Sac1 Lipid Phosphatase and Stt4 Phosphatidylinositol 4-Kinase Regulate a Pool of Phosphatidylinositol 4-Phosphate That Functions in the Control of the Actin Cytoskeleton and Vacuole Morphology

Michelangelo Foti,* Anjon Audhya,* and Scott D. Emr†

Division of Cellular and Molecular Medicine, The Howard Hughes Medical Institute, University of California, San Diego, School of Medicine, La Jolla, California 92093-0668

Submitted January 6, 2001; Revised May 15, 2001; Accepted May 31, 2001
Monitoring Editor: David Drubin

INTRODUCTION
Phosphoinositides (PIs) are key regulators of a wide variety of cellular processes including signal transduction, cell proliferation, vesicular trafficking, apoptosis, cytoskeletal organization, and transcription (reviewed by Fruman et al., 1998; Martin, 1998; Divecha et al., 2000). The reversible phosphorylation of these lipid head groups makes them ideally suited to function as temporal and spatial regulators of these processes. The synthesis and turnover of PIs are regulated by a set of recently identified kinases and phosphatases. We analyzed the primary role of the phosphoinositide phosphatase Sac1p in *Saccharomyces cerevisiae* with the use of a temperature-sensitive allele of this gene. Our analysis demonstrates that inactivation of Sac1p leads to a specific increase in the cellular levels of phosphatidylinositol 4-phosphate (PtdIns(4)P), accompanied by changes in vacuole morphology and an accumulation of lipid droplets. We have found that the majority of Sac1p localizes to the endoplasmic reticulum, and this localization is crucial for the efficient turnover of PtdIns(4)P. By generating double mutant strains harboring the *sac1*ts allele and one of two temperature-sensitive PtdIns 4-kinase genes, *stt4*ts or *pik1*ts, we have demonstrated that the bulk of PtdIns(4)P that accumulates in *sac1* mutant cells is generated by the Stt4 PtdIns 4-kinase, and not Pik1p. Consistent with these findings, inactivation of Sac1p partially rescued defects associated with *stt4*ts but not *pik1*ts mutant cells. To analyze potential overlapping functions between Sac1p and other homologous phosphoinositide phosphatases, *sac1*ts mutant cells lacking various other synaptojanin-like phosphatases were generated. These double and triple mutants exacerbated the accumulation of intracellular phosphoinositides and caused defects in Golgi function. Together, our results demonstrate that Sac1p primarily turns over Stt4p-generated PtdIns(4)P and that the membrane localization of Sac1p is important for its function in vivo. Regulation of this PtdIns(4)P pool appears to be crucial for the maintenance of vacuole morphology, regulation of lipid storage, Golgi function, and actin cytoskeleton organization.
In Saccharomyces cerevisiae, synthesis of PtdIns(4)P is mediated by two PtdIns 4-kinases, Pik1p and Stt4p. Both kinases are required for cell viability, regulating distinct and essential cellular processes (Audhya et al., 2000). Pik1p is a 125-kDa soluble protein residing at the nucleus and trans-Golgi compartments (Flanagan and Thorner, 1992; Garcia-Bustos et al., 1994; Walch-Solimena and Novick, 1999). It has been demonstrated to play a direct role in late events of the secretory pathway, secretory vesicle budding at the late Golgi, integrity of Golgi structure, and cytokinesis (Garcia-Bustos et al., 1994; Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000). Stt4p is a 216-kDa membrane-bound protein that is required for actin cytoskeleton organization, cell wall integrity, and maintenance of vacuole morphology (Yoshida et al., 1995; Audhya et al., 2000). Stt4p has also been implicated in the transport of an aminophospholipid from the endoplasmic reticulum (ER) to the Golgi/vacuole (Trotter et al., 1998).

Regulation of the distinct PtdIns(4)P pools generated by Stt4p and Pik1p is unclear, but recently, PI phosphatases have been proposed to inhibit or terminate PtdIns 4-kinase activity (reviewed by Majerus et al., 1999). In particular, a novel class of PI phosphatases containing a Sac1-like phosphatase domain have been identified in yeast, mammal, and plant cells (Srinivasan et al., 1997; Guo et al., 1999). Sac1p is the prototype of this phosphatase family, and proteins including Fig4p and two members of the synaptojanin-like (Sjl) protein family, Sjl2p and Sjl3p (also called Inp52p and Inp53p), all harbor a putative functional Sac1-like phosphatase domain (Guo et al., 1999). The Sac1-like domains of Sac1p, Sjl2p, and Sjl3p have been shown to predominantly dephosphorylate PtdIns monophosphates such as PtdIns(3)P and PtdIns(4)P in vitro. A weak phosphatase activity toward phosphatidylinositol (3,5)-diphosphate (PtdIns(3,5)P2) has also been shown, preferentially against the phosphate residue at the D-3 position of the inositol ring (Guo et al., 1999; Hughes et al., 2000). Sjl proteins also can dephosphorylate PtdIns(4,5)P2 because of the presence of a second phosphatase domain that recognizes the phosphate group at the D-5 position (Guo et al., 1999). Surprisingly, single or double sjl mutants appear to alter only PtdIns(3,5)P2 and PtdIns(4,5)P2 levels in vivo (Stoltz et al., 1998a, 1998b; Guo et al., 1999). In contrast, deletion of SAC1 leads to alterations in intracellular levels of several PI isoforms, but the greatest effect is on PtdIns(4)P levels (8- to 10-fold; Guo et al., 1998a, 1998b; Guo et al., 1999). Furthermore, genetic interactions have been described between mutations in the secretory pathway (SEC genes) and sac1 mutations (Cleves et al., 1989). Sac1p has been suggested to be required for proper transport of ATG and efficient translocation of preproteins into the endoplasmic reticulum (ER; Kochendörfer et al., 1999). The stabilization of PtdIns(4)P in Sac1p-deficient cells is likely responsible for the above cellular defects because overexpression of Sjl2p or Sjl3p, which also possess PtdIns(4)P phosphatase activity, are able to rescue some of the phenotypes associated with sac1 null mutations (Hughes et al., 2000). However, all of the previous work has been based on an analysis of sac1 null mutants, leaving open the possibility that abnormal phenotypes observed in these mutants are the result of secondary defects.

Therefore, to analyze the primary function of Sac1p in PtdIns(4)P turnover, we generated alleles of sac1 that are temperature sensitive for function (sac1Δ) and examined the immediate consequence of Sac1p inactivation. We found that ISac1p primarily turns over Stt4p-generated PtdIns(4)P and that membrane localization of Sac1p is crucial for its efficient function. High levels of PtdIns(4)P resulting from Sac1p inactivation are accompanied by changes in vacuole morphology and an accumulation of lipid droplets. Defects resulting from inactivation of Stt4p, but not Pik1p, could be partially rescued when Sac1p was also inactivated. Finally, analysis of sac1Δ mutants lacking one or more of the Sjl proteins showed that Sjl3p plays an essential compensatory role in Sac1p-deficient cells.

**MATERIALS AND METHODS**

**Strains and Media**

Sources of growth media for yeast and bacterial strains have been described elsewhere (Gaynor et al., 1994). Transformation into yeast was performed by a standard lithium acetate method (Ito et al., 1983) and Escherichia coli transformations were done as previously described (Hanahan, 1983). *S. cerevisiae* strains used in this study are listed in Table 1.

**Plasmids and DNA Manipulations**

Enzymes utilized for recombinant DNA techniques were purchased from commercial sources and used as recommended by the suppliers. Standard recombinant DNA and standard yeast genetic methods were performed as previously described (Sherman et al., 1979; Sambrook et al., 1989).

