Osmotic stress–induced increase of phosphatidylinositol 3,5-bisphosphate requires Vac14p, an activator of the lipid kinase Fab1p

Cecilia J. Bonangelino,1 Johnathan J. Nau,1 Jason E. Duex,1 Mikala Brinkman,1 Andrew E. Wurmser,2 Jonathan D. Gary,2 Scott D. Emr,2 and Lois S. Weisman1

1Department of Biochemistry, University of Iowa, Iowa City, IA 52242
2Division of Cellular and Molecular Medicine and Howard Hughes Institute, University of California at San Diego, School of Medicine, La Jolla, CA 92093

Phosphatidylinositol 3,5-bisphosphate ([PtdIns(3,5)P]2) was first identified as a nonabundant phospholipid whose levels increase in response to osmotic stress. In yeast, Fab1p catalyzes formation of PtdIns(3,5)P2 via phosphorylation of PtdIns(3)P. We have identified Vac14p, a novel vacuolar protein that regulates PtdIns(3,5)P2 synthesis by modulating Fab1p activity in both the absence and presence of osmotic stress. We find that PtdIns(3)P levels are also elevated in response to osmotic stress, yet, only the elevation of PtdIns(3,5)P2 levels are regulated by Vac14p. Under basal conditions the levels of PtdIns(3,5)P2 are 18–28-fold lower than the levels of PtdIns(3)P, PtdIns(4)P, and PtdIns(4,5)P2. After a 10 min exposure to hyperosmotic stress the levels of PtdIns(3,5)P2 rise 20-fold, bringing it to a cellular concentration that is similar to the other phosphoinositides. This suggests that PtdIns(3,5)P2 plays a major role in osmotic stress, perhaps via regulation of vacuolar volume. In fact, during hyperosmotic stress the vacuole morphology of wild-type cells changes dramatically, to smaller, more highly fragmented vacuoles, whereas mutants unable to synthesize PtdIns(3,5)P2 continue to maintain a single large vacuole. These findings demonstrate that Vac14p regulates the levels of PtdIns(3,5)P2 and provide insight into why PtdIns(3,5)P2 levels rise in response to osmotic stress.

Introduction

Phosphatidylinositol polyphosphates function in diverse cellular events. This diversity is facilitated by the variety of sterically distinct compounds generated via phosphorylation of inositol at any or all of its six hydroxyls. These compounds are rapidly synthesized and degraded via kinases, phosphatases, and lipases, and thus are ideal regulatory molecules (De Camilli et al., 1996; Michell, 1997). Phosphatidylinositol polyphosphates regulate cell growth and differentiation (Joly et al., 1995; Toker and Cantley, 1997), cytoskeletal rearrangements (Joly and Gertler, 2000), Ca2+ mobilization (Beridge, 1993), and membrane traffic (De Camilli et al., 1996; Roth, 1999; Odorizzi et al., 2000).

The involvement of phosphatidylinositol polyphosphates in membrane traffic has been demonstrated by the requirement of enzymes that synthesize specific phosphatidylinositol polyphosphates. For example, Ca2+-dependent secretory granule exocytosis requires both PEP1 and PEP3 activity (Hay et al., 1995), enzymes that together increase the local concentrations of phosphatidylinositol 4,5-bisphosphate ([PtdIns(4,5)P]2) (Liscovitch and Cantley, 1995; Kearsn et al., 1998). Likewise, synthesis of PtdIns(3)P is required for intracellular membrane transport. Mutations in the yeast phosphatidylinositol 3 kinase, VPS34, result in secretion of vacuolar proteins, such as carboxypeptidase Y (Herman et al., 1992; Schu et al., 1993; Stack et al., 1995). Moreover, the lysosomal protein, cathepsin D, requires mammalian Vps34p for proper sorting (Brown et al., 1995; Davidson, 1995). Thus, the ability to increase the local concentration of these phosphatidylinositol polyphosphates is intrinsic to some intracellular membrane traffic events.

Key words: VAC14; FAB1; PtdIns(3,5)P2; vacuole; phosphatidylinositol polyphosphates.

Abbreviations used in this paper: FM4-64, N-(3-triethylammoniumpropy)-4-(p-diethylaminophenylhexatrienyl); GFP, green fluorescent protein; GST, glutathione S-transferase; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate.
One of the primary roles for phosphatidylinositol polyphosphates in membrane traffic is recruitment of specific proteins to designated regions of membranes. Many of the proteins required for formation of clathrin-coated vesicles, dynamin, the α subunit of AP-2, and synaptojanin, contain PtdIns(4,5)P2 binding sites (Jost et al., 1998) and have been shown to bind PtdIns(4,5)P2 in vitro (Beck and Keen, 1991; Barylko et al., 1998; Bottomley et al., 1999). More recently, it has been shown that the epsin homology NH2-terminal domains of CALM (Ford et al., 2001) and epsin (Itoh et al., 2001) bind PtdIns(4,5)P2 as well. Likewise, PtdIns(3)P binds specifically to several proteins required for membrane traffic, including Vac1p, EEA1, and Vps27p. Each of these proteins contains a zinc-binding FYVE finger motif, which specifically binds PtdIns(3)P but not other phosphatidylinositols (Burd and Emr, 1998; Patki et al., 1998; Stenmark and Aasland, 1999). PtdIns(3)P is required for recruitment of these proteins to membranes and may also regulate protein function (Peterson et al., 1999; Tall et al., 1999; Gaullier et al., 2000).

The discovery of phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P2) has expanded the list of biologically relevant phosphatidylinositol polyphosphates (Dove et al., 1997; Rameh et al., 1997; Whiteford et al., 1997). In Saccharomyces cerevisiae, PtdIns(3,5)P2 is a low abundance phosphatidylinositol whose synthesis increases in cells exposed to osmotic stress (Dove et al., 1997). Synthesis of PtdIns(3,5)P2 in yeast requires Vps34p, demonstrating that PtdIns(3,5)P2 is produced by phosphorylation of PtdIns(3)P (Dove et al., 1997). The subsequent phosphorylation of PtdIns(3)P is catalyzed by Fab1p, the sole phosphatidylinositol (3)P 5-kinase in yeast (Cooke et al., 1998; Gary et al., 1998). Unlike vps34 mutants which are defective in membrane transport from the Golgi region to the vacuole, fab1 mutants have only a partial defect, demonstrating that PtdIns(3,5)P2 and PtdIns(3)P have distinct functions (Yamamoto et al., 1995; Gary et al., 1998).

