Phosphoinositides direct membrane trafficking, facilitating the recruitment of effectors to specific membranes. In yeast phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) is proposed to regulate vacuolar fusion; however, in intact cells this phosphoinositide can only be detected at the plasma membrane. In Saccharomyces cerevisiae the 5-phosphatase, Inp54p, dephosphorylates PtdIns(4,5)P₂, forming PtdIns(4)P, a substrate for the phosphatase Sac1p, which hydrolyzes (PtdIns(4)P). We investigated the role these phosphatases in regulating PtdIns(4,5)P₂ subcellular distribution. PtdIns(4,5)P₂ bioprobes exhibited loss of plasma membrane localization and instead labeled a subset of fragmented vacuoles in Δsac1Δinp54 and Δsac1Δinp54 mutants. Furthermore, sac1Δinp54 mutants exhibited vacuolar fusion defects, which were rescued by latrunculin A treatment, or by inactivation of Mss4p, a PtdIns(4)P 5-phosphatase that synthesizes plasma membrane PtdIns(4,5)P₂. Under these conditions PtdIns(4,5)P₂ was not detected on vacuole membranes, and vacuole morphology was normal, indicating vacuolar PtdIns(4,5)P₂ derives from Mss4p-generated plasma membrane PtdIns(4,5)P₂. Δsac1Δinp54 mutants exhibited delayed carboxypeptidase Y sorting, cargo-selective secretion defects, and defects in vacuole function. These studies reveal PtdIns(4,5)P₂ hydrolysis by lipid phosphatases governs its spatial distribution, and loss of phosphatase activity may result in PtdIns(4,5)P₂ accumulation on vacuole membranes leading to vacuolar fragmentation/fusion defects.

Phosphoinositide signaling molecules are phosphorylated derivatives of phosphatidylinositol (PtdIns)³ that play critical roles regulating the actin cytoskeleton, cellular proliferation, and vesicular trafficking (1). PtdIns can be reversibly modified by lipid kinase phosphorylation of the D-3, D-4, or D-5 positions of the inositol head group to create phosphorylated phosphoinositides that recruit and activate effectors containing phosphoinositide-binding domains (1). In yeast and mammalian cells, phosphatidylinositol 4-phosphate (PtdIns(4)P) regulates secretion from the Golgi, and PtdIns(4)P recruitment of specific effector proteins, including FAPP1 and FAPP2, is required for mammalian Golgi to plasma membrane trafficking (1–3). In Saccharomyces cerevisiae PtdIns(4)P is synthesized from PtdIns by three PtdIns 4-kinases, Pik1p at the Golgi, Stt4p at the plasma membrane, and Lsb6p at the plasma and vacuolar membrane (4–7). In yeast, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) is generated from PtdIns(4)P by the PtdIns(4)P 5-phosphatase, Mss4p (8). PtdIns(4,5)P₂ is involved in the regulation of endocytosis, actin cytoskeletal dynamics, and the maintenance of Golgi structural integrity (1).

Phosphoinositide signaling molecules are dephosphorylated by lipid phosphatases that regulate the temporal and spatial distribution of phosphoinositide signals. In yeast, PtdIns(4)P and PtdIns(4,5)P₂ are hydrolyzed by phosphoinositide phosphatases, including Sac1p and the inositol polyphosphate 5-phosphatases (5-phosphatases), Inp51-4p (9–12). Sac1p is a polyphosphoinositide phosphatase containing a C₅R catalytic motif, which is found in both SacI domain-containing lipid phosphatases, as well as dual specificity tyrosine and serine/threonine phosphatases. Four active SacI domain-containing lipid phosphatases, and Inp54p Leads to Accumulation of Phosphatidylinositol 4,5-Bisphosphate on Vacuole Membranes and Vacuolar Fusion Defects*⁵

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Phosphoinositide signaling molecules are phosphorylated derivatives of phosphatidylinositol (PtdIns)³ that play critical roles regulating the actin cytoskeleton, cellular proliferation, and vesicular trafficking (1). PtdIns can be reversibly modified by lipid kinase phosphorylation of the D-3, D-4, or D-5 positions of the inositol head group to create phosphorylated phosphoinositides that recruit and activate effectors containing phosphoinositide-binding domains (1). In yeast and mammalian cells, phosphatidylinositol 4-phosphate (PtdIns(4)P) regulates secretion from the Golgi, and PtdIns(4)P recruitment of specific effector proteins, including FAPP1 and FAPP2, is required for mammalian Golgi to plasma membrane trafficking (1–3). In Saccharomyces cerevisiae PtdIns(4)P is synthesized from PtdIns by three PtdIns 4-kinases, Pik1p at the Golgi, Stt4p at the plasma membrane, and Lsb6p at the plasma and vacuolar membrane (4–7). In yeast, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) is generated from PtdIns(4)P by the PtdIns(4)P 5-phosphatase, Mss4p (8). PtdIns(4,5)P₂ is involved in the regulation of endocytosis, actin cytoskeletal dynamics, and the maintenance of Golgi structural integrity (1).

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PtdIns(4,5)P$_2$ on the Vacuole Membrane

Phosphatidate phosphatases exist in *S. cerevisiae*, including Sac1p, Fig4p, and two of the four 5-phosphatases, Inp52p and Inp53p (also called Sjl2p and Sjl3p) (10, 13). The Sac1 domain of Inp51p/Sjl1p lacks the C$_{X,Y}$R motif and is therefore catalytically inactive. Sac1p hydrolyzes PtdIns(4,5)P$_2$ to PtdIns(4)P, whereas Sac1p hydrolyzes PtdIns(4)P to PtdIns. We have investigated whether these enzymes coordinately regulate PtdIns(4,5)P$_2$ metabolism. We demonstrate here that although the total cellular PtdIns(4,5)P$_2$ levels remain unchanged in *sac1 inps54* double mutants, the spatial distribution of PtdIns(4,5)P$_2$ is profoundly altered, accumulating on vacuole membranes. In these double mutants, vacuolar membrane PtdIns(4,5)P$_2$ is derived from the plasma membrane, and its accumulation on a subset of vacuole membranes is associated with defects in vacuolar fusion. These studies reveal tight regulation of PtdIns(4,5)P$_2$ levels at the plasma membrane is required to regulate vacuolar fusion.