**Disruption of SAC1.** SAC1, including 434 bp upstream of the start codon and 134 bp downstream of the stop codon, was amplified by polymerase chain reaction (PCR) from SEY6210 genomic DNA and ligated into a ScaI-SpeI–digested pBISK(--) to generate pBISK(--)–SAC1. The plasmid used to generate a chromosomal deletion of SAC1 was then constructed by inserting the TRPI gene into a ClaI-BamHI–digested pBISK(--)–SAC1–TRPI. A ScaI–SpeI fragment of pBISK(--)–SAC1–TRPI was then transformed into SEY6210I to delete 1540 bp of the SAC1-coding sequence (strain MYF62). The disruption was confirmed by PCR with the use of two different sets of primers.

**SAC1 and sac1ΔS22-623 Cloning.** SAC1 was subcloned from a pYEP24–based plasmid isolated from the yeast genomic library described by Carlson and Botstein (1982). Briefly, MYF62 was transformed with the pYEP24–based genomic library and plasmids containing the SAC1 open reading frame were isolated based on their ability to complement the GH18 sensitivity of MYF62 to generate pRSH18–SAC1, a BglII–SpeI SAC1 DNA fragment derived from a pYEP24–SAC1 was subcloned into pBS416 digested with BamHI–SpeI (Sikorski and Hieter, 1989). To generate sac1ΔS22-623, PCR primers were generated to amplify the SAC1 gene lacking sequence for the last 102 amino acids (with a NcoI restriction site upstream of the second codon and an Asel restriction site IS69 bp downstream of
the start codon), which was then digested with NcoI and Asel and ligated into pBluescriptSK+ (Stratagene, La Jolla, CA) containing the CPS1 promoter (pGO106). The resulting ligation product was then subcloned into a similar CEN-based pRS415 vector or a 2µ-based pRS425 vector.

**Generation of sac1** (Temperature-sensitive for Function) Conditional Alleles. A XbaI-PvuI SAC1 fragment (bp –6–1575) was amplified by error-prone PCR (Muhlrad et al., 1992) and cotransformed with Hsp150p-gapped pRS416-SAC1 into MFY62. Uracl auxotrophs were selected and initially screened for growth on G418 (25 µg/ml) containing YPD medium at 26 and 36°C. Mutants that grew at 26°C but not at 38°C were then also tested for growth on minimal media lacking inositol at both temperatures. From >10,000 transformants, three putative pRS416-sac1 plasmids were isolated, retransformed into MFY62 and tested for growth phenotypes and PI phosphatase activities in vivo at the restrictive temperature. For all studies, the sac1-23 allele was analyzed.

**Generation of Other sac1 Mutants.** To generate a stt4/sac1 double mutant (MFY55), AAY102 (stt4-ts) was transformed with Sphi/Sall-digested pBIISK(-)–SAC1:TRP1. Tryptophan auxotrophs were then tested by PCR for deletion of SAC1 with the use of two different sets of primers. MFY55 was then transformed with pRS416-sac1-ts. For all studies, the stt4-4 allele was analyzed.

For a pik1/sac1 double mutant (AAY131), MFY62, dissecting tetrads and isolating spores by growth on selective media. AAY131 was then transformed with pRS416-sac1-ts. For all studies, the pik1-83 allele was analyzed.

To generate sjl2-sac1 (AAY141), sjl3/sac1 (AAY142), and sjl2/sjl3/sac1 (AAY143) mutants, MFY62 carrying pRS416-sac1-ts was mated with SEY6210 sjl2, SEY6210 sjl3, and SEY6210 sjl2/sjl3 (J. Gary, unpublished data), respectively. Spores resulting from tetrad dissection were then selected for the appropriate markers, and disruptions were confirmed by PCR with the use of two different sets of primers for each gene.

**Generation of Green Fluorescence Protein (GFP)/myc-tagged SAC1 and sac1522-623 Strains.** Tagging of the Sac1p C-terminus with GFP or a 13Myc epitope was performed as described previously (Longtine et al., 1998). In brief, PCR products containing the tags and either a TRP1 or HIS3MX6 marker, flanked by homologous regions to SAC1, were generated as described by Longtine et al. (1998) with the use of the template described therein and transformed into SEY6210. Tryptophan or histidine auxotrophs were isolated. Both fractions were precipitated in the presence of 9% trichloroacetic acid.

**Metabolic Labeling and Immunoprecipitation**

Cell labeling and immunoprecipitations were performed as previously described (Gaynor et al., 1994) with minor modifications. In brief, log phase cultures were labeled with Tran 35S-label (DuPont NEN, Boston, MA) for 10 min and chased with cold methionine and cysteine for the indicated times; proteins were precipitated with 9% trichloroacetic acid. All temperature shifts, unless otherwise stated, were limited to 10 min at 38°C. Extracts were immunoprecipitated with antisera against carboxypeptidase Y (CPY), Hsp150p, or invertase, which have been previously characterized (Cowell et al., 1997; Gaynor and Emr, 1997). To assay internal and external fractions for the presence of invertase, cells harboring a plasmid expressing invertase (pCY1-20) were converted to spheroplasts after pulse-chase by adding a 2× buffer containing 50 mM Tris (pH 7.5), 2 M sorbitol, 40 mM NaF, 40 mM NaN 3, and 10 mM dithiothreitol and incubating on ice for 10 min. Zymolyase T100 (15 µg/OD600; Seikagaku Kogyo, Tokyo, Japan) was then added to the cell suspension and incubated for 30 min at 30°C. Cells were then subjected to centrifugation at 6000 rpm for 5 min, and the supernatant was removed. Both fractions were precipitated in the presence of 9% trichloroacetic acid.

---

### Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotypes</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEY6210</td>
<td>MATα leu2-3,112 ura3-52 his3 Δ200 trp1 Δ901 lys2-801 sec2 Δ9</td>
<td>(Robinson et al., 1988)</td>
</tr>
<tr>
<td>SEY6210.1</td>
<td>MATα leu2-3,112 ura3-52 his3 Δ200 trp1 Δ901 lys2-801 sec2 Δ9</td>
<td>(Robinson et al., 1988)</td>
</tr>
<tr>
<td>MFY62</td>
<td>SEY6210.1; sac1Δ:TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>AAY102</td>
<td>SEY6210; stt4Δ: HIS3 carrying pRS415stt4-4 (LEU2 CEN6 stt4-4)</td>
<td>(Audhya et al., 2000)</td>
</tr>
<tr>
<td>MFY55</td>
<td>AAY 102; sac1Δ:TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>AAY104.5</td>
<td>SEY6210; pik1Δ: HIS3 carrying pRS415pik1-83 (LEU2 CEN6 pik1-83)</td>
<td>(Audhya et al., 2000)</td>
</tr>
<tr>
<td>AAY131</td>
<td>AAY104.5; sac1Δ:TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>JGY130</td>
<td>SEY6210; sjl2Δ: HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>JGY131</td>
<td>SEY6210; sjl3Δ: TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>JGY132</td>
<td>SEY6210; sjl2Δ: HIS3 sjl3Δ: TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>MFY80</td>
<td>SEY6210.1; SAC1-GFP::HIS3MX6</td>
<td>This study</td>
</tr>
<tr>
<td>MFY107</td>
<td>SEY6210.1; sac1Δ-MCY13::TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>MFY109</td>
<td>SEY6210.1; sac1Δ522-623MYC13::TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>MFY111</td>
<td>SEY6210.1; sac1Δ522-623-GFP::TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>AAY141</td>
<td>SEY6210; sjl2Δ: HIS3 sac1Δ::TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>AAY142</td>
<td>SEY6210; sjl3Δ: TRP1 carrying pRS416sac1-23 (URA3 CEN6 sac1-23)</td>
<td>This study</td>
</tr>
<tr>
<td>AAY143</td>
<td>SEY6210; sjl2Δ: HIS3 sjl3Δ::TRP1 sac1Δ::TRP1 carrying pRS416sac1-23 (URA3 CEN6 sac1-23)</td>
<td>This study</td>
</tr>
<tr>
<td>EGY1181-10</td>
<td>SEY6210; sec18-1</td>
<td>(Sato et al., 1998)</td>
</tr>
</tbody>
</table>
In Vivo PI Analysis