Phenotypic analysis of two yeast mutants that cannot synthesize PtdIns(3,5)P2, fab1 and vac7, suggested that this phospholipid is required for selected vacuole membrane functions (Bonangelino et al., 1997; Gary et al., 1998). The most prominent phenotypes include an enlarged, unlobed vacuole and defects in both vacuole inheritance and acidification. A third mutant, vac14 shares all of these phenotypes.

Here we present the corresponding wild-type gene, VAC14, which encodes a newly discovered protein that, like Fab1p and Vac7p, resides on the vacuole membrane and is required for normal cellular levels of PtdIns(3,5)P2. We find that Vac14p is both a general activator and a specific osmotic response regulator of the PtdIns(3,5)P2 5-kinase, Fab1p. Moreover, we show that PtdIns(3,5)P2 levels are tightly correlated with vacuole morphology and that this ability to regulate the size and number of vacuole lobes, through increased PtdIns(3,5)P2, is a physiological response to a sudden increase in extracellular osmolarity. Furthermore, we find that after exposure to osmotic stress, the cellular levels of PtdIns(3,5)P2 increase 16–20-fold. These levels are much higher than previously reported and are similar to

![Figure 1. Proteins with identity to Vac14p exist in higher eukaryotes. Identical amino acids (black) and similar amino acids (gray) are highlighted. (Left) The NH2-terminal sequence of S. cerevisiae VAC14 and related ORFs were aligned using ClustalW (http://searchlauncher.bcm.tmc.edu:9331/multi-align/multi-align.html). Sequences were identified by searching the indicated databases via the BLAST algorithm (Altschul et al., 1990). The C. albicans database (http://sequence-www.stanford.edu/group/candida/search.html). ORFs from S. pombe (EMBL/GenBank/DDBJ accession no. CAB08779.1), A. thaliana (EMBL/GenBank/DDBJ accession no. AAD12702.1), C. elegans (EMBL/GenBank/DDBJ accession no. CAB00043.1) and D. melanogaster (EMBL/GenBank/DDBJ accession no. AF534829.1) were in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). The M. musculus sequence was identified in the Mouse EST database (http://www.ncbi.nlm.nih.gov/BLAST). The consensus sequence of 14 similar ESTs from chromosome 16 (EMBL/GenBank/DDBJ accession nos. AL527155, AL535971, AL555680, AL555602, BE409891, BE696780, BE728471, BE938100, BE901196, BE937614, BF081182, BF091052, BF325708, and BG107035) is shown. The sequences contain at least 25% global identity and 42% global similarity to S. cerevisiae VAC14. (Right) The COOH-terminal sequence of yeast Vac14p and similar ORFs were identified and aligned as in the left sequence. The M. musculus sequence is a consensus of 12 similar ESTs (EMBL/GenBank/DDBJ accession nos. AA036005, AA054023, AA058300, AA276168, AA497446, AA670618, BE862623, BF023070, BF237130, BF720417, BG079707, W09660). The H. sapiens sequence is hypothetical protein, FLJ10305 found on chromosome 16, deposited in the human genome database (http://www.ncbi.nlm.nih.gov/genome/seq). One of the mouse ESTs (clone ID 468926) had been mapped to chromosome VIII (106 cM offset) with an inferred position on human chromosome 16 (16q22-1qter).]
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the basal levels observed for PtdIns(3)P, PtdIns(4)P, and PtdIns(4,5)P2. Together, these findings suggest that PtdIns(3,5)P2 may function similarly to PtdIns(3)P and PtdIns(4,5)P2 (i.e., by recruiting membrane coat proteins) to regulate a specific vacuole-related membrane-trafficking pathway(s) that is required for the observed changes in vacuole morphology.

Results

Identification of the VAC14 gene

The Class III vac mutant, vac14-1, was isolated via fluorescence-activated cell sorting (for description of approach see Wang et al. [1996]). Like vac7 and fab1 mutants, vac14-1 cells are defective in vacuole inheritance, acidification, and morphology. These mutants have a single, unlobed, enlarged vacuole. Frequently, the vacuole spans both the mother and daughter cell resulting in an “open figure eight” vacuole morphology (Bonangelino et al., 1997).

We determined (see Materials and methods) that the VAC14 open reading frame is YLR386W (sequence deposited by the Yeast Genome Sequencing Project). The VAC14 2.64-kb open reading frame encodes a novel polypeptide of 880 amino acids. There are no notable motifs except for a putative transmembrane domain (see below). However, VAC14 displays a high degree of identity with open reading frames present in other eukaryotic organisms. The two regions of highest identity are near the NH2 terminus (residues 1–171) (Fig. 1, left) and COOH terminus (residues 578–746) (Fig. 1, right). Both mouse and human sequences matching either end were identified in the corresponding EST databases. Moreover, a human hypothetical protein, FLJ10305, found on chromosome 16, shows a high degree of identity with the COOH-terminal region of Vac14p and its sequence matches human EST sequences. Both the NH2- and COOH-terminal ESTs map to human chromosome 16, suggesting that they correspond to the same gene. No obvious Vac14p homologues were found in any of the published bacterial genomes.

Chromosomal deletion of VAC14

A heterozygous diploid containing a chromosomal deletion of VAC14 was created by replacing the VAC14 open reading frame with TRP1. The resulting diploid was sporulated. In all 20 tetrads examined the TRP1 gene cosegregated with the vac14 phenotypes. vac14/H9004 cells are viable and, like vac14-1, they display abnormal vacuole morphology (Fig. 2 a), are defective in vacuole inheritance, and have a vacuole acidification defect (Fig. 2 e). vac14-1 and vac14-1/H9004 cells have nearly identical growth rates, with a doubling time that is similar to that of wild-type cells (unpublished data). Because vac14-1 and vac14-1/H9004 behave identically in all tests performed, it is likely that the original vac14-1 allele is a loss of function mutation.