**EXPERIMENTAL PROCEDURES**

**Materials**—All restriction and DNA-modifying enzymes were obtained from Fermentas (Burlington, Canada), New England Biolabs (Beverly, MA), or Promega (Madison, WI). Oligonucleotides were obtained from GeneWorks (Adelaide, Australia). All other reagents were from Sigma or Invitrogen, unless otherwise stated. The constructs pEGFP-N1/PH-PLC and pEGFP-C1/PH-OSBP were kind gifts from Prof. Tamas Balla, NICHD, National Institutes of Health, Bethesda. GFP-PH-Nump1 construct was a gift from Prof. Mark Lemmon, University of Pennsylvania, Philadelphia. Vph1p antibody was from Prof. Tom Stevens, University of Oregon, Eugene. Antibody to Clc1p was a kind gift from Prof. Gregory Payne, UCLA. MFY72 (*sac1*ts) and AAY202 (*ms4*ts) strains and constructs encoding *ms4*-GFP and GFP-STOP were donations from Prof. Scott Emr, University of California, San Diego. Yeast strains used in this study are listed in Table 1, and plasmids are listed in Table 2.

**Disruption of SAC1 and/or INP54—Deletion of SAC1 or INP54 from the SEY6210 strain was as described previously (9, 15). Double null *sac1 inps54* and *fig4 inps54* mutants and *sac1*ts *inps54* mutants were created by replacing INP54 in the Δ*sac1*, Δ*fieg4* (ResGen/Invitrogen) and MFY72 strains, respectively, with a *LEU2* cassette as described previously (9). This resulted in the deletion of a segment of chr XV from coordinates 206,284–204,565, which spans the whole open reading frame of INP54 from nucleotides 1 to 1155, 400 bp upstream of the start codon and 166 bp downstream of the stop codon. The triple mutant *ms4*ts Δ*sac1 inps54* was generated by replacing SAC1 with a TRP1 cassette and INP54 with a URA3 cassette in the *ms4*ts (AAY202) strain. The TRPI sequence was amplified from pRS424 with the primers 5′-atacagtgctgtagtgccttaaacg-gagcaattgtcatctctgctttaaaatgaattc-3′ and 5′-tttaattctttttaaacgcgggagacatgatgatgat-3′. The PCR product was transformed into *ms4*ts strains resulting in the deletion of a segment from nucleotides 58 to 1830 or chr XI coordinates 34,601–36,373, creating a *ms4*ts *Δsac1* strain. INP54, including 1602 bp upstream of the start codon and 714 bp downstream of the start codon, was amplified from YE6210 genomic DNA using the primers 5′-gttttgctgtaacggcatggagctttcatcttttctgctggggagggc-3′ and 5′-gggggccgcttgactgtcagc-3′ and ligated into an Xhol-NotI-digested pBIKs (+) to generate pBINP54.
**TABLE 1**

| Yeast strains used in this study | Genotype | Source |
|---------------------------------|----------|--------|
| SEY6210                         | MATα ura3-32 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 | 63 |
| Δsac1 (ATY202)                  | SEY6210, sac1::TRP1 | This study |
| Δsac1 Δinp54                    | SEY6210, sac1::TRP1, inps4::LEU2 | 15 |
| sac1Δ (MFTY2)                   | MFTY2, inps4::LEU2 | This study |
| mss4Δ (AAY202)                  | SEY6210, mss4::HIS3MX, carrying YCplac111-mss4::102 | 41 |
| mss4Δ sac1 Δinp54               | AAY202, sac1::TRP1, inps4::URA3 | This study |
| Δgfp Δinp54                     | BY4741 (MATα his3-Δ1 leu2-0 met15-Δ0 ura3-Δ0), sac1::KanMX, inps4::LEU2 | This study |
| Δgfp55                          | BY4741, gfp::KanMX, inps4::LEU2 | This study |
| Δgfp27                          | BY4741, gfp::KanMX | This study |
| Δapm3                           | BY4741, apm3::KanMX | This study |

**TABLE 2**

| List of plasmids used in this study | Description | Source |
|-------------------------------------|-------------|--------|
| GFP-PH-NLIM1                        | 2μ URA3 vector expressing GFP at the N terminus of PH-NLIM1 | 44 |
| pPS130                              | 2μ URA3 vector expressing GFP under a GAL promoter | 64 |
| pPGK130                             | pPS130 with the GAL promoter replaced by a PGK promoter | This study |
| pPGK130/2xPH-PLC8                   | 2xPH-PLC8-GFP for localization of PtdIns(4,5)P2 | This study |
| pPGK130/PH-OSBP                     | PH-OSBP-GFP for localization of PtdIns(4)P | This study |
| pPGK130/SEC14                       | SEC14-GFP | This study |
| pPGK-Lys2/2xPH-PLC8                 | LYS2 plasmid for localization of PtdIns(4,5)P2, in sac1Δinp54 and mss4Δ sac1Δinp54 mutants | This study |
| pRS416-GFP/Pik1                     | CEN URA3 plasmid expressing Pik1-GFP under native promoter | This study |
| pRS416-GFP-STT4                     | 2μ URA3 plasmid expressing GFP-Stt4p under native promoter | S. Emr |
| pRS416-GFP-Mss4-GFP                 | 2μ URA3 plasmid expressing Mss4p-GFP under native promoter | S. Emr |
| pBINP54URA                          | For generation of mss4Δ sac1Δinp54 strains | This study |
| pPGK423/HA-INP54                    | 2μ HIS3 plasmid expressing HA-Inp54p under the PGK promoter | This study |

**URA3**, including its promoter, was amplified from pRS426 using the primers 5'-gactagtggctggcttcgtgac3' and 5'-catgacttactataatacag3' and cloned into PsiI-Spel-digested pB1NP54 to generate pB1NP54URA. The URA3 cassette flanked by the sequence upstream and downstream of INP54 was recovered from pB1NP54URA by Xhol-NotI digestion, and subsequently transformed into mss4Δ sac1Δ strain resulting in the deletion of INP54, including 400 bp upstream and 166 bp downstream of the open reading frame, creating a mss4Δ sac1Δ INP54 strain. Disruption of each gene was confirmed by PCR with the use of two unique sets of primers.