Analysis of PI levels was performed as previously described (Audhya et al., 2000). Briefly, cells from a log phase culture were shifted to the appropriate temperature for 10 min and then labeled with myo-[2-3H]inositol (Nycomed Amersham, Princeton, NJ). After 10 min, cells were unlabelled myo-inositol was added and cells were incubated for 30 min at the desired temperature. Cells were lysed and extracts were processed as previously described (Stack et al., 1995). Analysis of [3H]-labeled glycerophosphoinositols was performed with the use of a Gold HPLC (Beckman System, Fullerton, CA) coupled to an online radiomatic detector (Packard, Meriden, CT). All phospholipid data are expressed as percentages of the total number of counts loaded onto the high-performance liquid chromatography (HPLC) column for normalization.

Fluorescence and Electron Microscopy

FM4-64 Labeling. Labeling of vacuole membranes with the vital dye FM4-64 (Molecular Probes, Eugene, OR) was performed as previously described (Vida and Emr, 1995). Briefly, cells from a log phase culture were labeled with FM4-64 for 15 min, followed by a chase without dye for 45 min. For temperature shift experiments, cells were first pulse-chased with FM4-64 and then shifted to 38°C for the indicated time.

All the images were acquired with the use of a DeltaVision Deconvolving microscope (Applied Precision, Seattle, WA). Images were then processed with the use of Photoshop 4.0 (Adobe Systems, Mountain View, CA).

Immunofluorescence. Cells grown to midlog phase were spheroplasted and fixed with 4% formaldehyde. Fixed cells were then washed in 50 mM Tris-HCl, pH 7.5, 1 M sorbitol, permeabilized with 0.02% Triton X-100 for 10 min, washed again, and incubated on polylysine-coated slides for 20 min. Adherent cells were incubated for 30 min with blocking buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 containing 1% dry milk and 0.5 mg/ml bovine serum albumin) to prevent nonspecific binding of antibodies and then for 2 h with the indicated primary antibodies in WT buffer at room temperature (mouse monoclonal a-Myc 10 ng/ml OM-11-908 from Genosys [The Woodlands, TX], rabbit polyclonal a-Kar2 [1/10,000] kindly provided by Dr. M. Latterich [Silk Institute, La Jolla, CA], and rabbit polyclonal a-Kex2 [1/1000] kindly provided by Dr. R. Fuller [University of Michigan, Ann Arbor, MI]). Cells were washed three times with WT buffer and incubated again with the appropriate fluorochrome-conjugated secondary antibodies for 1 h at room temperature (Alexa Fluor568 goat anti-mouse A-11004 and Alexa Fluor488 goat anti-rabbit A-11008 from Molecular Probes). Cells were finally washed five times with phosphate-buffered saline/glycerol plus 0.02% Triton X-100 for 10 min and mounted in phosphate-buffered saline/glycerol plus 4’,6-diamidino-2-phenylindole (DAPI) for observation. For studies with the use of Sec7p-GFP as a medial Golgi marker, no antibody was required.

All the images were acquired with the use of a DeltaVision Deconvolving microscope (Applied Precision). Images were then processed with the use of Adobe Photoshop 4.0.

Actin Labeling. To analyze actin cytoskeleton organization, cells were grown to early log phase, shifted to the appropriate temperature for 2.5 h, fixed in 3.7% formaldehyde, and stained with rhodamine-phalloidin (Molecular Probes) as previously described (Benedetti et al., 1994). Polarization of actin in small budded cells was scored on >200 cells for each condition.

Electron Microscopy. Ultrastructural analysis was performed as previously described (Rieder et al., 1996). In brief, cells in a log phase growth were fixed with 3% glutaraldehyde for 1 h and spheroplasted before being processed for electron microscopy; >100 cells considered well preserved were examined for each analysis.

Nile Red Staining

Staining of lipid droplets with Nile red was performed on fixed cells as described by Greenspan et al. (1985) and images were acquired with the use of a DeltaVision Deconvolving microscope (Applied Precision). Images were then processed with the use of Adobe Photoshop 4.

RESULTS

sac1ts Mutant Cells Exhibit a Rapid Defect in the Turnover of Newly Synthesized PtdIns(4)P

Sac1p has been identified as a PI polyphosphatase (Guo et al., 1999). To examine the primary function of Sac1p in vivo, sac1 temperature-sensitive alleles (sac1ts) were generated by PCR-mediated random mutagenesis of the SAC1 phosphatase domain (coding region corresponding to aa 1–525 as shown in Figure 1A) and introduced into haploid yeast lacking the chromosomal copy of SAC1. Selection of sac1ts mutants was facilitated by the fact that deletion of SAC1 in the SEY6210 background confers hypersensitivity to low concentrations of the aminoglycoside antibiotic gentamicin (G418; 10 µg/ml) in rich media. From >10,000 transformants, three alleles were isolated that confer robust growth at 26°C, but not at the restrictive temperature of 38°C, on G418-containing rich media. Additionally, like sac1 cells, the sac1ts cells were found to be inositol auxotrophs at high temperature (Figure 1B). Among these three temperature-sensitive mutants, sac1-23ts exhibited the strongest defects at the restrictive temperature and was chosen for further analysis. Sequence analysis of the sac1-23 allele revealed six amino acid changes (Y111C, V190A, K223E, D336A, F366S, E416G). Interestingly, each of these mutations lie within or closely adjacent to the conserved Sac phosphatase domain of Sac1p (amino acids 114–503; reviewed by Hughes et al., 2000).

Labeling of cells with myo-[2-3H]inositol demonstrated that sac1Δ mutant cells show pleiotropic alterations of intracellular PI levels as compared with WT cells (Rivas et al., 1999; Stock et al., 1999; Hughes et al., 2000). We assessed steady-state intracellular PI levels in SEY6210 lacking the SAC1 gene. Cells were labeled for 12 h with myo-[2-3H]inositol and then processed for HPLC analysis of PIs as described in MATERIALS AND METHODS. We found that sac1Δ cells accumulated PtdIns(3)P (1.7-fold increase), PtdIns(4)P (19.8-fold increase), and PtdIns(3,5)P2 (4-fold decrease) as compared with WT cells. Because phospholipid metabolism is highly dependent on the cell growth phase (Paltauf et al., 1992), we also performed pulse-labeling experiments of PIs in the sac1Δ mutant. PIs were labeled with myo-[2-3H]inositol for 10 min and chased for 30 min, and the cellular levels of PIs were analyzed by HPLC. Under these conditions, where only PIs synthesized during a short pulse/chase were analyzed, the levels of PtdIns(3)P and PtdIns(3,5)P2 in sac1Δ cells were only slightly affected at 26°C, whereas PtdIns(4)P was increased by >12-fold. Similar to the steady-state labeling, PtdIns(4,5)P2 was found to be decreased by >75% (Figure 1C). When sac1ts cells were labeled at 26°C with the use of the same protocol, intracellular PI levels were only mildly affected as compared with those observed in WT cells (2-fold increase in PtdIns(4)P). However, when the labeling was performed at the restrictive
temperature of 38°C, sac1Δ mutant cells exhibited a dramatic increase in PtdIns(4)P levels (>7-fold), whereas the levels of PtdIns(3)P, PtdIns(3,5)P2, and PtdIns(4,5)P2 were only slightly altered as compared with WT cells (Figure 1C).

