and *vac7*, designated *vac14-1*, has been identified that shares the vacuole acidification and morphological defects observed in *fab1* and *vac7* mutants (Bonangelino et al., 1997). Furthermore, in the *vac14-1* mutant, both these defects are suppressed by the overexpression of *FAB1* (Bonangelino et al., 1997). Therefore, *Vac14p* represents another protein that is likely to function upstream of Fab1p. Consistent with this model, *vac14-1* mutant cells are also severely compromised in their ability to synthesize PtdIns(3,5)P₂ (unpublished observations). Efforts to clone *VAC14* are currently underway. Interestingly, overexpression of *FAB1* does not suppress the mutant phenotypes of *vac7Δ* cells (Bonangelino et al., 1997). Collectively, these data suggest that Fab1p, *Vac7p* and *Vac14p* function in a common signal transduction pathway which, through the activation of Fab1p, results in the localized production of PtdIns(3,5)P₂.

It has been previously demonstrated that a hyperos-

otic shock is required for maximal PtdIns(3,5)P₂ accumulation in yeast. This was suggested to be due to the activation of a PtdIns(3)P 5-kinase (Dove et al., 1997). Our data does not exclude the possibility that Fab1p, or its putative upstream activators Vac7p and Vac14p, are sensitive to hyperosmotic stress. However, enzymes dedicated to the turnover of PtdIns(3,5)P₂, possibly corresponding to one or more of the four PtdIns(5)P 5-phosphatase homologs in yeast (Srinivasan et al., 1997; Stolz et al., 1998), also represent candidate activities that may be sensitive to hyperosmotic stress. The further characterization of upstream activators, such as Vac7p and Vac14p, and the biologically relevant PtdIns(3,5)P₂ turnover activity should help in distinguishing between these possibilities.

Potential Intracellular Roles for PtdIns(3,5)P₂

With the identification of PtdIns(3,5)P₂ in yeast, it was suggested that this lipid, rather than PtdIns(3)P, plays a role in Golgi to vacuole trafficking (Dove et al., 1997). Although previous data indicates the essential nature of PtdIns(3)P in anterograde traffic to the vacuole (Schu et al., 1993), it was argued that the apparent requirement for Vps34p in this pathway reflects the need for PtdIns(3)P solely as a substrate for the 5-kinase activity. However, our data show that Fab1p, and therefore PtdIns(3,5)P₂ synthesis, appears not to play a role in anterograde protein traffic, as the delivery of proteins along all known vacuolar transport pathways are unaffected in *fab1* mutants. *fab1* mutant cells do give rise to several phenotypes including, temperature-sensitive growth, a vacuolar acidification defect, as well as an inability to properly segregate genomic and vacuolar material upon cytokinesis (Yamamoto et al., 1995). The later phenotypes appear to be secondary consequences resulting from the enlargement of the vacuole, which is the most immediate defect caused by the inactivation of the *fab1^{tsf}*. Indeed, the normal vacuole morphology observed in the *fab1^{tsf}* at the permissive temperature contrasts dramatically with the enlarged vacuole phenotype of the *fab1^{G2042/2045V}* strain, suggesting that a threshold level of PtdIns(3,5)P₂ production is required for proper vacuolar morphology.

We envision three possible models for how Fab1p activity is responsible for maintenance of vacuolar size. (a) PtdIns(3,5)P₂ may be required for vacuole ion homeostasis. Aside from its function as a site for macromolecular degradation, the vacuole is an important ion storage site which plays a role in regulating intracellular ion concentrations (Klionsky et al., 1990). Therefore, PtdIns(3,5)P₂ could be an activator of a vacuolar ion transporter and loss of this lipid could affect vacuolar morphology through changes in its osmolarity with respect to the cytoplasm. In mammalian cells, PtdIns(4,5)P₂ has been shown to be a positive regulator of inward-rectifying potassium channels (Huang et al., 1998; Sui et al., 1998). (b) Alternatively, PtdIns(3,5)P₂ could regulate the turnover of vacuolar membranes by mediating its invagination at the vacuole (or endosome), allowing for degradation within the vacuole lumen. Invaginations at the vacuole or endosome have been suggested by the presence of luminal vesicles within the vacuole (Wurmser and Emr, 1998) and endosome (multivesicular body; Gruenberg and Maxfield, 1995).