**Yeast Immunofluorescence**—For the detection of Vph1p, Pep12p, and Clc1p, yeast cells were fixed, spheroplasted, and stained as described previously (9). Anti-Vph1p and anti-Pep12p (Molecular Probes) were detected with anti-mouse Alexa-594 (Molecular Probes) and anti-Clc1p with anti-rabbit Alexa-594 (Molecular Probes). All other observations of GFP-tagged proteins were performed in live yeast cells. Fixed or live cells were placed on poly-L-lysine (2 mg/ml)-coated glass slides, and coverslips were mounted with SlowFade (Molecular Probes).

**Confocal Microscopy**—Yeast cells were visualized and analyzed using either an Olympus Fluoview confocal microscope or a Leica TCS NT confocal microscope, with green fluorescence collected in channel 1 (488 nm excitation, 530 ± 30 nm emission) and red fluorescence in channel 2 (568 nm excitation, LP590 nm). Images presented in the figures show either cells in a single field or from several different fields.

**Vacuole Labeling**—Yeast cells were grown to mid-log phase and metabolically labeled with 20 μM FM4-64 (Sigma) for 15 min at 28 °C in YPD (34), chased in fresh YPD without FM4-64 for 30–120 min as indicated, and viewed by confocal microscopy. For analysis of endocytosis (Fig. 5), cells were labeled with 2 μM FM4-64 and viewed by confocal microscopy at the indicated time points. For CMAC-Arg labeling, sac1Δ Δinp54 cells were incubated with 10 μM CMAC-Arg dye (Molecular Probes) for 4 h at 28 °C, shifted to 38 °C, further incubated for 2 h, and analyzed by confocal microscopy. To assay vacuolar fragmentation or fusion, yeast cells were grown in standard YPD medium, labeled with FM4-64 for 1 h, and then shifted to YPD + 0.4 M NaCl or H2O, respectively, for 30 min. All incubation steps were performed at 28 °C, except for sac1Δ Δinp54 cells that were incubated at 38 °C. Approximately 400 cells from three separate experiments were scored for vacuolar fragmentation, expressed as a percentage of the total cell population, and the mean ± S.E. was determined.

**Analysis of Vacuole Function**—YPD agar, pH 8.0, was prepared as described (35). Exponentially growing yeast cells were spotted onto agar plates in 10-fold serial dilutions, starting from a cell density of 10⁷ cells/ml. To visualize acidified compartments, yeast cells were incubated with 0.2 mM quinacrine (Sigma) in YPD, pH 8.0, for 5 min at room temperature, as described previously (36).

**Inhibition of Endocytosis**—To block endocytosis, sac1Δ Δinp54 cells expressing 2xPH-PLC-GFP were incubated with 33 μg/ml latrunculin A (Molecular Probes) at 28 °C for 1 h, shifted to 38 °C for 1–2 h, and labeled with 20 μM FM4-64 or 10 μM CMAC-Arg (Molecular Probes).

**Subcellular Localization of PtdIns(4)P and PtdIns(4,5)P2**— PH-OSBP was amplified from pEGFP-C1/PH-OSBP using the primers 5'-gatcataaacaagtcggctgatcagcc-3' and 5'-gatctctgctgcctacgactecc-3', and the resulting PCR product was cloned into the BglII site of pPGK1303. 2xPH-PLC81 was amplified from pEGFP-N1/PH-PLC81 using two different sets
TABLE 3
Total cellular phosphoinositide levels

| Strains                  | PtdIns(3)P | PtdIns(4)P | PtdIns(3,5)P₂ | PtdIns(4,5)P₂ |
|--------------------------|------------|------------|---------------|---------------|
| Wild-type                | 2.63 ± 0.32| 2.43 ± 0.12| 0.43 ± 0.03   | 1.23 ± 0.07   |
| Δsac1                    | 5.27 ± 0.35| 23.43 ± 0.98| 0.73 ± 0.09   | 1.0 ± 0.11    |
| Δsac1 Δinp54             | 5.53 ± 0.29| 25.07 ± 1.87| 0.93 ± 0.09   | 1.2 ± 0.21    |
| Δsac1 + Δ inp54          | 5.93 ± 0.12| 24.93 ± 1.18| 0.97 ± 0.07   | 1.13 ± 0.09   |

* Data were collected from three independent experiments, and the mean ± S.E. was determined.

of primers. The first set incorporated a BamHI site at the 5’ end (5’-ggtatccaaacaatgactggtggggccgggac-3’) and an EcoRI site at the 3’ end (5’-ggtatccgtttgtagggtctgc-3’), and the second set incorporated an EcoRI site at the 5’ end (5’-ggtatccgtttgtagggtctgc-3’) and an XhoI site at the 3’ end (5’-ctcgagactctcggggcgggct-3’). The two resulting PCR products were ligated together into BglII-XhoI-digested pPGK1303, and the two PH-2xPH-PLC-GFP constructs were subsequently transformed into yeast expression in Trans35S (Amersham Biosciences) per

Total cellular phosphoinositide levels

PtdIns(4,5)P₂ on the Vacuole Membrane

Analysis of Steady-state Levels of ALP—Extraction of ALP from yeast cells was performed as described previously (39). ALP was detected using a monoclonal ALP antibody (Molecular Probes).

Analysis of Total Cellular Phosphoinositides by HPLC—[14C]Inositol labeling of yeast, extraction and deacylation of lipids, and HPLC techniques were performed as described previously (15). Data were collected from three independent experiments, and the mean ± S.E. was determined.