Together these results indicate that Sac1p primarily functions in the turnover of a rapidly synthesized pool of PtdIns(4)P in vivo. Changes in the intracellular levels of other PIs previously observed in sac1Δ mutant cells are likely an indirect consequence of the long-term effect of Sac1p inactivation on PI metabolism.

Membrane Localization of Sac1p Is Required for Efficient PtdIns(4)P Turnover

Sac1p, in contrast to the other known yeast PI phosphatases, is an integral membrane protein (Whitters et al., 1993). Additionally, in vitro, Sac1p exhibits a higher phosphatase activity against PtdIns(3)P than PtdIns(4)P, its major in vivo substrate (Hughes et al., 2000). These features suggest that the intracellular localization of Sac1p is crucial for its biological specificity. To clarify this point, the chromosomal copy of SAC1 was tagged with GFP or a 13Myc epitope at the 3′-end (Sac1p-GFP and Sac1p-13Myc, respectively) to further examine intracellular localization of Sac1p. In addition, to assess the role of the C-terminal transmembrane domain of Sac1p, GFP- or 13Myc-tagged truncated forms of the protein were also generated by integration into the chromosome as described in MATERIALS AND METHODS (Sac1Δ522-623-GFP and Sac1Δ522-623-13Myc, respectively).

Tagging of full-length Sac1p with either GFP or a 13Myc epitope did not alter the activity of Sac1p in vivo. The fusion proteins were normally expressed, as assayed by Western blot analysis, and strains harboring this fusion as their sole copy of Sac1p did not display any growth defects (Foti, Audhya, and Emr, unpublished results). Furthermore, intracellular levels of PIs in cells expressing these fusion proteins were similar to WT cells (Figure 2A). In contrast, cells expressing the Sac1Δ522-623 proteins exhibited altered intracellular PI levels and showed growth defects similar to sac1Δ cells, although the truncated proteins were well expressed (Figure 2A). Interestingly, when Sac1Δ522-623 was overexpressed in sac1Δ cells, normal growth and near WT levels of PtdIns(4)P and PtdIns(4,5)P2 were restored, indicating that this truncated version of Sac1p was active (Figure 2A). Moreover, these data suggest that the Sac1p transmembrane domain is required for the efficient turnover of PtdIns(4)P.

Intracellular localization of GFP-tagged constructs (Sac1p-GFP and Sac1Δ522-623-GFP) were examined in living cells by fluorescence microscopy. Consistent with other studies in mammalian cells (Nemoto et al., 2000), the Sac1p-GFP fusion protein exhibited a fluorescence pattern typical of ER resident proteins, concentrating in a perinuclear ring and in a discontinuous juxtamembrane staining around the cell periphery (Figure 2B). Strikingly, the Sac1Δ522-623-GFP fusion protein was mislocalized and redistributed throughout the cytosol and the nucleus, with no evident membrane association (Figure 2B).

Identical results were obtained with the 13Myc-tagged Sac1p fusion proteins (Sac1p-13Myc and Sac1Δ522-623-13Myc) by immunofluorescence on fixed cells. Immunofluorescence detection of the full-length Sac1-13Myc protein produced an ER-staining pattern similar to that of Kar2p, an ER resident protein (Figure 2C). Localization of Sec7p, a

Figure 1. sac1Δ mutant cells display growth defects on selective media and generate increased levels of PtdIns(4)P at restrictive temperature. (A) The Sac1 PI phosphatase is a 623-amino acid protein with at least two distinct domains. The polyphosphoinositide phosphatase (PPIPase) domain (aa 114–503), also called the Sac phosphatase domain, contains seven highly conserved motifs, including the putative RXNCLDLRRT catalytic motif. The region between amino acids 522 and 573 encompasses two potential helical membrane-spanning domains predicted by hydropathy plot analysis according to Kyte and Doolittle. The region of Sac1p that was randomly mutagenized is indicated. (B) WT, sac1Δ, and sac1Δ mutant cells were streaked on YPD-rich media with or without 10 µg/ml G418 or minimal synthetic media lacking inositol at both the permissive temperature of 26°C and the restrictive temperature of 38°C for 3 d. (C) Cells were preincubated at either 26 or 38°C for 10 min, labeled with myo-[2-3H]inositol for 10 min, and then chased with excess unlabeled myo-inositol for 30 min at the indicated temperature. Lipids were then deacylated from cellular membranes, and glycerophosphoinositols were extracted and analyzed by HPLC. Quantitative comparisons of glycerophosphoinositols generated by WT, sac1Δ, and sac1Δ cells at 26 or 38°C are shown. These data represent the means ± SEM of at least three independent experiments.
Sac1 Phosphatase and Stt4 Kinase Regulate PtdIns(4)P

Sac1 Phosphatase and Stt4 Kinase Regulate PtdIns(4)P

The Stt4 PtdIns 4-Kinase Generates the Bulk of PtdIns(4)P That Accumulates in sac1ts Mutant Cells

Previous studies have suggested that the Sac1p phosphatase regulates the pool of PtdIns(4)P generated by the PtdIns 4-kinase Pik1p (reviewed by Hughes et al., 2000; Huijbregts et al., 2000). To define the PI kinase that synthesizes the pool of PtdIns(4)P that accumulates in sac1 mutant cells, double mutants harboring the temperature-sensitive sac1-23 allele together with a temperature-sensitive allele of the two known PtdIns 4-kinases in yeast, pkl-83 and stt4-4, were generated. We have previously shown that each kinase accounts for approximately half of the total PtdIns(4)P generated in cells (Audhya et al., 2000). We analyzed the intracellular levels of PtdIns(4)P in these double mutants at both the permissive and restrictive temperatures. At the permissive temperature, intracellular PtdIns(4)P levels were similar to sac1ts cells, in both double mutants, stt4ts/sac1ts and pklts/sac1ts (compare Figure 1C with Figure 3). Surprisingly, simultaneous inactivation of Sac1p and Stt4p results in few, if any, changes in PtdIns(4)P levels after the shift to restrictive temperature (1.1 ± 0.3% at 26°C vs. 1.7 ± 0.5% at 38°C), whereas inactivation of Sac1p and Pik1p results in a dramatic increase in PtdIns(4)P that is typically observed in sac1 mutant cells (1.6 ± 0.2% at 26°C vs. 6.2 ± 1.8% at 38°C). These results indicate that Stt4p, and not Pik1p, generates the bulk of PtdIns(4)P, which accumulates upon Sac1p inactivation.

Sac1ts Suppresses Phenotypes Associated with stt4ts But Not pklts Cells

To further investigate the role of Sac1p in PtdIns(4)P turnover, we assessed whether the phenotypes observed in PtdIns 4-kinase stt4ts and pklts mutant cells could be rescued by elimination of Sac1p activity. Although we could not detect any defects in actin cytoskeleton organization in pklts cells (Audhya et al., 2000), stt4ts cells fail to appropriately organize their actin cytoskeleton at restrictive temperature. Specifically, stt4ts cells display random cortical actin patches throughout both mother and daughter cells, instead of restricting these patches to the bud and septum as observed in WT cells (Audhya et al., 2000). To determine whether stabilization of the Stt4p-dependent PtdIns(4)P pool by Sac1p inactivation could prevent the defects in actin cytoskeleton organization displayed by stt4ts cells, stt4ts/sac1ts double mutant cells were incubated at the permissive or restrictive temperature, fixed, and then labeled with rhodamine-conjugated phalloidin. The distribution of actin patches and cables were then analyzed and quantified as described in MATERIALS AND METHODS. Consistent with a role for Sac1p in the turnover of PtdIns(4)P generated by Stt4p, we observed suppression of the defect in actin cytoskeleton organization in double stt4ts/sac1ts mutant cells as compared with stt4ts single mutant cells (Table 2), thus supporting a functional connection in vivo between Stt4p and Sac1p.