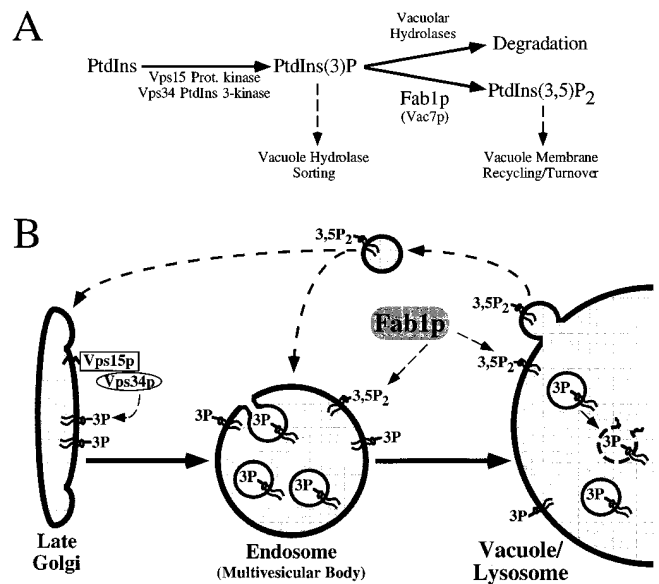


Figure 10. (A) The pathway for the biosynthesis of PtdIns(3,5)P₂ in yeast. The Vps15/34p-dependent phosphorylation of PtdIns leads to the production of PtdIns(3)P. A pool of this lipid then either traffics to the lumen of the vacuole for degradation or remains cytoplasmically exposed, serving as a substrate for a second, Fab1p kinase domain-dependent phosphotransfer reaction resulting in the synthesis of PtdIns(3,5)P₂. Below the phosphorylated lipid products, roles for these lipids are indicated. PtdIns(3)P is required in anterograde traffic from the Golgi to the vacuole, whereas PtdIns(3,5)P₂ may regulate the efflux of vacuolar membrane material. (B) A model for Fab1p-mediated regulation of vacuolar membrane recycling/turnover. Activation of Vps34p by the membrane associated lipid kinase Vps15p, leads to the site-specific synthesis of PtdIns(3)P at the Golgi/endosomal membrane. The production of PtdIns(3)P is not only required for this biosynthetic trafficking route (highlighted by the solid arrows), but also is used by the Fab1p lipid kinase for the synthesis of PtdIns(3,5)P₂. A large pool of PtdIns(3)P is turned over in a hydrolase-dependent manner, possibly by being sorted into the inner leaflet of endosomal invaginations which are transported to the lumen of the vacuole and, together with PtdIns(3)P, degraded. A separate pool of PtdIns(3)P must remain in the cytoplasmic membrane leaflet in order to be available to Fab1p. The presence of PtdIns(3,5)P₂ may then serve to recruit factors involved in the efflux of membrane to earlier compartments (*top, dashed lines*) or cause the internalization of vacuolar membrane into the lumen for degradation.

Consistent with this idea, *fab1Δ1* and *fab1^{D2134R}* kinase domain mutant strains have significantly fewer intravacuolar structures. (c) PtdIns(3,5)P₂ may direct the efflux of vacuolar membranes to earlier secretory compartments. According to this model vacuolar membrane homeostasis requires a balance between anterograde traffic (mediated by PtdIns(3)P) and retrograde traffic (perhaps through PtdIns(3,5)P₂; Fig. 10). Disruption of PtdIns(3,5)P₂ synthesis might prevent membrane recycling and result in the accumulation of membranes at the vacuole. This predicts that PtdIns(3,5)P₂ may be required for the formation or docking/fusion of retrograde carrier vesicles emanating from the vacuole. Indeed, the existence of a retrograde pathway from the vacuolar membrane to earlier secretory compart-

ments has also been suggested based on the recycling of a recombinant protein construct from the vacuole to the Golgi (Bryant et al., 1998).

