SAC1 Encodes a Regulated Lipid Phosphoinositide Phosphatase, Defects in Which Can Be Suppressed by the Homologous Inp52p and Inp53p Phosphatases*

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The yeast protein Sac1p is involved in a range of cellular functions, including inositol metabolism, actin cytoskeletal organization, endoplasmic reticulum ATP transport, phosphatidylinositol-phosphatidylcholine transfer protein function, and multiple-drug sensitivity. The activity of Sac1p and its relationship to these phenotypes are unresolved. We show here that the regulation of lipid phosphoinositides in sac1 mutants is defective, resulting in altered levels of all lipid phosphoinositides, particularly phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. We have identified two proteins with homology to Sac1p that can suppress drug sensitivity and also restore the levels of the phosphoinositides in sac1 mutants. Overexpression of truncated forms of these suppressor genes confirmed that suppression was due to phosphoinositide phosphatase activity within these proteins. We have now demonstrated this activity for Sac1p and have characterized its specificity. The in vitro phosphatase activity and specificity of Sac1p were not altered by some mutations. Indeed, in vitro mutant Sac1p phosphatase activity also appeared unchanged under conditions in which cells were drug-resistant. However, under different growth conditions, both drug sensitivity and the phosphatase defect were manifest. It is concluded that SAC1 encodes a novel lipid phosphoinositide phosphatase in which specific mutations can cause the sac1 phenotypes by altering the in vivo regulation of the protein rather than by destroying phosphatase activity.

The Saccharomyces cerevisiae gene SAC1 was originally isolated as a mutant allele able to suppress the phenotypes seen in strains containing the temperature-sensitive act1-1 actin allele (1). The gene was subsequently cloned when SAC1 was also identified as a mutant suppressor of mutations in the yeast phosphatidylinositol-phosphatidylcholine transfer protein, sec14-1 (2). In addition to the ability to suppress the effects of act1-1 and sec14-1 mutations, a variety of perplexing additional phenotypes have been attributed to yeast strains containing an assortment of mutations in the SAC1 gene (sac1-1 to sac1-29, mds1-sac1) (1–3). These include disruption to the localization of the actin cytoskeleton and chitin deposition, reminiscent of the act1-1 phenotype (1). Some, but not all, mutant sac1 strains also show inositol auxotrophy (3, 4); however, there are no apparent defects in de novo inositol biosynthesis or in utilization of inositol for phosphatidylinositol biosynthesis (4). Some, but not all, mutant sac1 strains also display multiple-drug sensitivity characterized by supersensitivity to a wide variety of drugs and detergents, including breafeldin A and novobiocin (3). Mutants also show secretory defects and deficiencies in ATP transport activity in the endoplasmic reticulum as well as an inability to translocate nascent prepro-a-mating factor and preprocarboxypeptidase Y (5, 6). In addition to these phenotypes, sac1 mutants also show cold sensitivity (1, 2) and synthetic lethality when in combination with the trp1 allele (3). Despite this considerable body of work, a function for Sac1p that relates to phenotypic changes has remained to be described.

New insight as to the function of Sac1p became possible during the cloning of the presynaptic inositol-5-phosphatase synaptotagmin-1 (7) when a region of considerable homology between this protein and Sac1p was identified. The synaptotagmin-1s are phosphatidylinositol-polyphosphate 5-phosphatases that remove phosphate from the d-5-hydroxyl position of phosphatidylinositol phosphates. They are members of a large group of 5-phosphatases (reviewed in Ref. 8) that also include the type I and II 5-phosphatases. All 5-phosphatases contain a 5-phosphatase domain; the type II 5-phosphatases additionally contain a type II domain; and the synaptotagmin-like proteins, in addition to the type II domain, contain a region of homology to Sac1p, the Sac domain (see Fig. 1). Synaptotagmin-1 represents one member of a family of Sac domain-containing inositol phosphatases (8) that also include the yeast proteins Inp51p, Inp52p, and Inp53p (also called Sj11p Sj12p, and Sj13p) (9, 10).

Despite the fact that Sac1p shows no homology to the domains in these proteins shown to catalyze phosphatase activity, we (3) and others (11, 12) had speculated that phosphatidylinositol (PtdIns) and its phosphorylated derivatives, the lipid phosphoinositides, could represent a medium by which Sac1p could administer its pleiotropic effects on actin function, secretion, inositol metabolism, and drug sensitivity. We describe here the direct demonstration that a function of Sac1p confers such control, albeit in a regulated manner.