We also assessed whether the rapid inactivation of Sac1p, which prevents turnover of a pool of PtdIns(4)P generated by Stt4p, could rescue defects associated with Pik1p inactivation. We have previously shown that secretion of Hsp150p, a high-molecular-weight glycoprotein, is impaired in pklts mutant cells at the restrictive temperature but not at the permissive temperature (Audhya et al., 2000). As shown in Figure 4A, inactivation of Sac1p neither affected protein secretion by itself nor rescued the Pik1p-dependent defect in Hsp150p secretion at the restrictive temperature. Furthermore, we previously described that pklts cells exhibit a kinetic delay of CPY maturation at the nonpermissive temperature, whereas at 26°C, CPY is processed normally (Audhya et al., 2000). CPY is converted from an ER-modified p1 precursor form to a Golgi-modified p2 precursor form and then transported to the vacuole where it is cleaved to generate the mature, active form of CPY (mCPY). In pikltts cells, CPY transport to the vacuole is delayed, resulting in a significant accumulation of the p2CPY precursor form. As shown in Figure 4B, sac1ts cells displayed normal processing of CPY at the restrictive temperature, and inactivation of Sac1p in pklts cells could not relieve the CPY maturation defect exhibited at the nonpermissive temperature. Interestingly, in contrast to sac1ts cells, we observed a kinetic delay of CPY maturation in sac1Δ cells, as has also been shown by others (Mayinger et al., 1995; Foti, Audhya, and Emr, unpublished results), suggesting again that deletion of SAC1 results in phenotypes indirectly related to the loss of Sac1p function.

Together, these results demonstrate that the rapid inactivation of Sac1p can rescue the actin defect associated with the stt4ts mutant. In contrast, Sac1p inactivation cannot rescue secretory defects exhibited by pikltts mutant cells, suggesting that the pool of PtdIns(4)P which accumulates in sac1 mutant cells cannot substitute for PtdIns(4)P generated by Pik1p to regulate secretion.

Sac1ts Cells Exhibit Altered Vacuole Morphology and Accumulate Lipid Droplets at the Restrictive Temperature

Inactivation of the two identified PtdIns 4-kinases in yeast, Stt4p and Pik1p, result in distinct alterations in vacuole morphology, suggesting that PtdIns(4)P plays a role in the maintenance of vacuole size and shape (Audhya et al., 2000). To determine whether high levels of PtdIns(4)P generated after Sac1p inactivation affect vacuole morphology, sac1ts mutant cells were labeled with the vacuolar vital dye FM4-64 (described in MATERIALS AND METHODS). At the permissive temperature, FM4-64 staining of sac1ts cells was similar to that observed in WT cells, highlighted by one mediol Golgi marker, yielded a completely distinct pattern of staining as compared with Sac1-13Myc, and we failed to observed any colocalization of these proteins (Figure 2C). Furthermore, Sac1-13Myc failed to colocalize with the late Golgi/endosome marker, Kex2p, again indicating that little, if any, Sac1p localizes to the Golgi. Similar to the Sac1Δ522-623–GFP localization, the Sac1Δ522-623–13Myc fusion protein showed a completely different and distinct pattern of distribution as compared with the full-length Sac1p-13Myc. Coimmunofluorescence studies with the use of Kar2p showed that Sac1Δ522-623 was no longer restricted to the ER, confirming that the C-terminal 102 amino acids of Sac1p are required for normal localization of Sac1p (Figure 2C).

Together these data demonstrate that the majority of Sac1p is localized to the ER and this localization is required for efficient turnover of PtdIns(4)P.
Figure 2 (facing page). Localization of Sac1p to the ER is crucial for the efficient turnover of PtdIns(4)P. (A) Cells were labeled with myo-[2-3H]inositol for 30 min. Lipids were then deacylated from cellular membranes, and the resulting soluble glycerophosphoinositols were extracted and analyzed by HPLC. Quantitative comparisons of glycerophosphoinositols generated by cells expressing the Sac1-13Myc or the Sac1Δ522-623–13Myc–tagged protein are shown. Additionally, glycerophosphoinositols from sac1Δ cells overexpressing the Sac1Δ522-623 truncation protein are shown. These data represent the means ± SEM of at least three independent experiments. (B) Full-length GFP-tagged Sac1p (Sac1p-GFP) or a truncated version of Sac1p lacking the last 102 amino acids, including the potential transmembrane domain (Sac1Δ522-623-GFP), were generated and observed by fluorescence microscopy in living cells. On the right, identical fields were observed with Nomarski optics. Pictures shown are representative of >95% of the cells observed. (C) Indirect immunofluorescence microscopy of Sac1-13Myc and Sac1Δ522-623–13Myc on paraformaldehyde-fixed cells. Cells expressing the Sac1-13Myc or Sac1Δ522-623–13Myc–tagged proteins were fixed, permeabilized, and incubated with α-Myc, α-Kar2p (ER marker), or α-Kex2p (Golgi/endocontrols marker) antibodies as described in MATERIALS AND METHODS. DAPI was used to stain DNA before mounting. For cells expressing Sec7p-GFP, no antibodies were required. On the right, identical fields were observed with Nomarski optics. Pictures shown are representative of >95% of the cells observed.

Table 2. Actin cytoskeleton organization in stt4Δ and sac1Δ mutants

<table>
<thead>
<tr>
<th>Actin cytoskeleton organization</th>
<th>Cells with normal actin morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast strain</td>
<td>26°C</td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
</tr>
<tr>
<td>stt4Δ</td>
<td>&gt;95</td>
</tr>
<tr>
<td>sac1Δ</td>
<td>&gt;90</td>
</tr>
<tr>
<td>stt4Δ/sac1Δ</td>
<td>&gt;90</td>
</tr>
</tbody>
</table>

Percentages are based on >200 cells observed for each strain at each condition. Wild type (SEY6210), stt4Δ (AAY102), sac1Δ (MFY62) carrying URA3 CEN6 sac1-23, and double mutant stt4Δ/sac1Δ (MFY55) carrying URA3 CEN6 sac1-23 cells at 26°C and 38°C are described. Comparison of stt4Δ and stt4Δ/sac1Δ cells is highlighted at 38°C.
uole that can be, at least in part, ameliorated by elimination of Stt4p activity.

