Retention of the yeast Sac1p phosphatase in the endoplasmic reticulum causes distinct changes in cellular phosphoinositide levels and stimulates microsomal ATP transport*

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Running title: Regulation of microsomal phosphoinositides by Sac1p

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The abbreviations used are: ER, endoplasmic reticulum; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein; PMSF phenylmethysulfonyl fluoride; PCR, polymerase chain reaction.
ABSTRACT

The yeast phosphoinositide phosphatase Sac1p localizes to endoplasmic reticulum (ER) and Golgi membranes and has compartment-specific functions in these organelles. In this study we analyzed in detail the topology of Sac1p. Our data show that Sac1p is a type II transmembrane protein with a large N-terminal cytosolic domain, which is anchored in the membrane by the two potential transmembrane helices near the C-terminus. Based on this topology we created a mutation which caused retention of Sac1p in the ER and as a consequence showed specific alterations in cellular phosphoinositide levels. Our results suggest that Sac1p controls a pool of phosphatidylinositol 3-phosphate and phosphatidylinositol 4-phosphate in the ER. Retention of Sac1p in the ER also stimulates ATP transport into the ER lumen but causes the same Golgi-specific defects that are seen in a sac1 null mutant. Taken together this study provides evidence that Sac1p is an important 4-phosphatase in the ER controlling different aspects of ER based protein processing and secretion.
INTRODUCTION

Phosphorylated derivatives of PtdIns\(^1\), also called phosphoinositides, play an important role in lipid based signal transduction. These molecules participate in the regulation of various cellular functions including membrane trafficking, cytoskeletal organization, cell proliferation and metabolism (1,2). Though it is well understood that generation of such phosphoinositides by specific lipid kinases is essential (3), there is increasing evidence that the regulated termination of these signals by phosphatases is equally important. Phosphoinositide phosphatases are consequently important mediators of phosphoinositide signaling events. Phosphatases belonging to a subclass, share a specific domain, which was first identified in the yeast Sac1 protein (4). Sac1 homology domain containing phosphatases catalyze the hydrolysis of a variety of phosphoinositide species and are found from yeast to mammals (4). \textit{In vitro} studies have shown that the Sac1 homology domain is capable of dephosphorylating PtdIns(3)P, PtdIns(4)P and PtdIns(3,5)P\(_2\), whereas PtdIns(4,5)P\(_2\) is not accepted as a substrate (5-7). In yeast, this protein family comprises 5 different members, Sac1p, Fig4p and in addition the three dual specificity phosphatases Inp51p, Inp52p and Inp53p (4). While the function of Fig4p is unclear, all other members of this family are polyphosphoinositide phosphatases and therefore perform important functions as regulators of phosphoinositide metabolism at various cellular locations (7-9).

Among these proteins Sac1p is the only integral membrane phosphatase (10). Sac1p is localized to ER and Golgi membranes (10), which indicates that this protein has compartment specific functions. Genetic and biochemical analyses defined Sac1p as an important regulator of ATP-uptake into the ER lumen, suggesting that one ER-specific function of this protein is the maintenance of the secretory function of this organelle by controlling the supply of ATP inside the ER lumen (11,12). A number of \textit{Sac1} mutants act as bypass suppressors for \textit{sec14}\(^{ts}\) mutants indicating that Sac1p has also a role in Golgi trafficking (13-15). We have recently presented evidence that Sac1p regulates a pool of PtdIns(4)P in the Golgi that is important for
forward trafficking to the cell periphery (16). The role of phosphoinositides in the ER is less well understood. A recent report indicates that lipid signals are also important regulators for vesicular transport at the yeast ER (17). In another study it was shown, that an ER resident portion of Sac1p plays a role in regulating phosphoinositide levels in this organelle (18). However, the exact cellular function of Sac1p in the ER remained unclear. It is also unknown how the differential localization of Sac1p to ER and Golgi membranes is mediated. Sequence analysis has revealed that Sac1p contains none of the characterized sequence motives that are required for retention in the ER. To date, the exact topology of the yeast Sac1 protein has not been studied in detail. An analysis using a mammalian version of Sac1p has shown that the N-terminal hydrophilic portion of this protein, which also contains the catalytic phosphatase domain, is oriented to the cytoplasm (19). However, it remains unclear which portion of the protein is integrated into the membrane.

In this work we analyzed the transmembrane arrangement of yeast Sac1p by proteolysis, specific antibodies and by expression of different truncated versions of this protein. We found that Sac1p is a type II membrane protein containing two potential transmembrane domains near the C-terminus. In addition, we analyzed two different mutations in SAC1 that both cause retention of the respective mutant protein in the ER. Our data suggest that Sac1p controls a distinct pool of phosphoinositides in the ER.
**EXPERIMENTAL PROCEDURES**