Figure 2. FAB1 suppresses the vacuole inheritance, morphology, and acidification defects of vac14-1. vac14-1 mutants expressing pRS426 (a and e), VAC14 on a low copy plasmid (b and f), FAB1 on a multicopy plasmid (c and g), or FAB1 from a low copy plasmid (d and h) were labeled with either FM4-64 to visualize vacuole membranes (A) or with 200 μM quinacrine to assess vacuole acidification (B). The photographs were taken with both fluorescence and a low level of transmitted light. Bar, 5 μm.
The growth defects of the double mutants, \textit{vac7-1}, \textit{vac14Δ} and \textit{fab1-1}, \textit{vac14Δ} were similar to \textit{vac7-1}, or \textit{fab1-1} mutants alone, consistent with \textit{VAC7}, \textit{VAC14}, and \textit{FAB1} functioning in the same pathway. We were unable to determine whether \textit{VAC7} functions upstream or downstream of \textit{FAB1} or \textit{VAC14} because \textit{VAC7} expressed from either a low-copy or multi-copy plasmid did not suppress the \textit{vac14-1}, \textit{vac14Δ} or \textit{fab1-1} mutant phenotypes (Bonangelino et al., 1997), and neither \textit{FAB1} nor \textit{VAC14} suppressed \textit{vac7-1}. However, a similar analysis suggested that \textit{VAC14} functions upstream of \textit{FAB1}. Overexpression of \textit{FAB1} suppressed the vacuolar morphology, inheritance (Fig. 2, c and d) and acidification defects of the \textit{vac14Δ1} mutant (Fig. 2, g and h), and the \textit{vac14Δ1} mutant (Bonangelino et al., 1997). This suppression required the kinase activity of Fab1p since overexpression of a kinase-dead \textit{fab1-D2134R} mutant (Gary et al., 1998) did not suppress the \textit{vac14Δ1} mutant phenotypes (unpublished data). These data suggest that Vac14p may be an activator of Fab1p kinase activity.

\textbf{Vac14p associates with vacuolar membranes}

Antibodies raised to a glutathione \textit{S}-transferase (GST)–Vac14p fusion protein recognized a polypeptide with a molecular weight of \(\sim 99\) kD, the predicted molecular weight of Vac14p (Fig. 3 A). (A second, 70-kD polypeptide is nonspecific as it appears in both wild-type and mutant extracts.) The majority of Vac14p was found in a membrane-related fraction that pelleted at 13,000 \(g\) (P13) (Fig. 3). The P13 fraction contains large organelles such as vacuoles, ER, and nuclei. A similar fractionation pattern was observed for other vacuolar proteins, such as Vac8p (Wang et al., 1998) (Fig. 3 C).

Vac14p was present on vacuoles isolated on a Ficoll flotation gradient (Fig. 4 A) and was enriched on purified vacuoles to a similar extent as the vacuolar membrane protein, Pho8p (Fig. 4 B). Sequence analysis of Vac14p revealed a putative transmembrane domain between residues 430 and 451 (determined by PSORT and hydropathy plot analysis). Despite this prediction, Vac14p was peripherally associated with vacuole membranes. Treatments with either 0.1 M \(\text{Na}_2\text{CO}_3\), pH 11.5, 1 M \(\text{NaCl}\), or 0.8 M \(\text{NH}_2\text{OH}\)-solubilized Vac14p, whereas Pho8p was not extracted by any of these treatments (Fig. 4 C).

Vac14p was also present in vacuoles isolated from \textit{vac7-1} and \textit{fab1-1} strains (Fig. 4 A). Thus, defects observed in \textit{vac7-1} cells are not due to a gross mislocalization of Vac14p. Interestingly, although \textit{fab1-1} vacuoles contained \(\sim 50\%\) less Vac14p than wild-type vacuoles (Fig. 4 A). The decrease of Vac14p on \textit{fab1-1} vacuoles was observed in four independent experiments and suggests that Vac14p may physically associate with Fab1p. Since some Vac14p remained associated with vacuoles in the absence of Fab1p, there are likely to be additional proteins that bind Vac14p and help mediate its association with vacuolar membranes. Vac7p is not a candidate because \textit{vac7-1} vacuoles contain normal levels of Vac14p. We were unable to detect a direct physical interaction between Vac14p and Fab1p by yeast two-hybrid, coimmunoprecipitation, or cross-linking experiments. Thus, any interaction is likely to be transient or indirect, and therefore difficult to detect by standard biochemical techniques. Cellular fractionation alone was used to determine Vac14p localization because we were unable to use the goat serum for indirect immunofluorescence.

\textbf{Proper GFP-Fab1p localization does not depend on Vac7p or Vac14p}

Fab1p, like Vac14p, is peripherally associated with vacuolar membranes (Gary et al., 1998). A GFP–Fab1p fusion protein was constructed to visualize Fab1p in various mutant strains. The GFP–Fab1p localized to endosomal and vacuolar membranes in wild-type yeast, \textit{fab1-1}, \textit{vac7-1}, and \textit{vac14Δ1} mutants (Fig. 5). This demonstrates that defects in \textit{PtdIns(3,5)P_2} levels in \textit{vac7-1} and \textit{vac14Δ1} mutants are not due to Fab1p mislocalization. Moreover, both Vac14p and Fab1p are localized on the vacuole, consistent with Vac14p serving as an activator of Fab1p.

\textbf{An improved method to quantify phosphatidylinositol polyphosphate levels in yeast}

Standard methods for analyzing phosphatidylinositol levels in yeast have consisted of lysing cells in acidified chloroform/methanol/HCl with the concomitant extraction...
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of total cellular lipids. We tested this method on wild-type yeast in the absence and presence of osmotic shock and obtained phosphatidylinositol polyphosphate levels similar to those reported previously (Dove et al., 1997; Gary et al., 1998) (Fig. 6 and Table I). However, we also noted that there were substantial fluctuations in the levels of PtdIns(3,5)P2 and PtdIns(4,5)P2 even in identically prepared samples. Therefore, we adapted a method described for analyzing PtdIns(3,5)P2 levels in tissue culture cells, where cells are lysed in 4.5% perchloric acid (Whiteford et al., 1997). This treatment precipitates most macromolecules, including proteins and lipids. The lipids are then deacylated with methylamine and the released glycerophospho-inositols are extracted with water. Notably, we obtained significantly higher quantities of the corresponding inositol monophosphates and biphosphates (Fig. 6 and Table I) with far less variability among identically prepared samples for the inositol bisphosphates. This allowed us to reproducibly detect basal levels of PtdIns(3,5)P2, which are ~15-fold higher than reported previously.