Therefore, this model suggests that PtdIns(3)P may itself act as an internal membrane marker which is synthesized early in the Golgi to vacuole transport pathway and imprints the carrier vesicles with a tag that is subsequently decoded/recognized at a distal site in the pathway. Fab1p may recognize and convert this anterograde-specific lipid tag into a second signaling lipid that then directs vacuolar membrane efflux or turnover. Recently, however, it has been shown that a significant fraction of PtdIns(3)P is delivered to the vacuole as cargo where it is degraded by luminal hydrolases (Wurmser and Emr, 1998). This necessitates that while a pool of PtdIns(3)P is transferred to the lumen of the vacuole for degradation, a separate or earlier pool of PtdIns(3)P remains available as a substrate for Fab1p in the cytoplasmic leaflet (Wurmser and Emr, 1998; Burd and Emr, 1998). This sequential signaling system at distinct organelles in the Golgi to vacuolar signaling pathway would allow the cell to maintain vacuole size/function (homeostasis).

The involvement of phosphoinositides in the spatial and temporal regulation of membrane trafficking argues that the various phosphorylated derivatives must be synthesized and degraded at specific sites along the pathway. For instance, the lipid PtdIns(4,5)P₂ may not only direct vesicle fusion (Hay and Martin, 1993; Hay et al., 1995), but its conversion by a PtdIns(5)P 5-phosphatase may also play a role in synaptic vesicle endocytosis (McPherson et al., 1994, 1996). Similarly, mutations within the PDGF tyrosine kinase receptor that prevent the recruitment of the mammalian phosphoinositide 3-kinase cause defects in the sorting and downregulation of this receptor (Kapeller et al., 1994). Interestingly, the internalization of the receptor occurs normally, indicating that the subsequent endosome to lysosome trafficking step is affected. Thus, the regulated synthesis and turnover of specific lipids at distinct organelle sites is required for the regulation of membrane trafficking. The isolation of Vps34p as a vacuolar protein sorting mutant lead to the hypothesis that the regulated production PtdIns(3)P on a specific membrane directly effects or recruits necessary factors for proper trafficking (Stack et al., 1993). Candidate downstream targets of PtdIns(3)P in yeast have already been identified, in part, based on the observation that the mammalian EEA1 gene product specifically interacts with PtdIns(3)P-containing liposomes (Patki et al., 1997). This interaction has now been determined to be mediated by the RING-FYVE finger domain (Burd and Emr, 1998) that is also present in Vps27p and Vac1p, proteins previously shown to be required for Golgi to vacuole protein trafficking (Piper et al., 1995; Burd et al., 1997). The binding of PtdIns(3)P by this domain may serve to recruit these proteins to a specific membrane site and/or modulate the activity of these and other transport factors. Interestingly, this domain is also present at the NH₂ terminus of Fab1p and may function to localize (and/or activate) Fab1p to membranes containing its substrate, PtdIns(3)P.

Specific downstream effects of phosphoinositides are often mediated by proteins which bind directly to specific lipid ligands (Martin, 1997). Regulation of membrane ho-

meostasis by Fab1p is likely to be mediated by a set of proteins that specifically interact with PtdIns(3,5)P₂. These PtdIns(3,5)P₂-specific binding proteins could correspond with proteins that commonly play roles in membrane trafficking events, such as coat proteins, SNAREs, or rab GTPases. As the ongoing VAC mutant screen has been successful in defining candidate upstream regulators of Fab1p activity, it also promises to be instrumental in identifying downstream effectors of Fab1p. Our results with Fab1p confirm the use of yeast in defining signal transduction pathways on the basis of an observable phenotype and ordering these factors within the pathway based on in vivo biochemical analyses.

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