*Plasmids*-Sac1 constructs for expressing proteins containing a c-myc epitope at their N-termini were created by PCR using the following primers: myc-Sac1p, 5'-CGT CTA GAA TGT GTG AAC AAA AGC TTA TTT CTG AAG AAG ACT TGG GTG GTG GTA TGA CAG GTC CAA TAG TG-3' and 5'-GCT CTA GAA GCT TGT TCT TCC CTC TG-3'; myc-Sac11-504, 5'-CGT CTA GAA TGT GTG AAC AAA AGC TTA TTT CTG AAG AAG ACT TGG GTG GTG GTA TGA CAG GTC CAA TAG TG-3' and 5'-GCT CTA GAT TAT TAT CCA CCA AGG AAT AAA TCG-3'; Sac1505-623, 5'-GCT CTA GAA TGT GTG AAC AAA AGC TTA TTT CTG AAG AAG ACT TGG GTG GTG GTA TGA GAC CAC ATA CCG CTT CTA TC-3' and 5'-GCT CTA GAA GCT TGT TCT TCC CTC TG-3'. The constructs were digested with XbaI and cloned into YCplac33 containing either the GAL1/10 promotor or the endogenous SAC1 promotor. The SAC1 promotor (500 bp upstream of AUG) was amplified by PCR using primers 5'-GCG AAT TCG CTA CCA CAT CCC TGA CGT C-3' and 5'-GCG GAT CCA TCT AGA CGA GAA AAT ATA C-3' and cloned into the EcoRI and BamHI sites of YCplac33.

A DNA fragment encoding the N-terminal portion of SAC1 including the potential transmembrane region TM1 (SAC11-552) was amplified by PCR using the primers 5'-CGT CTA GAA TGT GTG AAC AAA AGC TTA TTT CTG AAG AAG ACT TGG GTG GTG GTA TGA CAG GTC CAA TAG TG-3' and 5'-GCT CTA GAA GCT TGT TCT TCC CTC TG-3'. The XbaI digested PCR product was cloned into YCplac33 containing the GAL1/10 promotor resulting in pGK64. A DNA fragment encoding the C-terminus of SAC1 but lacking the coding region for potential transmembrane segment TM2 (SAC1574-623) was amplified by PCR using the primers 5'-GCA CGC GTC GAC AAG AAC GGT ATT CAG TTT-3' and 5'-CTA CGC GTC GAC AGC TTG TTC TTC CCT GTG-3'. The SalI digested PCR product was cloned into pGK64, resulting in pGK65, which now contains a version of SAC1 in which the
entire coding region for TM2 is replaced by a short sequence coding for the four amino acids, Ser Arg Val Asp (sac1ΔTM2).

To generate \textit{EYFP-SAC1} fusion constructs, a genomic fragment containing the Sac1 promotor (500 bp upstream of AUG) was amplified using the primers 5’-TTG CGG CCG CAC AGC TAC CAC ATC CCT GAC-3’ and 5’-GGC TCG AGA TCT AGA CGA GAA AAT ATA CG-3’ digested with \textit{NotI} and \textit{XhoI} and cloned into a \textit{YCplac33} vector containing the coding region of \textit{GFP} (pGK25), resulting in pGK26. A DNA fragment coding for \textit{EYFP} was obtained by digestion of pPS1891 (20) with \textit{BamHI} and \textit{XhoI}. This \textit{BamHI/XhoI} fragment was cloned into pGK26 replacing the coding region of \textit{GFP} with \textit{EYFP} (pGK66). For expression of N-terminal EYFP fusion proteins, DNA fragments containing the coding regions of \textit{SAC1}, \textit{sac1-22} and \textit{sac1-KKRD} were cloned into the \textit{XbaI} site of pGK66 resulting in pGK67, pGK68, and pGK69. To generate the KKRD motive at the C-terminus of Sac1p a L620K mutation was introduced into \textit{SAC1} by mutagenesis PCR using the primers 5’-CAA GCC GGA TCC TAA AAA AAG AGA TTA ATG AGT TCT-3’ and 5’-AGA ACT CAT TAA TCT CTT TTT TTA GGA TCC GGC TTG-3’. To create a \textit{SEC63-ECFP} fusion, a \textit{NotI/XhoI} fragment containing \textit{SEC63} and its promotor was obtained by digestion of pJK59 (21). This fragment was cloned into pRS314 (22), resulting in pGK59. A fragment coding for \textit{ECFP} was isolated from pPS1890 (20) by digestion with \textit{XhoI/KpnI} and ligated into pGK59, resulting in pGK60.

\textit{Preparation of yeast microsomes and proteolytic digests}-Yeast microsomes were prepared exactly as described previously (23). Microsomal preparations were adjusted to a concentration of 2 mg/ml in membrane buffer (250 mM sucrose, 50 mM potassium acetate, 20 mM HEPES, 1 mM DTT, pH 7.4). For complete digestion membranes were treated with 0.5 mg/ml proteinase K for 60 min on ice. Partial digests were conducted by incubation of membranes with 0.1 mg/ml trypsin for 30 min on ice. The proteolytic reactions were terminated by addition of 1 mM PMSF to the individual samples and further incubation for 10 min on ice. Integrity of the microsomal preparation was controlled by protection of the luminal factor.
Kar2p from proteolytic degradation. Proteolytic products of Sac1p were separated by SDS-PAGE (10% polyacrylamide). To identify small peptides below 10 kDa, high resolution Tricine SDS-PAGE was used (24).

Antibodies and Western analyses—Antibodies were raised in rabbits (New Zealand White) using the following peptides, which correspond to specific regions in Sac1p: amino acid 306-317, NQKGHELPVKEGC (anti-Sac1); amino acid 613-623, CKFSKPDPLKD (anti-Sac1-C). The peptides were coupled to Key limpet hemocyanin using glutaraldehyde as described in the literature (25). Transfer of proteins to nitrocellulose and decoration with antibodies were performed according to standard procedures (25).