Other PI Phosphatases Can Partially Compensate for the Loss of Sac1p Function

Sequence analysis of the yeast genome indicates the existence of other PI phosphatases that contain a conserved domain highly homologous to the Sac1p phosphatase domain. Of particular interest are the Sjl proteins, two of which have been shown to have similar enzymatic specificity to Sac1p in vitro (Guo et al., 1999). Notably, Sjl2p and Sjl3p have been identified as multicopy suppressors of sac1 mutant cells, suggesting that these phosphatases can partially compensate for the loss of Sac1p function (Hughes et al., 2000). Consistent with these findings, overexpression of either Sjl2p or Sjl3p suppresses the vacuole morphology defect we observe in sac1 mutant cells (Foti, Audhya, and Emr, unpublished results). Furthermore, after dissection of more than 24 tetrads generated from diploid strains harboring single chromosomal deletions of SJL3 and SAC1, we failed to isolate a single sac1/sjl3 double mutant strain, indicating that deletion of both genes results in lethality. To address whether Sac1p, Sjl2p, and Sjl3p may function in related pathways, sjl2/sjl3, sjl2/sac1, and sjl3/sac1 mutant cells harboring the sac1 allele were generated. These mutant strains exhibited surprising growth phenotypes as follows. sac1/sjl3 cells grew well on YPD-rich medium at 26°C but poorly at 38°C, a temperature at which both single mutants grow normally. sac1/sjl2 double mutant cells were not temperature sensitive for growth, consistent with our ability to isolate sjl2/sac1 double mutant cells. However, the sac1/sjl2/sjl3 triple mutant cells showed a clear growth defect, highlighted by slow growth at 26°C and cell death at 38°C. However, by performing a kill curve, we found that >90% of sac1/sjl2/sjl3 mutant cells were viable after a 3-h shift to nonpermissive temperature. Therefore, to ensure cell viability, experiments with these cells were limited to conditions where they were incubated at nonpermissive temperature for <1 h.

To determine how these different phosphatases cooperate in controlling PI metabolism, intracellular PI levels were analyzed by HPLC in these double and triple mutant cells.

Figure 4. Inactivation of Sac1p does not rescue transport defects associated with pik1 mutant cells. (A) Hsp150p secretion in WT, sac1, pik1, and pik1/sac1 cells. Cells were preincubated at 38°C for 10 min, metabolically labeled with an 35S-protein–labeling mixture during a 10-min pulse, and then chased in the presence of excess unlabeled methionine and cysteine for 30 min. Labeled cells were subjected to centrifugation and Hsp150p was immunoprecipitated from both the media (external) fraction and the cellular (internal) fraction and resolved by SDS-PAGE. (B) CPY processing in sac1, pik1, and pik1/sac1 cells. Cells were preincubated at 38°C for 10 min, metabolically labeled with a 35S-protein–labeling mixture during a 10-min pulse, and then chased in the presence of excess of unlabeled methionine and cysteine for the indicated times. CPY was then immunoprecipitated and resolved by SDS-PAGE. The migration positions of precursor and mature forms of CPY are indicated on the left. All data are representative of multiple experiments.

Figure 5. Sac1p phosphatase activity is required for normal vacuole morphology. sac1 cells were grown to early log phase at 26°C, and vacuoles were labeled with the vital dye FM4-64 for 15 min (see MATERIALS AND METHODS). After labeling, cells were chased for 45 min and shifted to the indicated temperature for 2 h. On the right, cells were observed by Nomarski optics. On the left, identical fields are shown under fluorescent illumination (rhodamine channel). sac1 cells at 26°C are shown on top, and sac1 cells after a 2-h shift to 38°C are shown below. These cells are representative of >90% of cells observed.
At the permissive temperature, PI levels in all mutant strains were similar to those observed in \textit{sac1}^{ts} cells (Figure 8A). At the restrictive temperature, we could not detect any changes in the intracellular PI levels of \textit{sac1}^{ts}/\textit{sjl2}/H9004 cells as compared with \textit{sac1}^{ts} cells. Interestingly, \textit{sac1}^{ts}/\textit{sjl3}/H9004 and \textit{sac1}^{ts}/\textit{sjl2}/H9004/\textit{sjl3}/H9004 mutant cells exhibited a dramatic accumulation of PtdIns(4)P, even when compared with \textit{sac1}^{ts} cells at the nonpermissive temperature. In these mutants, PtdIns(4)P represented 23–24% of the total newly synthesized PI, >2.5-fold greater than the level of PtdIns(4)P in \textit{sac1}^{ts} cells at the

Figure 6. \textit{sac1}^{ts} cells display morphologically abnormal vacuoles surrounded by vesicular structures reminiscent of lipid droplets at the restrictive temperature. \textit{sac1}^{ts} cells were incubated at either at 26 or 38°C for 2 h, fixed in 3% gluteraldehyde, and processed for electron microscopy (see MATERIALS AND METHODS). (A) \textit{sac1}^{ts} cells at 26°C. Bar, 1.0 μm. (B) \textit{sac1}^{ts} cells at 38°C. Bar, 1.0 μm. (C and D) Fivefold enlarged view of the framed vacuoles surrounded by lipid droplets in \textit{sac1}^{ts} cells at 38°C shown in B. All cells shown are representative of >90% of cells observed. v, vacuoles; ld, lipid droplets; n, nuclei.
nonpermissive temperature. This incredibly high amount of
PtdIns(4)P was also accompanied by a significant increase in
PtdIns(3,5)P2 which represented ~1% of the total newly
synthesized PIs in sac1ts/sjl2Δ/sjl3Δ cells (Figure 8B).
Together, our data demonstrate that Sjl2p and Sjl3p can
partially compensate for the loss of Sac1p function, likely by
dephosphorylating the PtdIns(4)P that accumulates in
sac1ts mutants.

Lack of Sjl2p and Sjl3p Activity in sac1 Mutant
Cells Leads to Defects in Early Golgi Function

PtdIns(4)P synthesis has been shown to regulate intracellular
traffic along the secretory pathway (Hama et al., 1998; Matsuoka et al., 1998; Walch-Solimena and Novick, 1999; Audhya et al., 2000). Because deletion of SJL genes in
sac1ts cells profoundly alters intracellular levels of
PtdIns(4)P, we assessed whether protein sorting also was
altered in these mutant cells.

Protein glycosylation and transport were monitored by
following the biosynthetic processing of CPY. At 26°C, CPY
maturation was normal in sac1ts/sjl2Δ and sac1ts/sjl3Δ cells,
whereas sac1ts/sjl2Δ/sjl3Δ cells displayed a minor kinetic
delay in processing of this hydrolase (Foti, Audhya, and Emr, unpublished results). However, at the restrictive
temperature, both sac1ts/sjl3Δ double and sac1ts/sjl2Δ/sjl3Δ tri-
ple mutant cells displayed defects in Golgi glycosylation,
illustrated by the lack of p2CPY after 5 min, whereas sac1ts/
slj2Δ cells processed CPY normally (Figure 9A). After 30 min
of chase, >90% of the labeled CPY remained in a ER-modi-
ified p1 form in sac1ts/sjl2Δ/sjl3Δ cells, whereas sac1ts/sjl3Δ
accumulated a hypoglycosylated form of mature CPY.

The integrity of the secretory pathway was also assessed in
sac1ts and sac1ts/sjl2Δ/sjl3Δ mutant cells by monitoring
the secretion of invertase. At the permissive temperature,
invertase was normally processed and secreted in both
strains (Foti, Audhya, and Emr, unpublished results). Strik-
ingly, sac1ts/sjl2Δ/sjl3Δ mutant cells exhibited a significant
defect in the secretion of invertase at the nonpermissive
temperature. This defect was not due to the retention of
invertase in the ER as is the case for sec18ts mutant cells,
which accumulate invertase exclusively in the ER core-modi-
fied form. Instead, sac1ts/sjl2Δ/sjl3Δ mutant cells accumu-
lated a hypoglycosylated Golgi-modified form of invertase,
consistent with a defect in Golgi-dependent modification of
this protein (Figure 9B). In sac1ts/sjl3Δ mutant cells, inver-
tase was secreted, but similar glycosylation defects were
observed (Figure 9C).

In summary, these data indicate that, in the absence of
Sac1p activity, the SJl proteins become essential for normal
Golgi function. Protein transport and glycosylation in the
Golgi are impaired in the sac1ts/sjl2Δ/sjl3Δ triple mutant.