RESULTS

Total Cellular Levels of PtdIns(4,5)P₂ Are Unaltered in Δsac1 Δinp54 Mutants—Inp54p and Sac1p may act sequentially to regulate PtdIns(4,5)P₂ and PtdIns(4)P levels, respectively, on specific subcellular membranes. To investigate this hypothesis we generated sac1 inp54 double null mutants (Δsac1 Δinp54) in the SEY6210 strain background. The total cellular phosphoinositide levels were determined in null mutant yeast strains by labeling cells to equilibrium with [14C]inositol. A dramatic increase in PtdIns(4)P levels was noted in Δsac1 cells (Table 3) (10), but all phosphoinositides, including PtdIns(4,5)P₂ and PtdIns(4,5)P₂, were normal in Δinp54 mutants (not shown). PtdIns(4)P levels were increased in Δsac1 Δinp54 mutants similar to Δsac1 mutants. PtdIns(4)P levels in Δsac1 mutants were also not significantly altered by Inp54p overexpression. PtdIns(3)P and PtdIns(3,5)P₂ levels were increased in Δsac1 compared with wild-type cells, but no further significant alteration was detected in Δsac1 Δinp54 mutants or when Inp54p was overexpressed in Δsac1 strains (Table 3). PtdIns(4,5)P₂ levels have been reported to decrease 4–5-fold in Δsac1 cells in some studies (40, 41), whereas in three other studies (10, 15, 19) and as shown here no significant alteration in the levels of PtdIns(4,5)P₂ in Δsac1 cells was observed (Table 3). These apparent discrepancies may relate to differences in the yeast strain and/or technical distinctions in the duration and/or methods of metabolic labeling. Overexpression of Inp54p in Δsac1 mutants did not alter PtdIns(4,5)P₂ levels, relative to wild-type, Δsac1, or Δsac1 Δinp54 strains. Therefore, Inp54p does not regulate PtdIns(4)P or PtdIns(4,5)P₂ cellular levels or interact with Sac1p to control the total cellular levels of these phosphoinositides.

PtdIns(4,5)P₂ Accumulates on Vacuolar Membranes upon Loss of Sac1p and Inp54p—When isolated vacuoles were labeled with a PtdIns(4,5)P₂ biosensor and stimulated to
undergo docking in vitro, PtdIns(4,5)P_2 was shown to be targeted to docking sites at vacuole vertices (28). However, in intact yeast and mammalian cells PtdIns(4,5)P_2 is detected predominantly at the plasma membrane (33, 42, 43), which is surprising given in vitro studies suggest PtdIns(4,5)P_2 may regulate vacuolar fusion (27, 28). To examine the spatial distribution of PtdIns(4,5)P_2 in intact yeast, we determined the localization of the PH domains of mammalian PLC-ζ1 and the yeast protein Num1p, which both bind PtdIns(4,5)P_2 with high affinity and specificity at the plasma membrane acting as PtdIns(4,5)P_2 biosensors (33, 42, 44). In both wild-type (Fig. 1A) and inp54 null mutant (not shown) strains, 2xPH-PLC-GFP localized intensely at the plasma membrane, with little cytosolic distribution. This suggests that the 5-phosphatases Inp51-3p may compensate for the loss of Inp54p in controlling the subcellular PtdIns(4,5)P_2 distribution. In contrast, 2xPH-PLC-GFP fluorescence was redistributed to the cytoplasm in ΔSac1 mutants, and little plasma membrane localization was detected (not shown), suggesting a decrease in PtdIns(4,5)P_2 levels at this site as reported (44). Strikingly, in ΔSac1 Δinp54 mutants the PtdIns(4,5)P_2 biosensor was not detected at the plasma membrane, rather fluorescence was either diffusely cytoplasmic and/or was intensely concentrated in ”ring-like” structures (Fig. 1A). Approximately 400 cells from four independent experiments were scored for 2x-PH-PLC-GFP distribution revealing that ~26 ± 3.0% (S.E.) of the double null mutant cell population exhibited PtdIns(4,5)P_2 biosensor vesicular accumulation with some cytosolic fluorescence, whereas the remaining cells showed only diffuse cytosolic fluorescence (see supplemental Fig. 1 for wide-field image), consistent with decreased plasma membrane PtdIns(4,5)P_2. The percentage of cells exhibiting vesicular accumulation of the biosensor may have been underestimated because cytosolic fluorescence may obscure faint vesicular accumulation of the biosensor as the fluorescence signal to noise ratio is low in cells expressing the biosensor at low to moderate levels. Vesicular fluorescence was not observed in the wild-type or any single null mutant. The distribution of the PtdIns(4,5)P_2-specific biosensor GFP-PH-Num1p in all strains, including wild-type, Δinp54, ΔSac1, and ΔSac1 Δinp54 (Fig. 1B, wild-type and single mutants not shown), was the same as that shown with 2xPH-PLC-GFP (Fig. 1A). Specifically the PtdIns(4,5)P_2 biosensor co-localized with FM4-64 staining of intracellular membranes in ΔSac1 Δinp54 mutants. These results suggest PtdIns(4,5)P_2 decreases at the plasma membrane in ΔSac1 Δinp54 mutants, associated with accumulation of PtdIns(4,5)P_2 on intracellular membranes.

We characterized PtdIns(4,5)P_2-positive vesicles by co-localization with membrane markers. In ΔSac1 Δinp54 mutants vesicular 2xPH-PLC-GFP fluorescence co-localized with FM4-64 staining of vacuolar membranes (Fig. 1A). In addition, FM4-64 staining revealed a fragmented vacuolar morphology in these double mutants, comprising a single large vacuole surrounded by multiple smaller vacuoles (see below). However, not all fragmented vacuoles exhibited PtdIns(4,5)P_2 biosensor fluorescence, and an average of 1.2 ± 0.1 vacuoles per 4.9 ± 0.6 total vacuoles within an individual cell (of 400 cells scored) exhibited 2xPH-PLC-GFP fluorescence. Deletion of SAC1 and INP54 in another yeast strain (BY4741) resulted in vacuolar fragmentation similar to that observed in the SEY6210 strain, and was associated with 2xPH-PLC-GFP localization to vacuolar membranes (not shown).