Lipid analysis—Since sac1 mutants are inositol auxotrophs, yeast cells were generally cultivated in 0.1 mM inositol before and during the labeling procedure. Early log phase cells were incubated with 5 µCi/ml [14C]myo-inositol for 3-4 doubling times. Labeling and extraction of lipids was performed as described earlier (16). Deacylation of lipids was performed as described in (26). HPLC analysis of glycerophosphoinositols was carried out on a 250 x 4.6 mm Partisil SAX column (Whatman) using a Jasco HPLC system equipped with a LB 508 Radioflow detector (Berthold, Bad Wildbach, Germany). Elution and quantification of glycerophosphoinositols was performed as described (16).

Phosphatase assay—1.3 ml of cytosol containing either soluble myc-Sac11-504 or myc-Sac1-221-504 was incubated with 40µl of a suspension containing monoclonal anti c-myc epitope antibodies covalently linked to Protein A Sepharose for 3h at 4°C. The beads were then washed 4 times in washing buffer (0.3% Triton X-100, 300 mM NaCl, 40 mM HEPES, pH 7.4, 1 mM PMSF). The Sac1p fragments were eluted from the beads with 140 µl of 100 mM glycine pH 3.5. The eluate was adjusted to pH 7.0 by addition of 0.5 M Tris, pH 8.0. For measuring phosphatase activity a modified version of a recently published protocol was used (27). 17 µl of reaction buffer (200 mM sodium acetate, 100 mM Bis-Tris, 100 mM Tris, 10 µg/ml porcine gelatine and 4 mM DTT, pH 6.0), 25 µl eluate containing 2 µg of recombinant...
soluble myc-Sac1\textsubscript{1-504} or myc-Sac1-22\textsubscript{1-504} and 8 µl of liposomes (250 µM phosphatidylyserine and 50 µM PtdIns(4)P in reaction buffer) were mixed and incubated at 30°C for the various times. Reactions were stopped by addition of an equal amount of 100 mM NEM, centrifuged at 150000 x g and the supernatants were transferred to fresh Eppendorf tubes. 25 µl of each supernatant was pipetted into ELISA plate wells. 50 µl of Malachite Green solution (1 volume 4.2% ammonium molybdate in 4 M HCl and 3 volumes of 0.045% Malachite Green, 0.01% Tween 20) were added to each well. After incubation for 20 min at room temperature the $A_{620}$ was measured.

*ATP Transport Assay*-Preparation of yeast microsomes and ATP transport assays were conducted exactly as described previously (23). Usually, 300 µl microsome suspensions (3 mg/ml protein) and 50 µM [\textsuperscript{14}C]ATP were used in the uptake reactions (23).

*Fluorescence microscopy*-Cells were grown in appropriate selective media to early log phase. EYFP and ECFP signals in living yeast cells were analyzed on a Olympus BX60 fluorescence microscope with a Zeiss UPlanAPO 100x oil immersion objective. Pictures were recorded using a Hamamatsu C4742-95 CCD camera and 'Openlab' software from Improvision (Heidelberg, Germany).
RESULTS

*Sac1p is a type II membrane protein containing two transmembrane segments*- When analyzed by secondary structure prediction programs, the sequence of Sac1p contains a large hydrophilic N-terminal domain and two hydrophobic stretches near the C terminus that could potentially serve as transmembrane regions. According to this model, the C terminus of Sac1p should be sufficient to anchor this protein in the membrane. To obtain experimental proof for such a topology we expressed myc-tagged truncated versions of Sac1p, containing either only the extreme C terminus comprising amino acids 505-623, or a major N-terminal portion from amino acid 1-504 which contained the entire Sac1-homology domain. Both fragments cofractionated with microsomal membranes but only the C-terminal fragment was resistant to high salt and sodium carbonate treatment (Fig. 1A). As a control we also probed the membrane association of Sec61p, which spans the membranes several times (Fig 1A). The C terminus of Sac1p therefore behaves like an integral membrane protein. In contrast, the N-terminal fragment could be completely released from microsomal membranes by sodium carbonate treatment (Fig. 1B) and accordingly has the characteristics of a peripheral membrane protein, such as Srp54p (28). This interaction with the membrane of a N-terminal fragment of Sac1p may reflect an interaction of Sac1p with peripheral or integral membrane proteins in the ER membrane.

To probe the topology of Sac1p in more detail, we expressed a version of this protein containing a c-myc tag at its N-terminus from a low copy vector in a *sac1Δ* strain under control of a *GAL1/10* promotor. Expression of the myc-Sac1 protein fully complemented *sac1* mutant phenotypes such as inositol auxotrophy and hypersensitivity towards Calcofluor White when expression was induced by galactose and can therefore functionally replace the wild-type protein (data not shown). Proteolytic treatment with proteinase K of microsomal membranes containing myc-Sac1p led to rapid degradation of the N-terminal myc-tag (Fig. 2A). In contrast, the luminal Kar2p was only susceptible to proteolytic degradation when the
membranes had been treated with detergent (Fig. 2A), showing that the membranes were intact and impermeable to the protease. This result indicated that the N-terminus of Sac1p faces the cytosol. Such a result was expected since the hydrophilic N-terminal domain of Sac1p contains also the phosphatase activity (6) and no luminal PtdIns kinase activity has been detected so far.