PtdIns(3,5)P2 levels rise 20-fold in response to osmotic stress

Using this improved method we found that osmotic stress results in much higher levels of PtdIns(3,5)P2 than reported previously (Table I). 10 min after exposure to osmotic stress, the levels of PtdIns(3,5)P2 and PtdIns(4,5)P2 are similar to PtdIns(3)P and PtdIns(4)P levels (Table I). Although the levels of each of the phosphorylated phosphatidylinositol underwent some change after exposure of the cells to osmotic stress, the change in PtdIns(3,5)P2 levels was the most dramatic, with the levels of this lipid rising 16–20-fold (Tables I and II). Quantification of the fold increase could not be performed on cells extracted with chloroform methanol HCl because with this method PtdIns(3,5)P2 was not detectable in cells grown in the absence of osmotic shock. Moreover, after a 10-min exposure to osmotic stress, ~8-fold more PtdIns(3,5)P2 and 7-fold more PtdIns(4,5)P2 were detected in cells lysed with perchloric acid compared with cells extracted with chloroform methanol HCL (Table I).
Fab1p, Vac7p, and Vac14p are required for steady-state levels of PtdIns(3,5)P2 during vegetative growth

We next reevaluated PtdIns(3,5)P2 levels in both vac7-1/H90041 and fab1-1/H90041. As reported previously (Gary et al., 1998), there was no detectable PtdIns(3,5)P2 in fab1-1/H90041, consistent with Fab1p being the sole 5-kinase that converts PtdIns(3)P to PtdIns(3,5)P2 (Table II). Likewise, no PtdIns(3,5)P2 was detected in the vac7-1/H90041 mutant. We also observed an elevation in PtdIns(3)P levels in these mutants following osmotic stress (see below).

We next analyzed PtdIns(3,5)P2 levels in the vac14-1/H90041 mutant. Like vac7 and fab1 mutants, in the absence of osmotic stress, no detectable PtdIns(3,5)P2 was observed (Table II). These results suggest that the Class III vac mutant phenotypes shared by vac7, fab1, and vac14 mutants are due to a deficiency in PtdIns(3,5)P2 levels.

vac14 mutants synthesize low levels of PtdIns(3,5)P2 after exposure to osmotic stress

Unlike vac7 and fab1 mutants, when vac14-1 cells were exposed to 0.9 M NaCl for 10 min, a small amount of PtdIns(3,5)P2 was observed (1.7 U) (Table II). This level is similar to the basal levels detected during vegetative growth (3.5 U), but much lower than the osmotic stress–induced levels (71 U) observed in wild-type cells.

We suspect that low, yet undetectable, levels of PtdIns(3,5)P2 are synthesized in vac14-1/H90041 cells in the absence of osmotic stress. First, vac14-1/H90041 mutants produce PtdIns(3,5)P2 during osmotic shock, demonstrating that Fab1p, the phosphatidylinositol (3)P 5-kinase, retains partial activity in the absence of Vac14p. Second, the doubling time of vac14 cells is similar to that of wild-type cells (unpublished data), in contrast to the greatly reduced growth.

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**Figure 5.** GFP-Fab1p is localized to the vacuole membranes in vac14-1 and vac7-1. Wild-type (A), fab1-1 (B), vac14-1 (C), and vac7-1 (D) strains expressing GFP-Fab1p from a 2μ plasmid (pRS426) were visualized using fluorescence light combined with low levels of transmitted light. Bar, 8 μm.

**Figure 6.** Use of 4.5% perchloric acid results in a significantly higher extraction of phosphatidylinositol monophosphates and bisphosphates. Cells were labeled with myo-[2-3H]inositol for 12 h at 24°C and exposed to 0.9 M NaCl for 10 min. (A) Cells were lysed with glass beads in 1.5 ml chloroform/methanol/1 N HCl (1:1:1) at room temperature. Phospholipids were extracted in the organic phase and dried under a N2 stream. (B) Cells were lysed with glass beads in 0.8 ml of 4.5% perchloric acid. Cell extracts were centrifuged at 14,000 rpm at 4°C to obtain a phospholipid-containing pellet. The phospholipids from both protocols were then deacylated and analyzed by HPLC. An online scintillation counter was used to detect the tritiated compounds. Approximately 3.2 × 106 cpm (A, chloroform/methanol/HCl) and 3.6 × 106 cpm (B, 4.5% perchloric acid) were injected, respectively.
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Table I. Cellular lipid extractions utilizing 4.5% perchloric acid provide a more representative measure of phosphatidylinositol levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PtdIns(3)P</th>
<th>PtdIns(4)P</th>
<th>PtdIns(3,5)P₂</th>
<th>PtdIns(4,5)P₂</th>
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<tr>
<td>0.9 M NaCl</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4.5% HClO₄</td>
<td>70 ± 15</td>
<td>100 ± 15</td>
<td>65 ± 10</td>
<td>90 ± 15</td>
</tr>
<tr>
<td>Chloroform: MeOH</td>
<td>38 ± 5</td>
<td>60 ± 5</td>
<td>43 ± 5</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>Chloroform: MeOH</td>
<td>8 ± 3</td>
<td>≤1.5</td>
<td>12 ± 5</td>
<td>10 ± 5</td>
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Cells were grown in isonitrol-free media in the presence of 0.01 mCi/ml ³H isonitrol. 0.9 M NaCl was added to half the cultures for 10 min prior to extraction. Cellular lipids were extracted and analyzed as described in Materials and methods. The cpm in each peak was corrected for the total number of cells analyzed by expressing each as a percentage of total cpm eluted. Then these values were normalized to PtdIns(3)P with no salt added for the 4.5% perchloric acid extraction. This peak was 2.0% of the total cpm eluted (80,000 cpm of 4 × 10⁷ total cpm). Values listed as ≤1.5 were areas where no peak above background was detected. The data (mean ± standard deviation) were calculated from results obtained in three independent experiments.