To ensure that PtdIns(4,5)P_2 accumulation on the vacuole was not a secondary phenotype arising from two deletion mutations, we employed the use of a sac1Δ mutant, in which INP54 was deleted. Yeast cells were grown at the permissive temperature to early log phase and then shifted to 38 °C for 1–2 h. At the permissive temperature, the PtdIns(4,5)P_2 biosensor localized to the plasma membrane in sac1Δ Δinp54 cells (Fig. 2A, see supplemental Fig. 1 for wild-field image); however, after a 1-h incubation at 38 °C, the intensity of the PtdIns(4,5)P_2 biosensor at the plasma membrane decreased significantly and instead was concentrated on intracellular vesicles that overlapped with FM4-64 labeling of endocytic intermediates (arrows) and the vacuole (arrowheads), respectively (Fig. 2A). By 2 h the PtdIns(4,5)P_2 biosensor exhibited no localization to endocytic intermediates and only co-localized with fragmented vacuoles (Fig. 2A, see supplemental Fig. 1 for wide-field image). Vacuolar fragmentation was noted only at the nonpermissive temperature.
We also examined whether \(\Delta sac 1 \Delta inp54\) inactivation regulated the spatial distribution of PtdIns(4,5)\(P_2\), the product of 5-phosphatase hydrolysis of PtdIns(4,5)\(P_2\). The localization of PtdIns(4)\(P\) was determined using the PH domain of oxysterol-binding protein (PH-OSBP) a bioreporter for PtdIns(4)\(P\) (42). In wild-type and \(\Delta inp54\) cells, PH-OSBP-GFP localized to punctate Golgi structures (supplemental Fig. 2). Despite the high levels of PtdIns(4)\(P\) in the \(\Delta sac 1\) and \(\Delta sac 1\Delta inp54\) mutants, PH-OSBP Golgi fluorescence was unchanged (supplemental Fig. 2). In additional control studies, we utilized the FYVE domain of early endosomal antigen 1 (EEA1) tagged to GFP to investigate the subcellular distribution of PtdIns(3)\(P\), a phosphoinositide implicated in vacuolar trafficking (45). FYVE-EEA1-GFP localized to punctate endosomal structures in wild-type and all mutant strains (not shown). Therefore, in \(\Delta sac 1\Delta inp54\) mutants only the spatial distribution of PtdIns(4,5)\(P_2\) is significantly altered.

To confirm vacuolar PtdIns(4,5)\(P_2\) localization, 2xPH-PLC-GFP-decorated vesicles in \(\Delta sac 1\Delta inp54\) and \(\Delta sac 1\)\(\Delta inp54\) mutants were co-localized with Vph1p, the 100-kDa subunit of the vacuolar ATPase (V-ATPase), a vacuolar membrane marker (Fig. 2B). We noted consistently that FM4-64 or V-ATPase Vph1p-labeled vesicles coincided with 2xPH-PLC-GFP fluorescence. To eliminate the possibility that 2xPH-PLC-GFP-labeled vesicles/vacuoles represent abnormal endosomal compartments, \(\Delta sac 1\)\(\Delta inp54\) cells, which had been preincubated at 38 °C for 2 h, were stained with antibodies against the late endosome resident protein Pep12p. Pep12p localized to late endosome/early endosome resident protein Pep12p. Pep12p localized to coated membranes in wild-type and all mutant strains (not shown). Single mutants not shown). To exclude the possibility that the mislocalization of 2xPH-PLC-GFP in \(\Delta sac 1\) and \(\Delta sac 1\Delta inp54\) mutants results from proteolysis of clathrin-coated vesicles (46), we investigated whether clathrin-coated vesicles accumulate/co-localize with PtdIns(4,5)\(P_2\)-coated membranes in \(\Delta sac 1\Delta inp54\) mutants by immunostaining yeast with clathrin light chain (Clc1p) antibodies. The subcellular localization of the clathrin light chain Clc1p was similar in wild-type and all mutant strains, and no accumulation of clathrin-coated vesicles or co-localization of Clc1p with 2xPH-PLC-GFP on internal membranes in \(\Delta sac 1\Delta inp54\) mutants was observed (Fig. 2D, single mutants not shown). To exclude the possibility that the mislocalization of 2xPH-PLC-GFP in \(\Delta sac 1\) and \(\Delta sac 1\Delta inp54\) mutants results from proteolysis of the GFP fusion proteins, anti-GFP immunoblot analysis of total cell lysates derived from wild-type, \(\Delta inp54\), \(\Delta sac 1\), or \(\Delta sac 1\Delta inp54\) mutants expressing 2xPH-PLC-GFP was per-
formed. Comparable expression of intact GFP fusion proteins was detected in all strains, with little proteolysis detected (not shown).

The mislocalization of PtdIns(4,5)P$_2$-binding 2xPH-PLC-GFP to the vacuolar membrane in the Δsac1 Δinp54 strain may result from mislocalization of the enzymes that generate PtdIns(4,5)P$_2$. To exclude this possibility, the intracellular localization of the PtdIns 4-kinases Stt4p and Pik1p and the PtdIns(4)P 5-kinase Mss4p was determined in the sac1, inp54 single and double null mutant cells. Pik1p and Stt4p produce ~95% of the total cellular PtdIns(4)P pool and are essential lipid kinases that contribute to the substrate pool used by Mss4p to produce PtdIns(4,5)P$_2$ (47). Therefore, the intracellular localization of the recently identified type II PtdIns 4-kinase Lsb6p (7) was not determined. The intracellular localization of Pik1p, Mss4p (supplemental Fig. 3, single mutants not shown), and Stt4p (not shown) was the same in wild-type and all null mutant strains. The phosphatidylinositol transfer protein Sec14p transfers phosphoinositides or phosphatidylcholine between membranes, an essential step in PtdIns(4)P and PtdIns(4,5)P$_2$ synthesis. Sec14p localized to punctate Golgi patches in wild-type cells, Δinp54, Δsac1, and Δsac1 Δinp54 mutants (supplemental Fig. 3, single mutants not shown).