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1 The abbreviations used are: PtdIns, phosphatidylinositol; GST, glutathione S-transferase; HPLC, high pressure liquid chromatography; GroPIns, glycerophosphoinositol; ER, endoplasmic reticulum.
Characterization of Monophosphorylated Phosphatidylinositol— Mutant sac1-deleted cells were labeled for lipid extraction, deacylation, and separation with [14C]inositol as described above. Fractions containing the monophosphorylated head groups were pooled and neutralized with triethylamine, diluted 10-fold in water before desalting (19). The GroIns3/P and GroPInsP standards were purified from 1H- radiolabeled yeast (as described). The GroIns5/P standards were generated from GroIns5/P isolated from 1H-radiolabeled yeast (18). The HPLC analyses were performed on a Partisil-5 μm strong-anion exchange column (250 × 4 mm) with a flow rate of 1 ml/min using the following gradient separation: 0 min, 0% solvent B; 5 min, 0% solvent B; 15 min, 2% solvent B; and 100 min, 2% solvent B (solvent A = H2O and solvent B = 1.25× (NH4)2HPO4, pH 3.8).

Phosphatase Assays—The phosphatase activity of proteins was determined using colorimetric or radioactive assays. Activity for yeast 5-phosphatase (Inp52p) was determined by incubation for 5 min at 37°C in the presence of 0.125 μM lipid phosphoinositide, 0.25% (w/v) octyl glucoside, 4 mM MgCl2, and 0.1 μM Tris (pH 7.4). The released phosphatase was then assessed colorimetrically as described before (20). Inp52p, 50 μl assay was stopped by adding 25 μl of malachite green/molybdate reagent, followed by 50 μl of water. The reaction was allowed to take place for 30 min before measurements were taken at 601 nm in an enzyme-linked immunosorbent assay reader (SpectraMax Plus, Molecular Devices). Basal phosphatase contents were determined by adding the enzyme after termination of the reaction with the malachite green/molybdate reagent.

The phosphatase activity of the Sac1p phosphatases was assessed in the presence of 10 μM lipid phosphoinositide, 0.25% (w/v) octyl glucoside, 2 mM MgCl2, and 100 μM Tris (pH 7.4) using radioactively labeled substrate that was prepared as described below. Phosphatidylinositol monophosphates were labeled with [γ-32P]ATP using PtdIns3s-3- and 4-kinases with PtdIns as a substrate (21). PtdIns5s/P was incubated in the presence of a 4-fold excess of phos- phatidylinositolphosphate with phosphatidylserine-3 kinase to produce PtdIns3s,5P2. The 32P-labeled lipids were separated by TLC (16), purified, and supplemented with unlabeled lipids (Echelon) before assay (21). The assay (50 μl) was stopped by adding 0.4 ml of methanol/CHCl3 (1:1), followed by 0.2 ml of 0.1 N HCl and 10 μl of Folch bovine brain extracts (Sigma) as carrier lipid (17). After phase separation, both phases were dried down under vacuum. The water phase was directly counted (scintillation), whereas the CHCl3 phase was subjected to TLC (17). After development, PtdIns3sP and PtdIns5s/P spots were scrapped and counted in scintillator. In both types of assay, phosphoinositides (Echelon) and phosphatidylinositol (Sigma) were first repurified by chromatography according to the water phase (Echelon) and subsequently quantified by ashing the lipids, followed by a colorimetric phosphate assay (25).

RESULTS

Inp52p and Inp53p Suppress sac1 Mutant Phenotypes—We had previously demonstrated that the mds1-sac1 allele of the gene SAC1 and some other sac1 mutants display multiple-drug sensitivity, a phenotype characterized by sensitivity to drugs such as novobiocin and brefeldin A (3). Using high- and low-copy wild-type yeast genomic libraries, we had screened for sequences able to suppress sac1 multiple-drug sensitivity and had identified a variety of sequences able to complement the mutant (3). However, the analysis was not comprehensive, and we had speculated that other open reading frames may be able to complement sac1 mutant phenotypes. Principally, we were interested in testing the genes that had been cloned as encoding proteins with homology to Sac1p, namely the S. cerevisiae genes INP51, INP52, INP53, and FIG4, and, in addition, human synaptotagmin-1. To examine the ability of these genes to complement sac1 mutant phenotypes, we transformed the genes, cloned into an inducible expression vector, into wild-type and sac1-deleted strains. Transformed strains were then tested for growth on plates containing a variety of drugs and