Sequence analysis revealed that the primary structure of Sac1p contains two hydrophobic stretches near the C-terminus from amino acid 524-544 and 554-573, both of which could potentially span the membrane. Placing the N-terminus of Sac1p towards the cytosol, therefore created three distinct hypothetical topologies depending on whether either one or both potential transmembrane segments were used. To distinguish whether Sac1p spans the membrane once or twice, we engineered sac1<sub>Δ</sub>TM2, a version of SAC1 in which the region encoding the second potential transmembrane domain from amino acid 554-573 was deleted. Sac1ΔTM2p was associated with microsomes and was not extractable by treatment of microsomal membranes with sodium carbonate and therefore, like Sac1p, behaved as an integral membrane protein (data not shown). We then used limited proteolysis and polyclonal antisera specific to various regions of Sac1p to obtain information on the respective topologies of Sac1p and Sac1ΔTM2p. Mild digestion of microsomal membranes containing Sac1p with trypsin generated a polypeptide of about 65 kDa, approximately 5 kDa shorter than the undigested protein, while Sac1ΔTM2p stayed largely intact under those mild conditions (Fig. 2B). Proteolytic treatment with proteinase K of microsomal membranes containing Sac1ΔTM2p with a N-terminal myc-tag led to rapid degradation of the myc-epitope, indicating that, like in myc-Sac1p, the N terminus of myc-Sac1ΔTM2p was oriented towards the cytosol (Fig. 2A). The 65 kDa proteolytic species of Sac1p was detected by antibodies raised against a peptide comprising amino acid 306-317 in the middle of Sac1p (anti-Sac1) (Fig. 2B). Importantly, this 65 kDa fragment was not recognized by antibodies specific to the last 11 amino acids of Sac1p (anti-Sac1-C), indicating that this C-terminal epitope of Sac1p was destroyed or removed by this treatment with trypsin (Fig. 2B). If the C terminus of Sac1p was
facing the ER lumen, either a 7.5 kDa or a 11 kDa fragment should be protected from proteolytic digestion in the lumen of the microsomal membranes. In contrast, if Sac1p would traverse the membrane twice a 5.5 kDa C-terminal portion of the protein would face the cytosol and be exposed to the protease. Since the N terminus of Sac1pΔTM2p is oriented towards the cytosol (Fig. 2A), the C terminus of this protein must face the microsomal lumen and consequently a 9 kDa protected fragment should be detectable (compare structural models in Fig. 3B). We therefore analyzed proteolyzed membranes containing either Sac1p or Sac1ΔTM2p using a Tricine SDS-PAGE system, which can resolve low molecular weight peptides (24). Again, the extent of proteolysis was analyzed using antibodies raised against the C-terminal peptide (anti-Sac1-C). Clearly, no protected low molecular weight peptide species could be detected by anti-Sac1-C antibodies after digesting membranes containing Sac1p, while a 9 kDa fragment was visible after protease treatment of membranes containing Sac1pΔTM2p (Fig. 3A). As a control, aliquots of the same samples were also analyzed by conventional SDS-PAGE and by Western blotting using antibodies against Kar2p to ensure that the membranes were intact (Fig. 2A). This experiment indicated strongly that the C termini of Sac1p and Sac1ΔTM2p face opposite sites of the microsomal membrane. As expected, the C terminus of Sac1pΔTM2 is located on the luminal side of the microsomes and therefore protected from proteolysis. Using identical conditions, the C-terminal epitope in Sac1p recognized by anti-Sac1-C antibodies is obviously accessible to the added protease. Consequently, the C terminus of Sac1p must face the cytosolic site of microsomal membranes. In summary our data are in favor of a topological model of Sac1p in which this protein spans the membrane twice with both the N-and the C-terminus facing the cytosol (Fig. 3B).