Fig4p like Sac1p, contains a SacI domain, and in vitro assays have revealed it functions as a PtdIns(3,5)P$_2$-specific phosphoinositide phosphatase (13). Fig4p localizes to the limiting membrane of the vacuole and plays a role in regulating the turnover of vacuolar PtdIns(3,5)P$_2$. Given Sac1p and Fig4p have overlapping substrate specificity, both hydrolyze PtdIns(3,5)P$_2$, we examined the localization of 2xPH-PLC-GFP in Δfig4 Δinp54 mutants (BY4741 strain background). This PtdIns(4,5)P$_2$ biosensor localized exclusively to the plasma membrane in the double mutant strain (supplemental Fig. 4). This suggests that Sac1p regulation of PtdIns(3,5)P$_2$ does not contribute to maintaining the proper subcellular distribution of PtdIns(4,5)P$_2$.

Loss of Sac1p and Inp54p Leads to Vacuole Fusion Defects—The cumulative results from the above experiments suggest Sac1p and Inp54p phosphatases regulate the flux of plasma membrane PtdIns(4,5)P$_2$. In the absence of these lipid phosphatases plasma membrane PtdIns(4,5)P$_2$ decreases and accumulates on a subset of fragmented vacuoles. Several questions arise from these data. First, we asked whether the fragmented vacuoles in Δsac1 Δinp54 mutants indicate a vacuole fusion defect. Vacuolar fragmentation is a marker of vacuole fusion defects (48). PtdIns(4,5)P$_2$ is implicated in regulating vacuolar fusion, based on in vitro studies (27, 28); however, it has never been detected on vacuole membranes in intact cells. Given that we had shown PtdIns(4,5)P$_2$ on a subset of vacuole membranes, we further characterized the vacuolar fragmentation phenotype of sac1 inp54 double mutants. Wild-type, Δinp54, and Δsac1 strains showed normal vacuole morphology at 28 °C, whereas the vacuoles of Δsac1 Δinp54 mutants were fragmented (see Figs. 1A and 5 (120 min)). This phenotype was also evident in sac1 Δinp54 cells when incubated at the nonpermissive temperature of 38 °C (Figs. 2A and 5 (120 min)), and correlated with the accumulation of PtdIns(4,5)P$_2$ on some vacuolar membranes (Figs. 2A and 4B). It is possible that PtdIns(4,5)P$_2$ becomes trapped on a subset of vacuole membranes that only fuse with each other, because of the high PtdIns(4,5)P$_2$ levels, but fail to fuse with other vacuoles, leading to PtdIns(4,5)P$_2$ accumulation on only a subset of membranes.

To analyze specifically for homotypic and/or heterotypic vacuole fusion defects in Δsac1 Δinp54 mutants, osmotic shift...
PtIns(4,5)P₂ on the Vacuole Membrane

A

2xPH-PLC-GFP  FM4-64  Merged  Nomarski
-LatA

+LatA

B

2xPH-PLC-GFP  CMAC-Arg  Merged  Nomarski
-LatA

+LatA

C

2xPH-PLC-GFP  FM4-64  Merged  Nomarski

28°C

38°C  1 h

MSG⁻¹ ΔSac1 ΔInp54

FIGURE 4. Retention of PtIns(4,5)P₂ at the plasma membrane rescues vacuolar fragmentation defects in sac1Δinp54 mutants. A, sac1ΔΔinp54 cells expressing 2xPH-PLC-GFP were either left untreated or treated with 33 μg/ml latrunculin A (LatA) for 1 h at 28°C, labeled with 20 μM FM4-64 for 15 min, and chased for 2 h at 38°C. B, untreated sac1ΔΔinp54 cells were incubated with 10 μg/ml CMAC-Arg for 4 h at 28°C and then shifted to 38°C for 2 h (top panel). Arrows indicate the presence of CMAC-Arg in the lumen of 2xPH-PLC-GFP-decorated vacuoles. Latrunculin A-treated cells were incubated for 2 h at 38°C in the presence of 10 μg/ml CMAC-Arg (lower panel). C, mss4⁻¹ ΔSac1 ΔInp54 cells expressing 2xPH-PLC-GFP were labeled with FM4-64, chased for 1 h at 28°C (top panel), and then shifted to 38°C for 1 h (bottom panel). Bar = 5 μm. Images presented are from multiple fields.

experiments were performed. Wild-type vacuoles fragment under hyperosmotic conditions and in contrast fuse in a hypo-osmotic environment (49). Wild-type, ΔSac1 ΔInp54, and sac1ΔΔinp54 cells were grown in YPD and then incubated in either 0.4 M NaCl YPD (hyperosmotic) or water (hypo-osmotic) for 30 min. Vacuoles were scored as either nonfragmented or multi-lobed/fragmented according to the guidelines described by LaGrassa and Ungermann (49). We first examined vacuole responses to hyperosmotic treatment. Less than 30% wild-type cells showed fragmented vacuoles in untreated conditions. Upon hyperosmotic treatment, wild-type cells underwent vacuolar fragmentation with more than 50% of cells showing multiple small vacuoles (Fig. 3, A and B). >80% of ΔSac1 ΔInp54 and ~65% of sac1ΔΔinp54 cells exhibited vacuolar fragmentation in normal YPD (untreated), which increased slightly upon hyperosmotic shock (Fig. 3, A and B). We next examined for vacuolar fusion defects. Following hypo-osmotic treatment, the majority of wild-type cells exhibited nonfragmented vacuoles that had fused into one single large vacule, so that <20% of cells exhibited fragmented vacuoles. In contrast, following hypo-osmotic treatment ~70% of ΔSac1 ΔInp54 and sac1ΔΔinp54 vacuoles remained fragmented, with no evidence of fusion (Fig. 3, A and B), indicating a general vacuolar fusion defect. Therefore, loss of the lipid phosphatases Sac1p and Inp54p leads to significant vacuolar fusion defects.

PtIns(4,5)P₂ on Vacuole Membranes Originates from the Plasma Membrane—We next asked whether the accumulation of PtIns(4,5)P₂ on a subset of vacuole membranes causes the observed vacuolar fusion defect. This is an important question as PtIns(4,5)P₂ is proposed to promote vacuole fusion; however, we noted vacuole fusion defects despite evidence of increased PtIns(4,5)P₂ on vacuole membranes. Second, we asked where the vacuolar PtIns(4,5)P₂ comes from, given there is no evidence for a substantial vacuole pool of PtIns(4,5)P₂ in normal yeast.