Vac14p is required for elevation of PtdIns(3,5)P₂ in response to osmotic stress

Because overexpression of FAB1 suppresses the vacuolar defects of the vac14ΔΔ mutant (Fig. 2), we tested PtdIns(3,5)P₂ levels in these strains. Expression of FAB1 from a low- or high-copy plasmid in the vac14ΔΔ mutant brought the levels of PtdIns(3,5)P₂ from undetectable to nearly the basal levels detected in wild-type cells (2 U) (Table III). These observations are consistent with Vac14p functioning upstream of Fab1p.

Notably, FAB1 expression in vac14ΔΔ mutants did not restore the elevation of PtdIns(3,5)P₂ normally observed in response to osmotic shock. The levels of PtdIns(3,5)P₂ after osmotic shock in vac14ΔΔ, or vac14ΔΔΔ with either a low or high copy FAB1, were statistically identical (2.4, 3.2, and 3.1 U, respectively) (Table III). As a control, vac14ΔΔ with VAC14 expressed from a low copy plasmid produced 30 U of PtdIns(3,5)P₂ in response to osmotic shock (Table III). Thus, Vac14p is required for the normal increase of PtdIns(3,5)P₂ in response to hyperosmotic stress. The small increase in PtdIns(3,5)P₂ observed after osmotic shock in the vac14ΔΔ and vac14ΔΔΔ mutants is most likely due to an osmotic stress–induced elevation of the biosynthetic precursor, PtdIns(3)P (see below).

Vps34p activity increases in response to osmotic stress

As PtdIns(3,5)P₂ levels rise, one would expect to see an equivalent decrease of the precursor, PtdIns(3)P. In fact, after exposure to osmotic stress, we observed a decrease in PtdIns(3)P in wild-type cells (Table II). However, our data suggest that the rate of PtdIns(3)P synthesis is elevated. First, in wild-type cells, the decrease in PtdIns(3)P by 32 U is not enough to account for the 71 U increase in PtdIns(3,5)P₂ (Table II). Second, if the rate of PtdIns(3)P synthesis rises due to osmotic stress, then one would expect to detect this rise in cells that are blocked in PtdIns(3,5)P₂ synthesis. Indeed, in fab1ΔΔ and vac7ΔΔ mutants, the levels of PtdIns(3)P after exposure to osmotic stress increased by 35 and 40 U, respectively (Table II). This suggests that after exposure to osmotic stress, PtdIns(3)P levels increase and are quickly depleted by the rapid production of PtdIns(3,5)P₂. However, in mutants that cannot synthesize PtdIns(3,5)P₂, the extra PtdIns(3)P accumulates.

In wild-type cells, the elevation of PtdIns(3)P in response to osmotic stress likely compensates for the rapid conversion of PtdIns(3)P to PtdIns(3,5)P₂. Lack of a compensatory mechanism would deplete the levels of PtdIns(3)P, which are required for membrane trafficking to the yeast vacuole (Schu et al., 1993). In addition, a rise in PtdIns(3)P in response to osmotic shock might also be responsible for producing an increase in PtdIns(3,5)P₂, as PtdIns(3)P could directly activate Fab1p or drive the reaction forward by mass action.

Although it is possible that Vac14p might indirectly affect PtdIns(3,5)P₂ levels by regulating Vps34p activity, our data demonstrate that this is not the case. If Vac14p activated Vps34p, then in a vac14ΔΔΔ, fab1ΔΔΔ double knockout there would be little or no PtdIns(3)P accumulation. However, in a vac14ΔΔΔ, fab1ΔΔΔ double mutant, the levels of PtdIns(3)P were virtually identical to fab1ΔΔΔ mutants, with a large accumulation of PtdIns(3)P in the presence of osmotic stress (an elevation of 40 and 35 U, respectively) (Table II). Thus, while osmotic stress activates Vps34p, Vac14p is not involved in this activation. Moreover, the defects in PtdIns(3,5)P₂ levels in vac14ΔΔΔ cells are not indirectly due to reduced PtdIns(3)P production.

Vac14p is an osmotic response activator of Fab1p

The observation that during osmotic stress vac14ΔΔ mutants have low levels of PtdIns(3,5)P₂ and accumulate PtdIns(3)P suggests either of two remaining hypotheses. First, Vac14p may function as an osmotic response activator of Fab1p. In the absence of its activator, Fab1p functions with reduced activity, leading to the accumulation of its substrate, Ptd-
Table III. *Vac14p* is required for normal elevation of PtdIns(3,5)P₂ in response to osmotic stress

<table>
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<th>PtdIns(3)P</th>
<th>PtdIns(3,5)P₂</th>
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<tr>
<td>+</td>
<td>100 ± 2</td>
<td>2.4 ± 0.3</td>
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<tr>
<td>−</td>
<td>100 ± 2</td>
<td>≤1.5</td>
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</table>

Cells were grown in inositol free media in the presence of 0.01 mM ³H inositol. 0.9 M NaCl was added to half the cultures for 10 min before extraction. The values were generated as described in Table II and normalized to wild-type (Table III). The data (mean ± standard deviation) were calculated from results obtained in four independent experiments.

Table IV. Overexpression of *VAC14* does not enable *fab1-2* to produce elevated levels of PtdIns(3,5)P₂ after exposure to osmotic stress

<table>
<thead>
<tr>
<th></th>
<th>PtdIns(3)P</th>
<th>PtdIns(3,5)P₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9 M NaCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>12 ± 2</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>−</td>
<td>150 ± 10</td>
<td></td>
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</tbody>
</table>

Cells were grown in inositol-free media in the presence of 0.01 mM ³H inositol. 0.9 M NaCl was added to half the cultures for 10 min prior to extraction. Cellular lipids were extracted and analyzed as described in Materials and methods. The cpm in each peak was corrected for the total number of cells analyzed by expressing each as a percentage of total cpm eluted. Then these values were normalized to PtdIns(3)P with no salt added in *fab1-2* with *VAC14* and *FAB1* 2μ (which was 2.1% of total cpm eluted; 84,000 cpm of 4 x 10⁶ total cpm). The data (mean ± standard deviation) were calculated from results obtained in three independent experiments.
Ins(3,5)P₂ levels in response to osmotic stress may serve to regulate vacuole morphology under conditions when the vacuole loses volume due to loss of water. Consistent with this hypothesis, we found that after a short exposure to hyperosmotic stress, the vacuole morphology of wild-type cells changed to smaller, more highly fragmented vacuoles (Fig. 8, a and e). However, the vacuole morphology of mutants unable to produce PtdIns(3,5)P₂ remained largely unchanged.