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media—The S. cerevisiae strain used in this study is FY833 (MATa his3Δ200 ura3-52 leu2Δ1 lys2Δ2 2013 rps15Δ GAL2) (13). The entire open reading frame of the gene SAC1 was deleted from FY833 strains by polymerase chain reaction as described previously (3). The bacteria used were Escherichia coli Top10 (Invitrogen). The genes INP51, INP52, INP53, and FIG4 were polymerase chain reaction-amplified from S. cerevisiae genomic DNA (Promega) using oligomers designed to amplify the open reading frames of these genes with restriction enzyme sites engineered at each end: INP51, GGATCCCATGGAACATTCCTCAGG (BanHI) and GTGGAACATTCTTTAACATGTTGG (SalI); INP52, GGATCCATGGAACATTCCTCAGG (BanHI) and GTGGAACATTCTTTAACATGTTGG (SalI); INP53, GGATCCATGGAACATTCCTCAGG (BanHI) and GTGGAACATTCTTTAACATGTTGG (SalI); and SAC1, GGATCCCATGGAACATTCCTCAGG (BanHI) and CCCCTGACATTTAATCTTTAAGG (SalI). Synaptotagmin-1 was polymerase chain reaction-amplified from a construct cloned by R. Woscholski using the oligomers GGATCCCATGGAACATTCCTCAGG (BanHI) and GTGGAACATTCTTTAACATGTTGG (SalI). Similarly, the Sac domain constructs (Sac1p, positions 141–531; Fig4p, positions 165–577; and synaptojanin, 56–445; Inp51p, positions 150–496; Inp52p, positions 166–556; and Inp53p, positions 166–531) were generated by polymerase chain reaction from cloned DNA: INP51, GGATCCCATGGAACATTCCTCAGG (BanHI) and GTGGAACATTCTTTAACATGTTGG (SalI) (17). These amplified DNAs were then blunt-cloned into the other construct to produce pGEX constructs were cut with BamHI and SalI fragments containing wild-type and Saclp deleted strains. Transformed strains were then tested for growth on plates containing a variety of drugs and
inhibition of growth (2 mg/ml, or kanamycin, (100 µg/ml) with or without a restrictive concentration of drugs (novobiocin, 1.8 mg/ml, or kanamycin, 100 µg/ml). Mutant strains complemented with SAC1, INP52, or INP53 showed wild-type drug resistance, whereas INP51, FY44, human synaptojanin-1 (SYN 1), and mutant mds1-sac1 (data not shown) continued to display drug sensitivity.

Inhibitors under conditions that would suppress (glucose) or promote (galactose) induction of the plasmid-borne gene. Expression of the genes in wild-type yeast appeared to have no effects on growth (data not shown), whereas expression of the genes in the sac1 mutant showed significant differences. Yeast sac1 mutants grew slightly slower than wild-type cells; however, mutants transformed with wild-type SAC1, INP52, or INP53 and grown on galactose grew at wild-type rates (Fig. 1). When grown on medium containing inhibitory concentrations of drugs, the effect was more pronounced. Yeast sac1 disruptants were drug-sensitive and did not grow; however, mutants transformed with wild-type SAC1, INP52, or INP53 and incubated on medium containing galactose grew at wild-type rates (Fig. 1). Therefore, the effects of sac1 mutations can be suppressed by some product contributed by overexpressed Inp52p and Inp53p phosphatases.

**Altered Lipid Phosphoinositide Levels of sac1-deleted S. cerevisiae**—The demonstration that sac1 phenotypes could be suppressed by phosphatidylinositol-phosphate 5-phosphatases strengthened our hypothesis that the lipid phosphoinositides may represent a part of the mechanism by which Sac1p is able to mediate its pleiotropic effects (3). Other observations such as the effects of Sac1p on actin function (1), which can be regulated by PtdIns(4,5)P2, the effects of Sac1p on Sec14p function, which regulates the ratio of PtdIns and phosphatidylcholine (24); and the fact that some SAC1 mutants show inositol auxotrophy (3) also suggested that the lipid phosphoinositides may be important for Sac1p function. Therefore, we sought to measure the levels of these lipids in wild-type and mutant sac1 strains. Wild-type and yeast strains containing a completely deleted SAC1 gene were incubated with [3H]inositol before total cell phospholipids were extracted and deacylated to allow separation of the resulting head groups by HPLC. The data in Fig. 2A indicate that cells lacking the SAC1 gene were characterized by reduced levels of GroPIns(4,5)P2 and an increase in GroPIns(3,5)P2. Most notably, the sac1-deleted strains showed a dramatic increase in a glycerophosphoinositide eluting at a time consistent with GroPIns(4)P. However, using the HPLC protocol described, the lipid deacylation products GroPIns(4)P and GroPIns(5)P cannot be efficiently separated (25). Having shown that Inp52p and Inp53p, both putative 5-phosphatases, could suppress the effects of sac1 mutants that appear to be characterized by a dramatic increase in a d-4-phosphorylated lipid, it remained a possibility that the increase in PtdIns(4)P was in fact a peak of PtdIns(5)P.