In yeast the bulk of PtIns(4,5)P₂ is found at the plasma membrane (33, 44). To evaluate whether vacuolar PtIns(4,5)P₂ originated from the plasma membrane, sac1ΔΔinp54 cells were treated with latrunculin A (1 h), which inhibits endocytosis before shifting to the restrictive temperature for 1 h (not shown) or 2 h (Fig. 4A). Under these conditions, FM4-64 was detected on the plasma membrane in latrunculin
A-treated cells indicating endocytosis was efficiently blocked (Fig. 4A). Significantly, the PtdIns(4,5)P₂ biosensor distribution was restricted to the plasma membrane, and no vacuolar membrane fluorescence was detected (Fig. 4A). Interestingly, latrunculin A treatment also rescued the vacuolar fragmentation defect in sac1Δ Δinp54 cells at 38 °C, as detected by the vacuole lumen stain CMAC-Arg (Fig. 4B, lower panel), consistent with the contention that PtdIns(4,5)P₂ accumulation on vacuolar membranes directly or indirectly causes the vacuolar fragmentation.

As latrunculin A also inhibits actin polymerization, which may indirectly affect vacuolar fusion, we also examined the role of plasma membrane-derived PtdIns(4,5)P₂ generated by Mss4p. MSS4 encodes the type I phosphatidylinositol 4-phosphate 5-kinase and is the only known phosphoinositide kinase in yeast responsible for the synthesis of plasma membrane PtdIns(4,5)P₂ from PtdIns(4)P (8). Mss4p localizes to the plasma membrane, but it may also undergo phosphorylation-dependent shuttling between the plasma membrane and the nucleus (8, 50). To determine whether the vacuolar membrane PtdIns(4,5)P₂ detected in the sac1Δ Δinp54 mutants was generated by Mss4p at the plasma membrane, and next to substantiate the hypothesis that PtdIns(4,5)P₂ accumulation on vacuole membranes causes vacuolar fragmentation, a triple mutant strain containing a temperature-sensitive allele of MSS4 was constructed, mss4ΔΔ sac1Δ Δinp54. Temperature-sensitive mss4ΔΔ mutants exhibit a 3-fold decrease in total cellular PtdIns(4,5)P₂ levels but no obvious vacuole fragmentation (33, 44). At the permissive temperature, 2xPH-PLC-GFP localized to a subset of fragmented vacuoles with faint plasma membrane staining in this triple mutant (Fig. 4C, see supplemental Fig. 5 for wide-field image). However, after 1 h at the nonpermissive temperature, both plasma membrane and vacuolar 2xPH-PLC-GFP fluorescence were significantly attenuated, and a cytosolic distribution of the PtdIns(4,5)P₂ biosensor was detected (Fig. 4C, see supplemental Fig. 5 for wide-field image). Significantly, under these conditions the FM4-64-labeled vacuoles appeared normal and not fragmented following inactivation of Mss4p, suggesting PtdIns(4,5)P₂ accumulation on the vacuole may trigger vacuolar fragmentation. This is concordant with our observation that latrunculin A-treated sac1Δ Δinp54 cells did not accumulate PtdIns(4,5)P₂ on the vacuole and displayed nonfragmented vacuoles. Collectively these studies suggest Sac1p and Inp54p control the flux of plasma membrane PtdIns(4,5)P₂ and in their absence PtdIns(4,5)P₂ may accumulate on some vacuolar membranes leading to vacuolar fragmentation.

**Delayed Endocytic Trafficking to the Vacuole in sac1Δ inp54 Double Mutants**—As we have shown evidence of significant redistribution of PtdIns(4,5)P₂ in sac1Δ Δinp54 double mutants, we examined the functional consequences. Because PtdIns(4,5)P₂ plays a role in endocytosis (1), we investigated whether endocytosis was delayed in sac1Δ Δinp54 mutants by examining the internalization of FM4-64.

At 0 min FM4-64 accumulated on the cell surface in wild-type cells and both single null mutants; by 30 min the dye was packaged into endocytic vesicles, and by 60 min FM4-64 reached the vacuole. Inactivation of Sac1p and Inp54p also delayed endocytic trafficking in the endosomal system (51, 52). We therefore examined whether endocytosis was delayed in sac1Δ Δinp54 and sac1Δ Δinp54 mutants by examining the internalization of FM4-64. At 0 min FM4-64 reached the vacuole in wild-type cells and both single null mutants; by 30 min the dye was packaged into endocytic vesicles, and by 60 min FM4-64 reached the vacuole. However, by 60 min FM4-64 remained localized on punctate endocytic structures, although in a few cells vacuolar staining was detected. By 120 min FM4-64 had reached the partially fragmented vacuoles in sac1Δ Δinp54 and sac1Δ Δinp54 mutants. Therefore, these double mutants exhibited evidence of delayed endocytic trafficking to the vacuole but normal internalization of endocytic vesicles from the plasma membrane. These studies suggest a role for Sac1p and Inp54p in the maintenance of vacuolar homeostasis.