![Figure 7. Simultaneous overexpression of VAC7 and VAC14 results in cells with a larger number of vacuole lobes and lobes of smaller size.](image-url)

Wild-type cells simultaneously expressing VAC7 and VAC14 from multicopy plasmids (A) or containing empty vectors, pRS424 and pRS423 (B). Vacuoles were labeled with 80 μM FM4-64. Several fields are shown for each. Bar, 8 μm.

![Figure 8. PtdIns(3,5)P₂ is required for osmotic stress induced fragmentation of the vacuole.](image-url)

Wild-type (a and e), vac14Δ1 (b and f), fab1Δ1 (c and g), vac7Δ1 (d and h) cells were labeled with FM4-64 to visualize vacuole membranes. Cells were incubated in YEPD alone (a–d) or YEPD containing 0.4 M NaCl (e–h) for 10 min. Bar, 8 μm.
(Fig. 8). This demonstrates that changes in vacuole morphology induced by exposure to osmotic stress are regulated via a molecular mechanism that requires the synthesis of more PtdIns(3,5)P2. Moreover, this observation provides the first insight into a physiological consequence of increased PtdIns(3,5)P2 production in response to osmotic stress.

Discussion

The discovery of a new biological phosphatidylinositol, PtdIns(3,5)P2, and the observation that PtdIns(3,5)P2 levels are acutely regulated by osmotic stress poses questions as to how this phospholipid is synthesized, how its cellular levels are regulated, where PtdIns(3,5)P2 production and turnover occur, and what functions PtdIns(3,5)P2 serves. Biosynthesis of PtdIns(3,5)P2 occurs through phosphorylation of PtdIns(3)P by Fab1p, the phosphatidylinositol(3)P 5-kinase (Huang et al., 1998; Sui et al., 1998). Its production also requires Vac7p, a novel vacuolar protein, whose function remains unknown (Bonangelino et al., 1997; Gary et al., 1998). Here we identify Vac14p, which functions in PtdIns(3,5)P2 synthesis by regulating Fab1p activity. Localization of Vac14p, Fab1p, and Vac7p, along with observations of the defects exhibited in strains lacking PtdIns(3,5)P2, suggests that this lipid is produced and functions at the late endosome and vacuole. The fact that the lipid levels rise 20-fold within 10 min of osmotic stress and cause the vacuole lobes to decrease in size and increase in number suggests that the increasing PtdIns(3,5)P2 levels and the concomitant change in vacuole volume may be a first line of defense against a sudden increase in osmolarity of the environment.

Changes in PtdIns(3,5)P2 levels could occur either via increased PtdIns(3,5)P2 synthesis, negative regulation of PtdIns(3,5)P2 turnover, or regulation of both (Fig. 9). However, analysis of selected fab1 mutants indicates that up-regulation of PtdIns(3,5)P2 synthesis, rather than negative regulation of its turnover, is the major contributor to the osmotic stress–induced increase in PtdIns(3,5)P2 levels. First, the fab1Δ mutant produces relatively normal levels of PtdIns(3,5)P2 under basal conditions, but is specifically defective in elevation of PtdIns(3,5)P2 synthesis in response to osmotic stress (Cooke et al., 1998, and Table IV). Second, when expressed in fab1Δ, Fab1p homologues from both Schizosaccharomyces pombe and mouse produce normal basal levels of PtdIns(3,5)P2 (McEwen et al., 1999). However, strains expressing these homologues as the sole copy of Fab1p are defective in the osmotic stress–induced elevation of PtdIns(3,5)P2. The only difference in these cells is the lack of wild-type Fab1p, and therefore the enzymes responsible for regulating PtdIns(3,5)P2 turnover should not be affected. This demonstrates that the dramatic elevation in PtdIns(3,5)P2 occurs via activation of Fab1p by accessory factors that are unable to act on the Fab1p homologues.

Vps34p activity is also regulated in response to osmotic stress in yeast. First, in wild-type cells there is a relatively small decrease of precursor, PtdIns(3)P, compared with the very large increase in product, PtdIns(3,5)P2. Moreover, in fab1Δ and vac7Δ mutants, exposure to osmotic stress produces elevated levels of PtdIns(3)P. Vps34p activity is not regulated by Vac14p, as levels of PtdIns(3)P rise in fab1ΔΔ, vac14ΔΔ double mutants; however, conversion of the additional PtdIns(3)P to PtdIns(3,5)P2 is dependent on the presence of Vac14p. Thus, while elevated Vps34p activity may be a prerequisite for elevated PtdIns(3,5)P2 levels, the activation of Fab1p via Vac14p is required for the 20-fold increase of PtdIns(3,5)P2.

Regulated turnover may also contribute to the control of PtdIns(3,5)P2 levels. PtdIns(3,5)P2 increases rapidly after osmotic shock and then disappears after 30–60 min (Dove et al., 1997). A concerted increase in the activity of the kinases combined with a delayed increase in the activity of the phosphatase.
phatase(s) and/or lipase could cause the rapid disappearance of PtdIns(3,5)P₂ that occurs within such a short period of time. Alternatively, it is possible that regulation of PtdIns(3,5)P₂ levels may occur solely via regulating Fab1p activity. Identification and characterization of enzymes that turnover PtdIns(3,5)P₂ will be necessary to determine whether turnover of PtdIns(3,5)P₂ is regulated as well.