To establish the precise identity of the monophosphorylated glycerolipid, seen at the GroPIns(4)P position, we labeled cells containing a completely deleted sac1 gene with [14C]inositol. As before, total cell phospholipids were then extracted and deacylated to allow separation of the glycerolipids by HPLC. Fractions containing the elevated levels of head group corresponding to the phosphatidylinositol monophosphate were collected and desalted. This purified [14C]inositol compound was then mixed with [3H]-labeled GroPIns(3)P, GroPIns(4)P, and GroPIns(5)P standards (26). The order of elution of the individual GroPInsPs had been previously confirmed using [14C]GroPIns(3)P and [3H]GroPIns(5)P standards and [3H]-labeled GroPIns(3)P, GroPIns(4)P, and Gro-P Ins(5)P standards (26). The data from HPLC analysis of [3H]inositol-labeled lipids from wild-type FY833 and sac1-deleted FY833 (corrected to A1 and) grown in YPD/glucose medium (cpm [3H] with error and % total lipid) showing a 7-fold increase in PtdIns(3,5)P2, a 1.5-fold increase in PtdIns(3)P, a 5-fold decrease in PtdIns(4,5)P2, and 8-fold increase in the putative PtdIns(4)P lipid. B, HPLC analysis of [14C]inositol-labeled FY833, sac1ΔURA3 lipids extracted from fractions containing the elevated peak of GroPIns(4)P or GroPIns(5)P (●) and comparison with authentic [3H]-labeled GroPIns(3)P, GroPIns(4)P, and Gro-P Ins(5)P standards (○). Therefore, the dramatic increase in monophosphorylated lipid is PtdIns(4)P.
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FIG. 3. SAC1 and INP52 restore PtdIns(3)P, PtdIns(4)P, PtdIns(3,5)P2, and PtdIns(4,5)P2 levels. Shown are data from HPLC analysis of [3H]inositol-labeled lipids from wild-type FY833, deletion mutant FY833::sac1::URA3, and the deletion mutant complemented with pRS315.SAC1 and pYX243.INP52. When grown in YPD/glucose medium, pYX243-borne SAC1 or INP52 had no effect on the disrupted lipid distribution (data not shown). Strains were grown in YPD/galactose medium to induce expression of protein. Sac1p and Inp52p were able to reduce the levels of PtdIns(3)P, PtdIns(4)P, PtdIns(3,5)P2, and to raise the level of PtdIns(4,5)P2, to near wild-type levels. Total lipid cpm were as follows: FY833, 111,438; deletion mutant FY833, sac1::URA3, 264,190; FY833::sac1::URA3+pRS315.SAC1, 908,359; and FY833::sac1::URA3-pYX243.INP52, 1,015,091.

TABLE I

Some Sac domains partially suppress sac1 deletion strain novobiocin sensitivity

| Medium | Sac1p | Mds1p | Fig4p | Inp51p | Inp52p | Inp53p | Synaptopojanin-1 |
|--------|-------|-------|-------|--------|--------|--------|-----------------|
| Glc    | ++    | ++    | ++    | ++     | ++     | ++     | ++              |
| Gal    | ++    | ++    | ++    | ++     | ++     | ++     | ++              |
| Glc + Nov** | -     | -     | -     | -      | -      | -      | -               |
| Gal + Nov | +     | -     | -     | -      | -      | -      | -               |
| Glc + Kan | -     | -     | -     | -      | -      | -      | -               |
| Gal + Kan | +     | -     | -     | -      | -      | -      | -               |

** Nov, novobiocin; Kan, kanamycin.

arating the species by HPLC, confirming that the lipid species proposed to be PtdIns(4)P was indeed PtdIns(4)P (Fig. 2B). This confirmed that the 10-fold increase in lipids seen in sac1 mutants was indeed an increase in PtdIns(4)P.

We have consistently seen a −7-fold increase in PtdIns(3,5)P2, a 1.5-fold increase in PtdIns(3)P, a 5-fold decrease in PtdIns(4,5)P2, and a 10-fold increase in PtdIns(4)P. Recently, similar analyses of lipid phosphoinositide levels in sac1 mutants also indicated that levels of PtdIns(4)P increase, by −6−10-fold (11, 12). However, although there is agreement with respect to the elevation of PtdIns(4)P, PtdIns(3)P, and PtdIns(3,5)P2, we show here a 5-fold decrease in PtdIns(4,5)P2 not reported before. Whether this distinction is due to strain differences or labeling protocols remains to be resolved; however, we have observed the decrease in PtdIns(4,5)P2 levels in sac1 deletions in two different strain backgrounds (FY333 and YPH499; data not shown).