The sac1-22 mutant protein displays compromised phosphatase activity and is restricted to the ER-Detailed analysis of different point mutations in SAC1 such as sac1-22 revealed compartment specific differences in the strength of the respective phenotypes (12,29). While both sac1-22 and sac1Δ strains show defects in actin cytoskeletal arrangement and Golgi
trafficking, *sac1Δ* strains additionally display impaired ATP transport into the ER, a defect that is absent in the *sac1-22* mutant (12). To address whether the absence of ER-specific defects in this mutant was a consequence of an altered cellular localization of the mutant protein, we compared the intracellular localization of N-terminal EYFP-fusion proteins of wild-type Sac1p and Sac1-22p. To ensure that comparable levels of the different YFP-fusion proteins were synthesized, all fusion proteins were expressed under control of the endogenous Sac1 promoter from *CEN* plasmids. There is some disagreement in the literature regarding the intracellular localization of Sac1p. A study using immunofluorescence and biochemical fractionations had shown that wild-type Sac1p localizes to both ER and Golgi membranes (10). In a recent analysis, it was shown that the vast majority of Sac1p versions containing GFP or multiple myc-epitopes at their C-termini were restricted to the ER (18). Since Sac1p has a Golgi-specific role (16) it was important to explicitly prove that our fusion of EYFP to the N terminus of Sac1p does not disturb this specific function of Sac1p. In control experiments it was therefore confirmed that expression of EYFP-Sac1p in a *sac1Δ* strain fully complemented the hypersensitivity towards Calcofluor White (Fig. 8A), which is a Golgi-specific *sac1Δ* mutant phenotype (16). Other phenotypes such as inositol auxotrophy were also complemented by expression of Sac1p fused N-terminally to EYFP (data not shown). Since a Sec63p-GFP fusion protein has been reported to be a useful marker for yeast ER, we used a Sec63p-ECFP derivative for *in vivo* colocalization with EYFP-Sac1 proteins in the respective strains. More than 50% of the cells expressing EYFP-Sac1p showed weak or no perinuclear staining and EYFP-Sac1p was instead confined to punctate structures resembling the localization reported for non-tagged Sac1p (Fig. 4A) (10). Clearly, these punctate structures did not colocalize with Sec63-ECFP indicating that a substantial portion of Sac1p is not restricted to the ER. In striking contrast, EYFP-Sac1-22p was exclusively confined to perinuclear and peripheral regions and no punctate localization could be detected (Fig. 4B). Essentially, the localization of EYFP-Sac1-22p was very similar to the one observed for Sec63p-ECFP with a slightly more
pronounced peripheral localization of EYFP-Sac1-22p (Fig 4B). This result strongly indicated that the *sac1-22* mutation caused retention of the mutant protein in the ER. Since the point mutation in *sac1-22* lies outside of the consensus region known to be essential for phosphatase activity (Fig. 3B) (5), the Sac1-22 mutant protein may therefore retain some catalytic activity. Such a residual activity may be sufficient for suppressing ER-specific defects in ATP-transport since the retention of Sac1-22p in the ER should cause an increased level of the mutant protein in this organelle. To investigate this possibility directly, we purified a recombinant fragment of Sac1p containing either the entire wild-type Sac1 homology domain or the corresponding domain of Sac1-22p (Fig. 5A). These purified soluble Sac1p fragments were then used to assay their respective phosphatase activity. Consistent with published results the recombinant wild-type Sac1 domain actively degraded PtdIns(4)P to PtdIns (Fig. 5B) (27). In contrast, the recombinant domain containing the *sac1-22* mutation displayed a severely diminished phosphatase activity (Fig. 5B). To test how this defect in Sac1-22p would influence the cellular levels of phosphoinositides, we quantified the relative amount of these lipids in *sac1Δ* cells expressing Sac1-22p. While a deletion of *SAC1* in our strain background resulted in a 9-fold elevation of cellular PtdIns(4)P levels (16), expression of Sac1-22p in this strain caused an only 3-fold increase in PtdIns(4)P (Fig 6). This result was consistent with our finding that the Sac1-22p phosphatase domain had reduced but measurable enzymatic activity towards PtdIns(4)P. Interestingly, the levels of PtdIns(3)P in the *sac1-22* mutant were identical to wild-type levels, indicating that expression of Sac1-22p was sufficient for maintaining correct cellular levels of this lipid.

*Retention of Sac1p in the ER causes a decrease in PtdIns(4)P and stimulates ATP-uptake*- The Sac1-22 mutant protein displayed both a severe defect in phosphatase activity and an incorrect retention in the ER. It was consequently difficult to estimate how much the accumulation of Sac1-22p in the ER contributed to the observed phospholipid phenotype. To more specifically investigate the ER-specific function of Sac1p, we expressed a Sac1p mutant
with an ER retention signal but containing a wild-type phosphatase domain. The C terminus of Sac1p consists of the amino acid sequence LKRD. The cytosolic orientation of this C-terminal region of Sac1p (Fig. 3B) allowed us to create an ER retention signal at this position. We changed the LKRD motif at the C terminus of Sac1p and of an EYFP-tagged version of Sac1p to KKRD, which was shown before to confer strong ER retention to a model protein (30). Using fluorescence microscopy we tested whether the KKRD mutant of EYFP-Sac1p was indeed confined to the ER. As seen in Fig. 4C, the mutant protein was exclusively found in a perinuclear region and at the cell periphery, practically identical to the localization of Sec63p-ECFP. Different from EYFP-Sac1p (Fig. 4A) the EYFP-Sac1p-KKRD was not localized to punctate structures confirming that the KKRD motive was sufficient to retain the protein in the ER (Fig. 4C).

Our earlier observations pointed towards a regulatory role of Sac1p in microsomal ATP transport (12). One possibility, of how Sac1p could control the ATP-transport activity in the ER, was by regulating the level of a specific phospholipid signal which may influence the activity of the ATP transporter. Alternatively, Sac1p may directly interact with components of the ATP transport machinery. It was therefore of interest to explore the consequences on ATP transport of Sac1p retention in the ER. Consequently, we prepared microsomal membranes from a strain expressing Sac1p-KKRD from a \textit{CEN} based plasmid under control of the endogenous \textit{SAC1} promotor. Consistent with the regulatory role of Sac1p in this process, we found a remarkable increase in ATP transport activity in microsomes prepared from a \textit{sac1Δ} strain expressing Sac1p-KKRD (Fig. 7). We also determined the cellular phosphoinositide levels in this strain. Significantly, the expression of the ER-retention mutant of Sac1p did not only repair the phosphoinositide phenotype of the null mutant, but resulted in a remarkable decrease of PtdIns(4)P below wild-type levels (Fig. 6). The cellular amount of PtdIns(3)P was also somewhat lower compared to a wild-type strain (Fig. 6). This data indicated strongly that considerable portions of the PtdIns(4)P and PtdIns(3)P accumulating in
sac1Δ cells were localized to ER membranes and therefore susceptible to degradation by the ER-retained Sac1p mutant protein.