DISCUSSION

In this study, we generated a temperature-sensitive allele of the
SAC1 gene to characterize the primary function of the
Sac1 PI phosphatase. Our data demonstrate that Sac1p
primarily metabolizes PtdIns(4)P synthesized by the Stt4p
PtdIns 4-kinase and that membrane localization of Sac1p is
crucial for its efficient function. The rapid inactivation of Sac1p leads to a dramatic alteration in vacuole morphology and an accumulation of lipid droplets. Furthermore, loss of Sac1p function rescues defects associated with stt4ts but not pik1ts mutant cells. Additionally, function of Sjl3p, an Sjl phosphatase homologous to Sac1p, was found to be essential for viability in Sac1p-deficient cells. We suggest that Sjl3p partially compensates for the loss of Sac1p PtdIns(4)P phosphatase activity. Our results indicate that deficiency in both Sac1p and Sjl3p activities leads to Golgi dysfunction and cell death. Together our data support a model in which Stt4p and Sac1p control the synthesis and turnover of a pool of PtdIns(4)P spatially and functionally distinct from the pool of PtdIns(4)P generated by Pik1p (Figure 10). Inactivation of Sac1p induces an accumulation of PtdIns(4)P, which may rapidly traffic to more distal compartments in the secretory pathway like the Golgi and vacuole. To prevent improper signaling in sac1 mutant cells, PtdIns(4)P must be metabolized/inactivated by other phosphatases such as Sjl3p. Con-

Figure 9. The dramatic accumulation of PtdIns(4)P in sac1ts/sjl2Δ/sjl3Δ mutant cells alters Golgi-dependent glycosylation pathways. (A) The indicated strains were preincubated at 38°C for 10 min, metabolically labeled with an 35S-protein–labeling mixture during a 10-min pulse, and then chased in the presence of excess unlabeled methionine and cysteine for the indicated times. CPY was then immunoprecipitated and resolved by SDS-PAGE. The migration positions of precursor and mature forms of CPY are indicated on the

Figure 10. Stt4p- or Pik1p-dependent phosphorylation of PtdIns leads to the production of distinct pools of PtdIns(4)P. Sac1p specifically turns over a pool of PtdIns(4)P pool generated by Stt4p, whereas Sjl proteins are potential candidate phosphatases for Pik1p-generated PtdIns(4)P turnover in the Golgi. The proposed roles for the different PtdIns(4)P pools are indicated. The Stt4p/Sac1p-dependent PtdIns(4)P pool is required for vacuole morphology, actin cytoskeleton organization, and regulation of neutral lipids storage, whereas the Pik1p-dependent PtdIns(4)P pool is essential for secretory vesicle formation from the Golgi and maintenance of Golgi/endosome morphology.

Figure 9 (cont). left. The star indicates the migration position of underglycosylated mature CPY. (B) WT, sac1ts, and sac1ts/sjl2Δ/sjl3Δ mutant cells were preincubated at 38°C for 10 min, metabolically labeled with an 35S-protein–labeling mixture during a 10-min pulse, and then chased in the presence of excess unlabeled methionine and cysteine for 30 min. Cellular transport was stopped by the addition of NaN3 and NaF after the pulse-chase, and the cells were converted to spheroplasts. Internal and external fractions were separated by centrifugation and analyzed for the presence of invertase by immunoprecipitation. (C) sac1ts and sac1ts/sjl3Δ mutant cells were preincubated at 38°C for 10 min, metabolically labeled with an 35S-protein–labeling mixture during a 10-min pulse, and then chased in the presence of excess unlabeled methionine and cysteine for 30 min. Cells were then treated as in B. All data are representative of multiple experiments.
consistent with this hypothesis, deletion of SFL3 in sac1Δ cells results in a dramatic accumulation of PtdIns(4)P at the restrictive temperature, accompanied by defects in cell growth and Golgi function.

PtdIns(4)P Is the Primary Substrate for Sac1p
Analysis of PI levels in sac1Δ cells has led us to conclude that the main substrate of Sac1p in vivo is PtdIns(4)P. Two of the six mutations in the sac1Δ-23 allele lie within a conserved motif found in all Sac phosphatase domains. However, the highly conserved CX9R(T/S) motif remains intact. This is not surprising, because mutations in this motif result in steady-state defects in Sac1p function (reviewed by Hughes et al., 2000). Previous studies have clearly shown that Sac1p exhibits phosphatase activity toward all monophosphorylated PIs in vitro, especially PtdIns(3)P (Guo et al., 1999; Hughes et al., 2000). These observations support the idea that the in vivo specificity of Sac1p for PtdIns(4)P is related to the accessibility of its substrate. Our studies indicate that the majority of Sac1p localizes to the ER, where levels of PtdIns(3)P and PtdIns(3,5)P2 are likely to be very low. Indeed, PtdIns(3)P synthesis is essential for transport from the Golgi to the endosome/vacuole (Schu et al., 1993), suggesting that PtdIns(3)P is generated at the Golgi/endosome. PtdIns(3)P localization studies in yeast with the use of FYVE domain-GFP fusion proteins as a reporter also revealed a PtdIns(3)P localization studies in yeast with the use of FYVE domain-GFP fusion proteins as a reporter also revealed a distribution pattern for PtdIns(3)P reminiscent of the Golgi/endosome (Burd and Emr, 1998). One explanation for the accumulation of PtdIns(3)P or PtdIns(3,5)P2 in sac1Δ mutants is that the high amount of PtdIns(4)P in these cells can compete as a substrate for PtdIns(3)P- or PtdIns(3,5)P2-specific phosphatases, thereby altering the normal turnover of these lipids.

The discrepancy between our data showing the localization of Sac1p to the ER and previous results from Whitters et al. (1993) reporting a localization of Sac1p to both the Golgi and the ER is still unresolved. Our data are consistent with the exclusive localization of the rat homologue of Sac1p to the ER in different types of mammalian cells, but we cannot rule out the existence of a small pool of Sac1p in the yeast Golgi (Nemoto et al., 2000). If a small Golgi pool does exist, it may rapidly recycle back to the ER.

Localization of tagged Sac1p lacking the last 102 amino acids (Sac1Δ522-623) revealed a striking loss of ER association in contrast to the full-length protein, demonstrating that the C-terminal transmembrane domain of Sac1p is required for its ER localization. Mislocalization of truncated Sac1Δ522-623 resulted in alterations in the metabolism of PIs and a growth phenotype similar to that observed in sac1Δ cells, suggesting that localization of Sac1p to ER membranes is crucial for its efficient activity. However, when overexpressed, the Sac1Δ522-623 fusion protein is capable of complementing defects exhibited by sac1Δ mutant cells. Thus, the Sac1Δ522-623 truncation protein possesses phosphatase activity in vivo, which is not unexpected because its Sac phosphatase domain remains intact, but its inappropriate localization likely prevents accessibility to a pool of PtdIns(4)P that Sac1p normally regulates.