Restored Lipid Phosphoinositide Levels of Complemented sac1-deleted S. cerevisiae—Having shown that yeast sac1 mutants have dramatically altered levels of lipid phosphoinositides, yeast mutants transfected with vectors containing SAC1 and INP52 were grown in galactose to induce production of Sac1p and Inp52p and were labeled with [3H]inositol to test the lipid phosphoinositide profiles of these yeast mutants. As shown in Fig. 3, in galactose, sac1 mutants continued to show defects in the levels of all of the lipid phosphoinositides compared with wild-type yeast. Transformation of the yeast mutants with a vector containing the wild-type SAC1 gene restored the level of lipids to almost wild-type levels. Similarly, the gene INP52 conferred a restoration of lipid levels to the mutant on induction, a feature not seen when grown on glucose (data not shown).

The Sac Domain Is Sufficient to Restore Partial Drug Resistance—Although the restoration of the level of lipid phosphoinositides by Inp52p and Inp53p could have been due to the carboxyl-terminal 5-phosphatase domain, we speculated that suppression was in fact due to the only region of homology between these proteins, the amino-terminal Sac domain (Fig. 1). To examine this, the Sac domains from Sac1p, Inp52p, and Inp53p had a slight growth advantage (+++) over those without (+) when grown on galactose. These strains also showed growth (+) on drug-containing gene induction plates, whereas all noninduced (glucose) and INP51, FIG4, synaptopojanin-1, and mutant mds1-sac1 Sac domain-containing strains showed no growth (−).
phatase—Recently, it has been demonstrated that the Sac domains of Inp52p and Inp53p, but not that of Inp55p, contain phosphatase activity (11). The differential effect of the Sac domains on the rescue of Sac1p-depleted cells questioned whether the properties ascribed to Inp52p and Inp53p Sac domains were also retained by Sac1p. This issue is emphasized by the finding that Fig4p was unable to rescue despite being the closest Sac1p-related sequence (data not shown). To determine if the Sac domain of Sac1p displayed phosphatase activity, we cloned SAC1 and a construct consisting of only the Sac domain from Sac1p into GST-tagged vectors for expression in yeast. Using a variety of radiolabeled lipid phosphoinositides, we determined that Sac1p has phosphatidylinositide phosphatase activity. GST-tagged Sac1p was expressed and purified from yeast and tested for phosphatase activity against 32P-labeled PtdIns(3)P, PtdIns(4)P, PtdIns(3,5)P2, and PtdIns(4,5)P2. The protein showed activity principally against monophosphorylated phosphatidylinositides, and no activity could be detected for PtdIns(4,5)P2 (data not shown).

**FIG. 4.** Sac1p has phosphatidylinositide phosphatase activity. GST-tagged Sac1p was expressed and purified from yeast and tested for phosphatase activity against 32P-labeled PtdIns(3)P, PtdIns(4)P, PtdIns(3,5)P2, and PtdIns(4,5)P2. The protein showed activity principally against monophosphorylated phosphatidylinositides, and no activity could be detected for PtdIns(4,5)P2 (data not shown).

**FIG. 5.** Mutations at Leu-246 within the Sac domain confer drug sensitivity. A, sequences containing the Sac domain were compared using Clustal X (42) and were then optimized by visual inspection. The Sac domain (~350 amino acids long) contains several regions (data not shown) of particular identity, of which two containing the mds1-sac1 mutations are shown. All the full-length sequences contained within the DNA sequence databases have been included except splice variants of the same gene, many of which have not been characterized. Amino acids showing 100, >67, and >50% identity are indicated (asterisks, colons, and periods, respectively) and conserved (>50%) hydrophilic, polar, basic, and aromatic amino acids are also shaded. S.c., S. cerevisiae; S.p., Schizosaccharomyces pombe; H.s., Homo sapiens; B.t., Bos taurus; R.n., Rattus norvegicus; C.e., Caenorhabditis elegans; A.it., Arabidopsis italiana. Abbreviated GenBank accession numbers are as follows: 22599, AL022599; 22103, AL022103; Q9256, Q9256.5; Q9652, Q9652; Z8320, Z8320; Z8046, Z8046; Z81072, Z81072; Z81072; 1.8 mg/ml) or inositol (ino). Growth (+ + +) occurred on novobiocin only when cells were transformed with vectors containing wild-type SAC1 or mutant sac1-F97L. Cells transformed with vector containing the sac1-L246P or mds1-sac1 construct did not grow on novobiocin, indicating that the L246P mutation is responsible for the phenotype. Inositol auxotrophy, not a phenotype of mds1-sac1 alleles, was suppressed by all versions of SAC1.