**Retention of Sac1p in the ER causes cell wall defects**—Since both Sac1-22p and Sac1p-KKRD are retained in the ER, we speculated that sac1-22 strains as well as sac1Δ strains expressing Sac1p-KKRD would exhibit the Golgi specific defect seen after a complete loss of Sac1p. The function of Sac1p in the Golgi is not completely understood but genetic evidence suggested that Sac1p is important for secretion (13-15). We have recently investigated this aspect in detail and found that Sac1p is an important regulator for transport of chitin synthases from a Golgi associated storage compartment to the cell periphery (16). As a consequence, sac1Δ strains have specific cell wall defects and are hypersensitive towards the drug Calcofluor White, a widely used indicator for detecting cell wall defects in yeast mutants (16). We therefore analyzed the mutants expressing Sac1-22p or Sac1p-KKRD for cell wall defects by assaying their growth on plates containing Calcofluor White. While a SAC1 strain grew well under these conditions, a sac1Δ strain in which Sac1-22p or Sac1p-KKRD was expressed, showed severely impaired growth on media containing Calcofluor White. This growth defect was as strong as the one observed in a sac1Δ strain only containing a control vector (Fig. 8B). This result provided additional evidence that Sac1-22p and Sac1p-KKRD are indeed retained in the ER and as a consequence are depleted from the Golgi to such an extent that Sac1p-dependent regulation of trafficking of cell wall specific enzymes was impaired.
DISCUSSION

The proper function of phosphoinositide phosphatases depends on their specific association with intracellular membranes. Of the four yeast 5-phosphatases only Inp54p directly interacts with phospholipid bilayers via a C-terminal tail-anchor (17). The only confirmed example of an integral membrane phosphatase containing a Sac1 domain is represented by Sac1p itself (10). Our study now demonstrates that the membrane localization of yeast Sac1p is mediated via two potential transmembrane regions near the C terminus of this protein. Both the large N-terminal domain that contains the phosphatase activity and a C-terminal hydrophilic region of this protein are facing the cytosol. The rat homolog of Sac1p was also shown to contain a large cytosolic N-terminal domain but the topology of the C-terminal portion of this protein was not tested (19). However, since the two potential transmembrane regions in yeast Sac1p are conserved in the rat protein, the overall topology of rat and yeast Sac1p are likely to be the same.

Sac1p was shown before to localize to both ER and Golgi membranes (10). This differential localization was in line with the proposed compartment specific functions of Sac1p. Our recent work indicated that Sac1p controls a pool of PtdIns(4)P in the Golgi that is required for forward trafficking to the plasma membrane (16). In the ER, Sac1p regulates the uptake of ATP into this organelle (11,12). It was therefore of particular interest to analyze specific sac1 mutants in which only one compartment was affected. One example of such a mutant was sac1-22, which showed wild-type ATP-uptake into the ER but displayed the same Golgi-specific trafficking defects that are also observed in a sac1Δ strain (12,29). Our data demonstrate that this compartment specific phenotype is based on a combination of a severe defect in 4-phosphatase activity and the aberrant accumulation of Sac1-22p in the ER. It remains unclear how the sac1-22 point mutation causes retention of the mutant protein in the ER. According to our topological model of Sac1p, the region containing the amino acid change in Sac1-22p is placed in relative proximity to the membrane. Therefore, this portion of the protein may be
critical for interacting with an unknown factor at the ER membrane required for trafficking of Sac1p out of the ER. Alternatively, the sac1-22 mutation may lead to destabilization or unfolding of certain regions in Sac1-22p thereby causing aberrant retention in the ER. Such destabilization of a cytosolic domain of Sac1-22p could also explain the defect in phosphatase activity observed in this mutant protein.