Given that PtdIns(3,5)P₂ levels rise so dramatically after a short exposure to osmotic stress leads to questions of how a rise in PtdIns(3,5)P₂ levels could mitigate problems caused by a sudden change in osmolarity. One of the first consequences of exposing yeast to osmotic stress is the loss of water from the cells, producing a hypertonic cytoplasm. This in turn results in loss of water from the vacuole. It is possible that this water release is regulated by PtdIns(3,5)P₂ through control of a vacuolar-specific water channel. Although a vacuolar aquaporin has not yet been demonstrated in S. cerevisiae, at least two aquaporins have been identified, AQP1 and AQP2 (Carbery et al., 2001; Meyrial et al., 2001). Either of these may localize, in part, to the vacuole membrane. Further, most plant cells contain both plasma membrane and vacuolar aquaporins (Martinoia et al., 2000; Maurel and Chrispeels, 2001). These prevent the rapid dehydration and cell lysis that could occur when the osmolarity of the external environment changes. As yeast are exposed to similar fluctuations in their external environment, it is likely that they also have similar mechanisms to regulate both water and ion uptake and exit.

PtdIns(3,5)P₂ may also regulate a vacuolar ion channel and thereby control the internal osmotic strength of the vacuole lumen. A decrease in ion flux out of the vacuole would help retain water that would otherwise be lost to the cytoplasm. Notably, other phosphatidylinositols have been implicated in the regulation of several types of channels. For instance, PtdIns(4,5)P₂ serves as a positive regulator of inward-rectifying potassium channels (Huang et al., 1998; Sui et al., 1998). PtdIns(4,5)P₂ also plays a role in the regulation of TRP channel in Drosophila photoreceptors (Hardie et al., 2001). Studies on the purified yeast vacuolar ATPase have suggested that this enzyme requires phospholipids for maximal activity (Uchida et al., 1988). In addition, the yeast phosphatidylinositol–specific phospholipase C (Plc1p) is required for activation of a plasma membrane ATPase, thus implicating phosphatidylinositol phospholipid metabolism in its regulation (Coccetti et al., 1998). Importantly, all of the yeast mutants defective in PtdIns(3,5)P₂ levels are also defective in vacuolar acidification, despite the normal localization of the vacuolar ATPase to the vacuole membrane (Bonangeli et al., 1997). This suggests that PtdIns(3,5)P₂ may regulate the vacuolar ATPase.

PtdIns(3,5)P₂ also regulates vacuole membrane fission. In the absence of PtdIns(3,5)P₂, vacuoles are extremely large and unlobed. In contrast, wild-type vacuoles are complex and contain multiple lobes. In addition, production of elevated levels of PtdIns(3,5)P₂ in the absence of osmotic stress, by simultaneous overexpression of Vac14p and Vac7p, results in a larger number of smaller vacuolar lobes, consistent with an increase in vacuole membrane fission.

Allowing the vacuole membrane surface area to remain constant during a rapid change in vacuole volume enables cells to adjust to a dynamic environment. Hypo-osmotic stress induced vacuole fusion maintains vacuole membrane surface area in the face of a rapid influx of water into the vacuole (Wang et al., 2001). Conversely, exposure to high osmolarity causes vacuoles in wild-type cells to become smaller and more highly fragmented. In addition, an increase in retrograde vesicle traffic, which requires PtdIns(3,5)P₂ (Bryant et al., 1998), removes a higher proportion of vacuole membranes than vacuole volume. By simultaneously regulating water and ion flux, and increasing vacuolar fission and retrograde traffic, PtdIns(3,5)P₂ may provide a concerted mechanism to prevent vacuole lysis during osmotic stress.

### Materials and methods

#### Media, strains, and molecular biology techniques

Yeast extract-peptone-dextrose (YPED) media, synthetic minimal media with the necessary nutritional auxotrophic supplements, sporulation media, synthetic complete media without inositol, and synthetic media without uracil or inositol (Sherman, 1991; Schu et al., 1993) were prepared as described. High pH YEPD plates with 1.8 M ethylene glycol contained: 1% yeast extract, 2% Bacto-peptone, 1.5% agar, 2% glucose, 1.8 M ethylene glycol, and 100 mM potassium phosphate (pH 7.6). The last three ingredients were not autoclaved and added separately. The strains used in this study are listed in Table V.

#### Labeling yeast vacuoles with FM4-64 or quinacrine

Yeast vacuoles were visualized in vivo by labeling log-phase cells with 80 µM N-3-(3-ethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) (FM4-64) (Bonangeli et al., 1997; Vida and Emr, 1995) or with quina-
crine (Weisman et al., 1987). Cells were viewed with a 100× objective on an Olympus BX-60 fluorescence microscope (FM4-64: excitation, 560 nm, dichroic mirror at 595 nm, emission, 630 nm; quinacrine: excita-
tion, 470 nm; dichroic mirror at 495 nm; emission, 425 nm) combined with a low level of transmitted light to reveal cell outlines. Images were captured digitally with a Hamamatsu ORCA CCD camera controlled with IP spectrum software (Sc Analytics). Images were processed using Adobe Photoshop™.

#### Cloning VAC14

VAC14 was cloned by complementing an inability of vac14-1 cells to grow in high pH media with ethylene glycol at 33°C (Bonangeli et al., 1997). 12 overlapping clones that complemented the growth and vacuolar morphology defects of vac14-1 cells were obtained. A 3.9-kb SpeI fragment containing a single complementing open reading frame was identified and partially sequenced. Confirmation that YLR386W (sequence deposited by the Yeast Genome Sequencing Project) is VAC14 was demonstrated by creating a strain with LEU2 linked to the wild-type YLR386W locus (see below).

#### Construction of a strain with LEU2 integrated adjacent to the VAC14 locus and a strain with a chromosomal deletion of VAC14

To construct a LEU2-marked strain, a 3.9-kb SpeI fragment containing VAC14 was cloned into pRS315 (Sikorski and Hieter, 1989) (pCB48). This plasmid was linearized with XhoI and transformed into wild-type (v14A) and the T7 universal primer. Diploid cells were generated by crossing the LEU2-marked strain (VAC14) with LNY4552 (leu2-3,112, vac14-1). Of 16 tetrad analyzed, all LEU2 colonies were wild-type, whereas all leu2 cells displayed vac14-1 phenotypes.