**Mutation of Sac1p at Leu-246 Confers Drug Sensitivity—**We have previously isolated the mds1-sac1 allele consisting of two mutations within the SAC1 gene, F97L and L246P (3). Both of the mds1 mutations occur within the highly conserved Sac domain motifs (Fig. 5A), although these mutations do not occur within the proposed catalytic RXNXCXDLRTN motif (11). It appeared likely that one of the two mds1 mutations may be sufficient to produce the mds1 phenotypes by destroying Sac1p phosphatase activity. To investigate this possibility, we transformed wild-type and mutant Sac1p constructs with only one of the mds1 mutations, F97L or L246P, into Sac1p-deleted strains, and we examined the ability of these strains to grow on medium containing inhibitory concentrations of various drugs with or without inositol. Some sac1 alleles confer inositol auxotrophy upon mutant strains (3, 4); indeed, complete deletion of sac1 confers inositol auxotrophy on strains, whereas the mds1-sac1 allele has been shown to cause no defect in inositol metabolism (3). The data in Fig. 5B confirm these findings within the strain FY833. sac1ΔURA3 and show

**FIG. 5.** Mutations at Leu-246 within the Sac domain confer drug sensitivity. A, sequences containing the Sac domain were compared using Clustal X (42) and were then optimized by visual inspection. The Sac domain (~350 amino acids long) contains several regions (data not shown) of particular identity, of which two containing the mds1-sac1 mutations are shown. All the full-length sequences contained within the DNA sequence databases have been included except splice variants of the same gene, many of which have not been characterized. Amino acids showing 100, >67, and >50% identity are indicated (asterisks, colons, and periods, respectively) and conserved (>50%) hydrophilic, polar, basic, and aromatic amino acids are also shaded. S.c., S. cerevisiae; S.p., Schizosaccharomyces pombe; H.s., Homo sapiens; B.t., Bos taurus; R.n., Rattus norvegicus; C.e., Caenorhabditis elegans; A.it., Arabidopsis italiana. Abbreviated GenBank accession numbers are as follows: 22599, AL022599; 22103, AL022103; Q9256, Q9256.5; Q9652, Q9652; Z8320, Z8320; Z8046, Z8046; Z81072, Z81072; 1.8 mg/ml) or inositol (ino). Growth (+ + +) occurred on novobiocin only when cells were transformed with vectors containing wild-type SAC1 or mutant sac1-F97L. Cells transformed with vector containing the sac1-L246P or mds1-sac1 construct did not grow on novobiocin, indicating that the L246P mutation is responsible for the phenotype. Inositol auxotrophy, not a phenotype of mds1-sac1 alleles, was suppressed by all versions of SAC1.
that wild-type SAC1 and mutant sac1-F97L genes conferred wild-type drug resistance, whereas mutant sac1-L246P and mds1-sac1 genes did not restore drug resistance. Additionally, all the mutant alleles tested restored inositol prototrophy. Thus, the L246P mutation is likely to be solely responsible for the multiple-drug sensitivity seen in mds1 mutants.

The mds1-sac1 Mutations Do Not Destroy Phosphatase Activity—To establish whether the mutation responsible for drug sensitivity was also responsible for the changes in lipid levels, SAC1 containing either one or both of the mds1 mutations, F97L or L246P, was cloned into a GST-tagged vector for expression in yeast. These vectors were transformed into yeast, and proteins were extracted, purified, and tested for phosphatase activity as described above. As shown in Fig. 6, the in vitro activity of mutants Sac1p-F97L, Sac1p-L246P, and Sac1p-mds1 did not differ from that of wild-type Sac1p (see Fig. 4 for comparison). This was rather surprising since the inability to rescue the drug sensitivity suggested that mutation at Leu-246 would destroy, reduce, or at least alter specificity of the activity of the Sac1p phosphatase to produce the observed multiple-drug sensitivity.

The mds1-sac1 Mutations Confer Sac1p Phosphatase Regulatory Defects—To further assess the consequences of the mutations within Sac1p, [3H]inositol-labeled yeast cells containing a completely deleted SAC1 gene were transformed with wild-type and mutant sac1 clones. Cells were grown in YPD medium, and as described above, total cell phospholipids were then extracted and deacylated to allow separation by HPLC. The complemented mutant showed restored levels of lipid phosphoinositides (Fig. 7A) with both wild-type and mutant sac1 genes. However, we did observe that mutant cells containing the sac1 genes with the L246P mutation showed slightly less restoration of the level of lipid phosphoinositides (Fig. 7). We also [3H]inositol-labeled yeast mutants grown in SD medium and again extracted total phospholipids for examination by HPLC. Under these conditions, sac1-L246P and mds1-sac1 were unable to restore the levels of all of the lipid phosphoinositides to those seen with wild-type and sac1-F97L clones (Fig. 7, B and C). Indeed, although in minimal medium the level of PtdIns(4)P in the sac1 mutant increased only 2-fold (Fig. 7B), both Sac1p-L246P and double-mutant Sac1p were completely unable to reverse this increase. It appears that the phosphatase activity of the SAC1P-L246P mutants was only slightly decreased in mutants grown in YPD medium; however, when grown in SD medium, sac1 deletion strains transformed with these mutants showed an inability to restore the levels of the phosphoinositides.