Our results indicate that accumulation of the defective phosphatase in the ER is sufficient to complement ER-specific defects of the null mutant. sac1Δ cells expressing the partially active Sac1-22p showed a normal level of PtdIns(3)P and displayed only three-fold elevated PtdIns(4)P level, versus 9-fold elevation of PtdIns(4)P in a sac1Δ strain (16). These results therefore suggest that Sac1p regulates a distinct pool of phosphoinositides at the ER. Such a role for Sac1p was supported by our observation that expression in a sac1Δ strain of a version of Sac1p containing a canonical KKXX ER retention motive at its C-terminus resulted in reduction of cellular PtdIns(4)P to about half the level of a wild-type strain. As expected, expression of Sac1-22p or Sac1p-KKRD did not complement the Golgi trafficking defects of the null mutant. Though it was not possible to directly measure ER versus Golgi PtdIns(4)P levels in vivo, our results clearly indicate that there exists a substantial ER-specific pool of PtdIns(4)P that is regulated by Sac1p. Since expression of Sac1-22p in a sac1Δ strain showed wild-type levels of PtdIns(3)P and expression of Sac1p-KKRD caused even a slight decrease of this lipid, our study also provides evidence that the accumulation of PtdIns(3)P in sac1Δ strains occurs at the ER. Recently, it was shown that the majority of PtdIns(3)P in yeast cells was localized to endosomal compartments with only negligible amounts present in other organelles (31). Sac1p may therefore be required to maintain a low basal level of this lipid in ER membranes. Similar low ER levels of PtdIns(3)P were also observed in mammalian cells (31), yet a number of studies have shown that this lipid is an important regulator of ER-dependent processes in mammals (32,33). It remains therefore quite possible that the exact regulation of PtdIns(3)P in the ER of yeast cells is also of physiological importance.
The role of PtdIns(4)P in the yeast ER is also not well established. It was reported recently that the PtdIns 4-kinase Stt4p is required for trafficking of phosphatidylserine from the ER to the Golgi (34). Though the intracellular localization of Stt4p has not been determined, the mammalian PtdIns 4-kinase PI4Kα, which shows high homology to Stt4p has been shown to localize to ER membranes (35). In addition, there is genetic evidence, that the PtdIns(4)P level in the yeast ER depends on Stt4p but not on the other yeast PtdIns 4-kinase Pik1p (18). The ER-specific pool of PtdIns(4)P in yeast is also affected by the 5-phosphatase Inp54p which is specifically localized to ER membranes (17). Inp54p hydrolyzes PtdIns(4,5)P2 to PtdIns(4)P (17) and is therefore expected to contribute to changes in PtdIns(4)P levels in the ER. Interestingly, inp54 null mutants showed increased trafficking of a marker protein out of the ER (17). These results therefore suggest that either PtdIns(4,5)P2 is a positive regulator or that PtdIns(4)P is an inhibitor of ER-based vesicular transport. The latter possibility would be consistent with the observation that ER to Golgi transport is considerably slowed in sac1Δ strains which display a dramatic accumulation of PtdIns(4)P (12). The three Sac1-domain containing 5-phosphatases in yeast may theoretically also contribute to regulating PtdIns(4)P levels in the ER and the Golgi, though no specific association of these proteins with these organelles has been detected (9). However, it has been shown recently that a sac1Δ inp53Δ double mutants display synthetic defects resulting in enhanced accumulation of cellular PtdIns(4)P levels. Consequently, Sac1p and Inp53p are likely to have in part overlapping functions (18).

The accumulation of PtdIns(4)P and PtdIns(3)P in sac1Δ strains also parallels the significant inhibition of microsomal ATP transport in this mutant (11). Importantly, expression of Sac1p-KKRD which is retained in the ER led to a substantial increase in ATP-transport activity while at the same time causing a significant reduction of PtdIns(4)P levels. Our findings are therefore consistent with the possibility that microsomal ATP-transport is regulated via phosphoinositides. Alternatively, Sac1p may directly interact with components of
the ATP-transporting machinery and therefore regulate uptake of ATP in a fashion that is independent from its phosphatase activity.

In summary this work provides strong evidence that Sac1p plays a crucial role in the ER by regulating specific pools of phosphoinositides in this compartment. This work also indicates that Sac1p is an important 4-phosphatase in ER and Golgi membranes. Future work should now be directed towards the identification of the processes that are regulated by ER-specific phosphoinositide signals.

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Fig.1. **Two potential transmembrane segments between amino acids 505-623 are required to anchor Sac1p in the membrane.** A, microsomes prepared from a strain expressing mycSac1<sub>505-623</sub> were sequentially washed with 500 mM potassium acetate, pH 7.4 (KOAc); 100 mM sodium carbonate, pH 9.5 (Carb); 2% Triton X-100. Association of myc-Sac1<sub>505-623</sub> and Sec61p with microsomal membranes was analyzed by Western blotting using anti-myc and anti Sec61p antisera. B, microsomes prepared from a strain expressing myc-Sac1<sub>1-504</sub> were sequentially washed with 500 mM potassium acetate, pH 7.4 (KOAc); 100 mM sodium carbonate, pH 9.5 (Carb); 2% Triton X-100. Association of myc-Sac1<sub>1-504</sub> and Srp54p with microsomal membranes was analyzed by Western blotting using anti-myc and anti-Srp54p antisera.

Fig.2. **The N termini of Sac1p and Sac1ΔTM2p face the cytosol.** A, microsomes were treated with 0.5 mg/ml proteinase K for 60 min on ice. Where indicated, membranes were permeabilized by 2% Triton X-100 before incubation with the protease. Digest of the N-terminal myc-tag of Sac1p and Sac1ΔTM2p and protection of the luminal protein Kar2p were analyzed by Western blotting using anti-myc and anti-Kar2p polyclonal antisera. Integrity of the microsomal preparation is indicated by protection of Kar2p from proteolysis in the absence of detergent. B, microsomes containing Sac1p or Sac1ΔTM2p were treated with 0.1 mg/ml Trypsin for 30 min on ice. Where indicated, membranes were permeabilized by 2% Triton X-100 before incubation with the protease. Proteolytic products of Sac1p and Sac1ΔTM2p were analyzed by Western blotting using antibodies recognizing a C-terminal fragment (anti-Sac1-C) or a portion in the middle of Sac1p (anti-Sac1).
Fig. 3. **Deletion of potential transmembrane region TM2 in Sac1p changes orientation of the C terminus.** A, microsomes containing Sac1p or Sac1ΔTM2p were treated with 0.5 mg/ml Proteinase K for 60 min on ice. Where indicated, membranes were permeabilized by 2% Triton X-100 before incubation with the protease. The presence of a protected C-terminal fragment was analyzed by Western blotting after separation of the digests on high resolution Tricine SDS-PAGE (24) using antibodies recognizing a C-terminal fragment of Sac1p (anti-Sac1-C). B, topological model of the transmembrane arrangement of Sac1p and Sac1ΔTM2p.