Role of Sac1p in the Control of Vacuole Morphology and Neutral Lipid Storage
We previously demonstrated that stt4Δ and pkl1Δ mutants cells both exhibit distinct, but dramatic, alterations in vacuole morphology, suggesting that PtdIns(4)P plays a crucial role in the appearance and/or function of this organelle (Audhya et al., 2000). The rapid inactivation of Sac1p in sac1Δ mutant cells is also accompanied by dramatic changes in vacuole shape. Thus, strict control of distinct pools of PtdIns(4)P appears to be crucial to maintain normal morphology and function of the vacuole. We also observed an accumulation of lipid droplets in sac1Δ cells, which often appeared to cluster around the vacuole and associate with vacuolar invaginations. These lipid droplets, consisting of a hydrophobic core of neutral lipids (steryl esters and triglycerides) surrounded by a phospholipid monolayer, are thought to be derived from ER membranes. It has been suggested that formation of these lipid droplets could provide a transport route of sterol esters to the plasma mem-

Sac1p Acts on a Pool of PtdIns(4)P Synthesized by the Stt4p Kinase
Our analysis of PI levels in stt4Δ and pkl1Δ mutants carrying the sac1Δ allele demonstrates that Stt4p, but not Pkl1p, generates the pool of PtdIns(4)P that is metabolized by Sac1p. Previously, Pkl1p was thought to function as the kinase that produces high levels of PtdIns(4)P in sac1Δ mutant cells (Hama et al., 1999; Rivas et al., 1999). Indeed, Pkl1p activity has been shown to be crucial for protein secretion out of the Golgi (Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000) and overexpression of Pkl1p can weakly suppress the growth defect exhibited by the sec14Δ secretory mutant cells at semirestrictive temperature (Hama et al., 1999). Because mutations in the SAC1 gene can bypass the requirement for Sec14p (Cleves et al., 1989; Stock et al., 1999), it was assumed that stabilization of PtdIns(4)P generated by Pkl1p in the Golgi could suppress secretory defects associated with sec14Δ mutant cells (Hughes et al., 2000; Huijbregts et al., 2000). In contrast to these assumptions, our data establish a functional link between Sac1p and Stt4p, both biochemically and genetically. Strikingly, loss of Sac1p function partially rescues the actin cytoskeleton defect exhibited by stt4Δ cells at the nonpermissive temperature. Consistent with this finding, Sac1p was initially identified in a screen for "suppressor of actin" mutants (Novick et al., 1989), but a mechanism for this suppression has been elusive. Together with our data, we suggest that loss of Sac1p function may rescue specific mutant alleles of act1 by affecting levels of PtdIns(4)P generated by Stt4 PtdIns 4-kinase.

Although, the intracellular location of Stt4p is unknown, our results would predict that Stt4p could localize, at least in part, to Sac1p-containing membranes like the ER. Consistent with this, Stt4p has been shown to play a role in the trafficking of aminophospholipids from the ER to the Golgi/vacuole (Trotter et al., 1998), and PtdIns 4-kinase α, the mammalian homologue of Stt4p, is associated with ER membranes (Wong et al., 1999). However, it is possible that PtdIns(4)P generated by Stt4p may be produced elsewhere and be rapidly transported to Sac1p-containing membranes for turnover. Further studies are underway to determine the localization of Stt4p.
brane, and it is feasible that Sac1p plays a role in this process (reviewed by Zweytick et al., 2000).

**Role of PI Phosphatases containing a Sac1-like Domain in Golgi Function**

Elimination of Sjl3p activity, in contrast to Sjl2p activity, in sacΔ cells results in lethality. Analysis of PI metabolism in sacΔ/sjl3Δ suggests that this growth defect is likely due to the additional increase in PtdIns(4)P levels in the double mutant as compared with sacΔ single mutant cells. The double mutant also exhibits defects in secretion and Golgi-specific glycosylation. Consistent with the observed defect in protein glycosylation, Sjl3p has been shown to play a role in trafficking from the Golgi (Luo and Chang, 1997; Bensen et al., 2000) and thus may localize to the Golgi where it can metabolize PtdIns(4)P. Recent studies of Sjl2p and Sjl3p show the proteins to be diffusely distributed throughout the cell under normal conditions, leaving open the possibility that these phosphatases regulate PI pools at the Golgi (Ooms et al., 2000). Triple sacΔ/sjl2Δ/sjl3Δ mutant cells exhibited additional defects in Golgi glycosylation when compared with sacΔ/sjl3Δ double mutant cells. Thus, even though deletion of Sjl2 in sacΔ cells has no observable effect on PI metabolism as compared with sacΔ cells alone, expression of the Sjl2 phosphatase became essential in the sacΔ/sjl3Δ background at elevated temperatures. Additionally, we observed an increase in PtdIns(3,5)P2 levels at the restrictive temperature in sacΔ/sjl2Δ/sjl3Δ triple mutant cells. Although the defect in invertase secretion observed in these cells correlates with the increase in PtdIns(3,5)P2, we do not favor a role for this isomer in the alteration of Golgi secretory function. Indeed, several lines of evidence argue against this possibility. First, synthesis of PtdIns(3)P and PtdIns(3,5)P2 have been shown to be involved in the regulation of Golgi to vacuole-trafficking events but not in Golgi glycosylation or secretion (Schu et al., 1993; Odorizzi et al., 1998). Second, Fab1p, the only known yeast PtdIns(3)P 5-kinase, has been localized to prevacuolar and vacuolar compartments (Gary et al., 1998). Third, the bulk of PtdIns(3)P in yeast was shown to be localized to endosomes, multivesicular bodies, and vacuoles (Stenmark et al., 1996; Gillooly et al., 2000), and finally, sacΔ/sjl2Δ/sjl3Δ cells do not display defects in secretion of other secretory cargo such as Hsp150p. Therefore, it is likely that the dramatic increase of PtdIns(4)P levels subsequent to Sac1p inactivation in sacΔ/sjl2Δ/sjl3Δ cells causes the observed Golgi glycosylation defect, perhaps by altering the localization/trafficking of Golgi-specific glycosyltransferases. These results suggest that Stt4p-generated PtdIns(4)P that escapes degradation by Sac1p activity is, at least partially, turned over by Sjl proteins, presumably in the Golgi. Moreover, when sac1 mutant cells also lack the phosphatase activities of Sjl2p and Sjl3p, increased PtdIns(4)P levels negatively impact on normal Golgi function, and this results in a loss in cell viability. However, under normal conditions, Sjl3p does not turnover Stt4p-generated PtdIns(4)P because of the efficient activity of Sac1p. Consistent with this, deletion of SJL3 in stt4Δ cells fails to stabilize PtdIns(4)P produced by this lipid kinase and fails to rescue any of the phenotypes associated with stt4Δ cells (Foti, Audhya, and Emr, unpublished results).

The importance of tight regulation of both PI synthesis and turnover by PI kinases and phosphatases for proper signaling is clear. However, very little is known about the downstream effectors for PtdIns(4)P. The identification of downstream effectors of PtdIns(4)P as well as additional genetic and biochemical analysis of enzymes involved in the metabolism of PtdIns(4)P should help to elucidate the role of this particular isomer and its derivatives in protein and lipid trafficking, as well as in membrane dynamics and cytoskeletal organization.

**ACKNOWLEDGMENTS**

We thank Chris Hofeditz and Tammie McQuistan for assisting with the electron microscopy analysis, Immunelectron microscopy Core B of Program Project grant CA58689 headed by M. Farquhar. We would also like to thank Patricie Burda, Chris Stefan, and Andrew Wurmsner for critical reading of the manuscript and Perla Arcaira for technical assistance. We thank Jonathan Gary for generating the yeast strains JGY130, JGY131, and JGY132 and Greg Odorizzi for providing pGO106. This work was supported by a grant from the National Institutes of Health (CA58689 to S.D.E.). S.D.E. is supported as an investigator of the Howard Hughes Medical Institute. M.F. is supported by the Swiss National Funds for the Scientific Research (fellowship 823A-053440).

**REFERENCES**


Seron, K., Tieaho, V., Prescianotto-Baschong, C., Aust, T., Blondel, M., Guillaud, P., Devilliers, G., Rossanese, O.W., Glick, B.S., Riez-