DISCUSSION

The study here demonstrates that in Sac1p-depleted cells, the regulation of lipid phosphoinositides is defective and that this affects all phosphoinositides, including PtdIns(4,5)P2. The work also indicates that Sac domains from Inp52p and Inp53p exhibit phosphatase activity that can suppress drug sensitivity and phosphoinositide regulation defects in sac1 mutants. In
addition, we have demonstrated that Sac1p exhibits lipid phosphoinositide activity and that the enzyme will dephosphorylate monophosphorylated phosphatidylinositol and PtdIns(3,5)P₂, but not PtdIns(4,5)P₂. Most significantly, we have shown that mutations conferring sac1 phenotypes, which had been predicted to be a result of loss of phosphatase function, show an unchanged in vitro phosphatase activity and specificity. This appeared to be the case in vivo; however, we went on to identify conditions under which the mutant protein conferred phosphoinositide defects and drug sensitivity. We therefore conclude that the conserved motifs within the Sac domain mutated in Mds1p-Sac1p confer critical in vivo regulation of Sac1p activity.

As Sac1p appears to exhibit a similar phosphatase activity to Sac domains from Inp52p and Inp53p, it is perhaps not surprising that these proteins are able to suppress sac1 mutant phenotypes. The fact that Inp51p is unable to suppress sac1 defects is not unexpected. Although clearly containing a well-defined Sac domain, Inp51p contains a variety of amino acid substitutions (in the proposed catalytic RXNCDCLDRTN motif) that might be expected to destroy activity. The evidence from this work and that of Ref. 11 confirms this prediction, although this remains to be proven formally. Fig 4p, on the other hand, shows no significant mutations within the proposed catalytic motif or other motifs within the Sac domain (data not shown) and might therefore be expected to exhibit phosphatase activity. However, deletion of the gene does not cause significant defects in lipid phosphoinositide levels (11), perhaps indicating that the protein does not represent a major activity under these conditions. The protein is induced upon pheromone stimulation (26), and perhaps activity is regulated under these conditions much as demonstrated here for Sac1p. In contrast, the results obtained with synaptojanin were unexpected. The Sac domain of synaptojanin-1 probably shows phosphatase activity comparable to that of Inp52p and Inp53p (11), and we would have therefore expected synaptojanin to suppress sac1 defects. We have confirmed that the domain has phosphatase activity, which is significantly higher than that of Sac1p. The confirmation of expression of the protein and characterization of the phosphatase activity and the lipid phosphoinositide specificity of synaptojanin should resolve these differences.

Characterization of the phosphatase activity and the lipid phosphoinositide specificity of Sac1p has established that Sac1p displays phosphatidylinositol monophosphatase and some PtdIns(3,5)P₂ phosphatase activity. The protein clearly has an important role in the regulation of cellular lipid phosphoinositide levels as is seen by the 10-fold increase in the levels of PtdIns(4)P in (YPD medium). The substrate specificity of the protein would indicate that Sac1p is also important in regulating levels of PtdIns(3)P; however, as the levels of PtdIns(3)P increase only slightly on deletion of the gene, this perhaps indicates that other enzymes are also involved in the regulation of this lipid. Yeast cells also contain two homologues of the mammalian pTEN phosphatase (27, 28), which has been demonstrated to dephosphorylate PtdIns(3)P as well as PtdIns(3,4,5)P₃ (29). Although the specificities of these pTEN homologues remain to be established, it is clear that they encode proteins with phosphatase activity (28). Thus, Sac1p may not be exclusively involved in the regulation of β,γ-phosphorylated phosphoinositides.