To create a chromosomal deletion of VAC14, the 3.9-kb SpeI fragment, containing VAC14 and both 5' and 3' untranslated regions, was subcloned into pRS424 (pCB49). VAC14 was removed with StyI and BclI and replaced by the PCR-amplified TRP1 gene (digested with StyI and BamHI). The primers used to amplify TRP1 were V41 (5'-GCTACCCCTGGGCTACCTACGTAATCCCTTGGC-3') and V42 (5'-GGGATCCCTACCTAACCTTCTCCTCAGTC-3'). The resulting plasmid (pCBS2) was digested with SpeI and the 2.5-kb fragment (containing the VAC14 knockout cassette)
Table V. Strains used in this study

<table>
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<th>Strains</th>
<th>Genotype</th>
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<td>MATA leu2-3::112 ura3-52 his3-D200 trp1-D901 lys2-801 suc2-D9 pep4-D1137</td>
<td>Gomes de Mesquita et al., 1996</td>
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<td>JBY007</td>
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<td>This study</td>
</tr>
</tbody>
</table>

was transformed into LWY3143. Colony PCR (with the primers v14A; see above) and V43 5’-GACGTGAAATTCTCCCTGTCG-3’ identified transformants containing TRP1 at the VAC14 locus.

Preparation of antibodies to Vac14p
A GST-VAC14 chimera was created by subcloning VAC14 into the IPTG inducible GST vector pMAL (Marshall et al., 1996). To introduce the appropriate restriction sites (XmaI at the 5’ end and XhoI at the 3’ end), VAC14 was amplified with the primers 5’-CCGCCCCGGGGGACCATGGGAAAAATCGATGTGAATTTTTTATATACGGG-3’ and 5’-GGGCTAGCTAGGAAGTATTTTTTTATATATATACGGG-3’. The PCR product was digested and ligated to pET21a and digested with Xmal and SalI. The resulting plasmid (pCB69) was transformed into Escherichia coli DE3 cells. The soluble GST-Vac14 fusion protein was isolated on glutathione-Sepharose beads and antibodies were raised in goats (Elmira Biological). Serum was depleted of GST antibodies by passing over a GST-Affi gel column (Bio-Rad Laboratories) and further purified by passage over a total yeast protein column prepared from vac14A cells.

Subcellular fractionation and Western blot Analysis
Yeast cell extracts and subcellular fractionations were performed as described (Bonangelino et al., 1997). Briefly, yeast strains were grown in YEP medium at 24°C to an O.D. of 0.6. Cells were harvested, washed with cytosol buffer (20 mM Hepes, pH 6.8, 0.15 M potassium acetate, 10 mM MgCl₂, and 0.25 M sorbitol), resuspended to ~100 OD/ml and lysed with glass beads. Cell extracts were centrifuged at 500 g at 4°C for 4 min. The SS supernatants were centrifuged at 13,000 g at 4°C for 10 min and the resulting S13 supernatants were centrifuged at 100,000 g at 4°C for 30 min. The pellets (P13 and P100) were resuspended in an equal amount of cytosol cocktail as the supernatants. Laemmli sample loading buffer was added to equivalent OD units of each fraction. Samples were heated to 80°C for 10 min before loading onto a 7.5% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose (ECL High Bond; Amersham Life Science, Piscatway, NJ) in Tris-glycine-methanol transfer buffer at 4°C at 35 V for 2 h.

Vacuole isolation on a Ficoll gradient

Yeast vacuoles were isolated as described previously on discontinuous Ficoll gradients (Conradt et al., 1992). Equal micrograms of vacuoles (found as a band at the interface between the 4% and 0% Ficoll) from each strain were separated on a 7.5% SDS polyacrylamide gel, transferred to nitrocellulose, and immunoblotted as described above.

Extraction of Vac14p from the membrane
Vacuoles from wild-type (LWY7235) cells were diluted to 1 μg/ml with 0% Ficoll buffer (10 mM Pipes, pH 6.8, 200 mM Sorbitol) and 100 μl aliquots were treated with one of the following conditions: 2% Triton X-100, 1.4 M urea, 0.1 M NaCl, 1 M NaCl, 0.8 M NH₄OH, or left untreated (buffer added to the appropriate volume). Samples were incubated on ice for 30 min and centrifuged at 100,000 g at 4°C for 30 min and the pellets were resuspended with an equal volume as the corresponding supernatant fraction. Equal amounts were analyzed by SDS-PAGE and immunoblotting as described above.

Analysis of GFP-Fab1p in vivo
GFP was inserted into the FAB1 ORF in pRS426 by homologous recombination. To introduce FAB1 sequences at both the 5’ and 3’ ends, GFP was amplified by PCR from pGOGFP (Cowles et al., 1997) using the primers FABGP-N (5’-GCT CAC ATG TCC GTG CTTCC CCA CTG GTA CTT CAT CTG TGA TGG GTA AAG GAG AAC TTC TT-3’) and FABGP-C2 (5’-GGG ACG CAG TCC GGA CAG TAC GAT TTG ATG TCG TGG CTG ACC CCG GGC CCG CCG TAC GTG C-3’). The PCR product and AatII-linearized pRS426-FAB1 were cotransformed into yeast and resultant colonies were screened for GFP fluorescence. The pRS426-GFP-FAB1 plasmid was then transformed into various yeast strains and fluorescence was visualized with the same filter set used for quinacrine.

Analysis of phosphatidylinositols
Cells were grown in YEP or synthetic media to an O.D. of 0.6 and 0.8. 1 OD₅₆₀ U of cells was harvested, washed, and resuspended in synthetic media lacking inositol. 0.14–0.4 OD₅₆₀ U were used to inoculate 5 ml of media lacking inositol containing 50 μM of myo-[2-³H]-inositol. Cells were labeled for 12 h at 24°C, harvested by centrifugation, washed, and resuspended in 100 μl of inositol-free media. For hypotonic shock, an equal volume of 1.8 M NaCl was added to cells for a final concentration of 0.9 M NaCl and the resulting suspension was incubated at 24°C for 10 min. Then, 300 μl of ice cold 4.5% perchloric acid (Whiteford et al.,...
Vacc14p regulates Fab1p PtdIns(3)P 5-kinase activity | Bonangelino et al. 1027


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References