Fig. 4. **The sac1-22 mutation and a KKRD motive at the C terminus cause retention of the Sac1 protein in the ER.** Colocalization of ECFP and EYFP signals is indicated by magenta colors. A, Nomarski optics and fluorescence colocalization of a N-terminal EYFP fusion with Sac1p and a C-terminal ECFP fusion with Sec63p. The fusion proteins were expressed form CEN based plasmids under control of the endogenous SAC1 promotor in a sac1Δ strain. B, Nomarski optics and fluorescence colocalization of a N-terminal EYFP fusion with Sac1-22p and a C-terminal ECFP fusion with Sec63p. The fusion proteins were expressed form CEN based plasmids under control of the endogenous SAC1 promotor in a sac1 strain. C, Nomarski optics and fluorescence colocalization of a N-terminal EYFP fusion with Sac1p-KKRD and a C-terminal ECFP fusion with Sec63p. The fusion proteins were expressed form CEN based plasmids under control of the endogenous SAC1 promotor in a sac1Δ strain.

Fig. 5. **The sac1-22 mutation causes a severe defect in phosphatase activity.** A, cytosolic fragments encompassing amino acids 1-504 of myc-Sac1p or myc-Sac1-22p were expressed in yeast cells and immunopurified using anti-myc antibodies covalently coupled to protein A sepharose beads. Aliquots of the beads were boiled in SDS-PAGE sample buffer and the purity of the immunopurified fragments was analyzed by SDS-PAGE (10% polyacrylamide). B, phosphatase activity was determined in a similar manner as published recently (27). Data are
from three independent phosphatase measurements. The error bars correspond to the standard
deviation of the respective mean values.

Fig. 6. **Retention of Sac1p in the ER causes alterations in the cellular levels of PtdIns(3)P and PtdIns(4)P.** Quantification of cellular phosphoinositide levels in *sac1Δ* strains expressing either *SAC1*, *sac1-22* or *sac1-KKRD* from *CEN* based plasmids. Yeast strains were incubated with 5 µCi/ml [14C]inositol for 3-4 doubling times. Lipids were extracted, deacylated and the [14C]-labeled glyceroinositol phosphate species were analyzed by HPLC.

Fig. 7. **Retention of Sac1p in the ER stimulates ATP-uptake.** Microsomes prepared from *sac1Δ* strains expressing either *SAC1*, *sac1-22* or *sac1-KKRD* from *CEN* based plasmids were assayed for microsomal ATP transport. Data are triplicates from three independent microsomal preparations for each strain. The error bars correspond to the standard deviation of the respective mean values.

Fig. 8. **Expression of EYFP-Sac1p but not of Sac1-22p and Sac1p-KKRD complements the cell wall specific defect in a sac1Δ strains.** A, cultures from *sac1Δ* strains containing either a *CEN* based plasmid carrying the coding region of EYFP under control of the *SAC1* promotor or *CEN* based plasmids carrying *SAC1* or *EYFP-SAC1* under control of the *SAC1* promotor were plated on YPD plates and on plates containing 0.02 mg/ml Calcofluor White and incubated at 25°C for 5 days. B, cultures from *sac1Δ* strains containing either an empty vector or *CEN* based plasmids carrying *SAC1*, *sac1-22* or *sac1-KKRD* under control of the *SAC1* promotor were plated on YPD plates and on plates containing 0.02 mg/ml Calcofluor White and incubated at 25°C for 5 days.
Figure 1

A

\( \text{myc-Sac}_1^{505-623} \)

| KOAc | Carb | Triton X-100 |
|------|------|-------------|
| P    | S    | P           |
| S    | P    | S           |
| P    | S    |             |

- anti-myc
- anti-Sec61p

B

\( \text{myc-Sac}_1^{1-504} \)

| KOAc | Carb | Triton X-100 |
|------|------|-------------|
| P    | S    | P           |
| S    | P    | S           |
| P    | S    |             |

- anti-myc
- anti-Srp54p
Figure 2

A

anti-myc

\[ \text{myc-SAC1} \quad \text{myc-sac1ΔTM2} \quad \text{myc-SAC1} \quad \text{myc-sac1ΔTM2} \]

\[ \begin{array}{cccc}
  - & + & + & + \\
  - & - & + & + \\
\end{array} \]

\[ \begin{array}{cccc}
  - & + & + & + \\
  - & - & + & + \\
\end{array} \]

\text{Proteinase K}

\text{Triton X-100}

B

anti-Sac1

\[ \text{myc-SAC1} \quad \text{myc-sac1ΔTM2} \]

\[ \begin{array}{cccc}
  - & + & + & + \\
  - & - & + & + \\
\end{array} \]

\[ \begin{array}{cccc}
  - & + & + & + \\
  - & - & + & + \\
\end{array} \]

\text{Trypsin}

\text{Triton X-100}
Figure 5

A

B

Phosphate released (μmol/g protein)

Time (min)
Retention of the yeast Sac1p phosphatase in the endoplasmic reticulum causes distinct changes in cellular phosphoinositide levels and stimulates microsomal ATP transport

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