Mutants also show significantly decreased levels of PtdIns(4,5)P₂, which was surprising, especially considering the elevated levels of PtdIns(4)P and as Sac1p would not seem to directly regulate PtdIns(4,5)P₂, having no detectable phosphatase activity for it. PtdIns(4)P 5-kinase activity, producing PtdIns(4,5)P₂, must therefore be either tightly regulated (30) or compartmentally isolated from the PtdIns(4)P increase in sac1 mutants. Sac1p is located in the endoplasmic reticulum (ER) and Golgi apparatus in yeast (4), whereas Mss4p (31), the PtdIns(4)P 5-kinase (32, 33), is proposed to be located on the plasma membrane (33). The reduction in the lipid seen could be due to the action of phosphatases (such as Inp52p) overexpressed to counteract the accumulation of PtdIns(4)P. This is perhaps confirmed by the observation that PtdIns(4,5)P₂ levels remain quite low in sac1 mutants complemented with Inp52p. The precise mechanisms by which these phosphatases regulate cellular lipid phosphoinositides remain to be established. However, these observations provide a model by which Sac1p is able to mediate its many effects.

Regulation of the actin cytoskeleton and thus cell wall chitin has been shown to occur via the effects of PtdIns(4,5)P₂ on some of the many actin-binding proteins such as profilin (34) and cofilin (35). The effects of SAC1 deletion on PtdIns(4,5)P₂ could well account for the actin defects seen in sac1 mutants (1–3). Sac1p mutants that confer reduced levels of PtdIns(4,5)P₂ show defects in actin cable polymerization and cortical actin patch localization, phenotypes also seen in mss4-depleted cells, which are severely compromised in their ability to synthesize PtdIns(4,5)P₂ (31). Guo et al. (11), who did not demonstrate significant changes in PtdIns(4,5)P₂ levels, proposed a novel role for PtdIns(3)P, PtdIns(4)P, or PtdIns(3,5)P₂ in regulating the actin cytoskeleton. This mechanism is unlikely to be via D-3-phosphorylated lipids, as deletion of VPS34, the only PtdIns 3-kinase in S. cerevisiae, affects vacuolar function (36) rather than actin function.

The gene encoding Sac1p was originally cloned after it was identified as a mutant suppressor of defects in Sec14p, the yeast Golgi phosphatidylinositol-phosphatidylcholine transport protein. The mechanism by which Sac1p inactivation bypasses the requirement for Sec14p function has been discussed previously (6, 12, 37), and it is proposed to involve diacylglycerol produced as a result of accumulation of PtdIns(4)P.

The effects of mutations in SAC1 on the lipid phosphoinositides may also mediate the effects seen on ER protein translocation and folding (5, 6). ATP transport into the ER is essential for the multiple reactions occurring within the ER lumen to enable proteins to cross the ER membrane and to initiate transport (38). Cells containing deleted sac1 genes have a greatly reduced ability to transport ATP into the ER lumen, whereas cells overexpressing Sac1p have enhanced ATP transport (5). These observations are not directly due to Sac1p, which does not resemble transporters of any kind and has been demonstrated to have no intrinsic ATP transport activity (6). ATP transporters in the plasma membrane have been shown to be regulated by PtdIns(4)P (39, 40), and it is possible that similar proteins in the ER could have reduced activity in the presence of elevated PtdIns(4)P levels. Additionally, PtdIns(4,5)P₂ has been shown to activate ER ATPases (41); the reduction in PtdIns(4,5)P₂ levels seen in sac1 mutants could account for the phenotypes seen.

The mechanisms of inositol auxotrophy and drug sensitivity seen in some sac1 mutants are perhaps more difficult to explain. The deletion mutants tested in this work and those in other studies (11, 12) are inositol-auxotrophic and show changes in lipid phosphoinositides and drug sensitivity. The mds1-sac1 mutant can display drug sensitivity, but is not an inositol auxotroph. The sac1-22 mutant is an inositol auxotroph and drug-resistant (3) and only suppresses sec14-1 mutants when grown on inositol (36). Whether the phenotypes are in

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anyway linked or how Sac1p phosphatase defects conferring changes in phosphoinositides cause these phenotypes is not clear. It is evident, however, that mds1-sac1 mutations within highly conserved motifs of the Sac domain, presumed to be intrinsic for phosphatase activity, actually alter regulation of the enzyme activity. Thus, the protein must interact with other regulatory growth condition-dependent factors to mediate phosphatase activity. It is possible that these interactions may have implications for the regulation of inositol metabolism and for cellular drug resistance.

*S. cerevisiae* probably contains only four significant phosphorylated lipid phosphoinositides: PtdIns(3)P, PtdIns(3,5)P$_2$, PtdIns(4)P, and PtdIns(4,5)P$_2$. No PtdIns(3,4,5)P$_3$ has been detected in S. cerevisiae (16), this is likely to be insignificant. Yet there appear to be many lipid phosphoinositide phosphatases with a variety of specificities. Having defined Sac1p as a regulated lipid phosphoinositide phosphatase, the identification of the factors controlling its activity and in what cellular location is the aim of future work.

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