**Sac1p and Inp54p Mutants Exhibit Defects in Biosynthetic Vacuolar Trafficking**—The fragmented phenotype we observed in sac1Δ Δinp54 double mutants was not unlike that exhibited by some vps mutants (51, 52). We therefore examined whether Sac1p and Inp54p function in directing vesicle-mediated trafficking in the endosomal system. Defects in the endocytic pathway intersect with the Golgi-to-vacuole trafficking pathway at the level of the late endosome. ER-Golgi-to-vacuole trafficking was analyzed specifically for the ability to sort and mature the vacuolar hydrolases. The transport of CPY to the vacuole was determined by metabolic labeling of cells with trans-[35S]Cys, Met label, and chased with excess nonradiolabeled methionine and cysteine. In wild-type and Δinp54 strains, CPY commenced conversion to the mature form within 5 min of chase,
and by 10 min the prominent species was the mature CPY (Fig. 6A). However, Δsac1 mutants displayed predominantly the ER p1 form at 10 min, with little mature CPY. After 30 min of chase, mature CPY (61 kDa) was the dominant form, although the p1 form still persisted (Fig. 6A). Yeast cells were labeled with \( ^{35} \text{S} \)-Cys, Met and chased with excess nonradiolabeled methionine and cysteine at 25 °C. At each time point cell extracts were prepared, and CPY was immunoprecipitated using a CPY antibody and analyzed by SDS-PAGE and fluororadiography. p1, ER precursor of CPY; p2, Golgi precursor of CPY; m, vacuolar mature form of CPY. B, yeast cells spotted on YPD agar plates were overlaid with a nitrocellulose filter and incubated at 30 °C for 48 h, followed by immunoblotting with a CPY antibody. Δinp54 and Δinp55 strains were included as positive controls. WT, wild-type; C, yeast cell extracts were immunoblotted using an ALP antibody to analyze ALP maturation. Δapm3 mutant cells were included as a control. p, precursor; m, mature form; s, soluble form. D, yeast cells were labeled with \( ^{35} \text{S} \)-Cys, Met and chased with excess methionine and cysteine for 30 min. Medium was collected from each sample and protein precipitated with 10% trichloroacetic acid. An equivalent of 1 A\(_{\text{soc}}\) unit for each sample was analyzed by SDS-PAGE and fluororadiography. Proteins secreted by all strains are indicated by arrows, and proteins not secreted by Δsac1 Δinp54 mutants are indicated by asterisks.

FIGURE 6. inp54p contributes to the function of Sac1p in regulating the classical vacuolar trafficking pathway. A, yeast were metabolically labeled with trans-\( ^{35} \text{S} \)-Cys, Met and chased with excess nonradiolabeled methionine and cysteine at 25 °C. At each time point cell extracts were prepared, and CPY was immunoprecipitated using a CPY antibody and analyzed by SDS-PAGE and fluororadiography. p1, ER precursor of CPY; p2, Golgi precursor of CPY; m, vacuolar mature form of CPY. B, yeast cells spotted on YPD agar plates were overlaid with a nitrocellulose filter and incubated at 30 °C for 48 h, followed by immunoblotting with a CPY antibody. Δinp27 and Δinp55 strains were included as positive controls. WT, wild-type; C, yeast cell extracts were immunoblotted using an ALP antibody to analyze ALP maturation. Δapm3 mutant cells were included as a control. p, precursor; m, mature form; s, soluble form. D, yeast cells were labeled with trans-\( ^{35} \text{S} \)-Cys, Met and chased with excess methionine and cysteine for 30 min. Medium was collected from each sample and protein precipitated with 10% trichloroacetic acid. An equivalent of 1 A\(_{\text{soc}}\) unit for each sample was analyzed by SDS-PAGE and fluororadiography. Proteins secreted by all strains are indicated by arrows, and proteins not secreted by Δsac1 Δinp54 mutants are indicated by asterisks.
PtdIns(4,5)P$_2$ on the Vacuole Membrane

The results of the study described here have identified PtdIns(4,5)P$_2$-vacular membrane accumulation following inactivation of two lipid phosphatases, Sac1p and Inp54p. Evidence to support this contention was demonstrated by the vacuole membrane localization of two PtdIns(4,5)P$_2$-specific binding domains, which co-localized with vacuole membrane markers. We demonstrated vacuole membrane accumulation of PtdIns(4,5)P$_2$ in both Δsac1 Δinp54 and sac1$^{ts}$ Δinp54 mutants, indicating this is not merely a secondary effect of these null mutations. Prior to this study, PtdIns(4,5)P$_2$ has not been detected previously on vacuole membranes in intact yeast cells, although in vitro studies reported the recruitment of PtdIns(4,5)P$_2$, PtdIns(3)P, ergosterol and DAG to the vertices of purified vacuoles in docking reactions (28). In mammalian cells although PtdIns(4,5)P$_2$ has not been detected on the mammalian homologue of the vacuole, the lysosome, several lipid phosphatases that hydrolyze PtdIns(4,5)P$_2$ including the OCRL 5-phosphatase, and two novel PtdIns(4,5)P$_2$ 4-phosphatases localize to lysosomal membranes, suggesting the levels of this phosphoinositide are tightly regulated at this site (29, 30).

We propose PtdIns(4,5)P$_2$-vacuole membrane accumulation results from the trafficking of PtdIns(4,5)P$_2$-coated vesicles from the plasma membrane via the endocytic route. The results that support this hypothesis include firstly, the PtdIns(4,5)P$_2$ biosensor accumulated initially on endocytic structures in sac1$^{ts}$ Δinp54 cells at the non-permissive temperature prior to vacuole accumulation. Secondly, latrunculin A treatment which blocks endocytosis, resulted in the restriction of 2xPH-PLC-GFP fluorescence to the plasma membrane in sac1$^{ts}$ Δinp54 mutants at the non-permissive temperature. Thirdly, the introduction of a temperature sensitive allele of MSS4, which synthesizes all PtdIns(4,5)P$_2$ at the plasma membrane, into the Δsac1 Δinp54 strain resulted in redistribution of 2xPH-PLC-GFP fluorescence from vacuole membranes to the cytosol at the non-permissive temperature. Therefore, PtdIns(4,5)P$_2$ generated by Mss4p at the plasma membrane is regulated in part by Sac1p and Inp54p, and in the absence of these phosphatases PtdIns(4,5)P$_2$ may accumulate on a subset of vacuole membranes.

PtdIns(4,5)P$_2$ Turnover Is Necessary for Proper Vacuole Fusion

PtdIns(4,5)P$_2$ has been proposed to regulate vacuole fusion, specifically priming and docking of vacuoles (27). However, the accumulation of PtdIns(4,5)P$_2$ on vacuole membranes of sac1 inp54 double mutants was associated with vacuole fusion defects. Osmotic shift experiments revealed enhanced fragmentation in Δsac1 Δinp54 and sac1$^{ts}$ Δinp54 strains under hyperosmotic conditions, plus a failure of the fragmented vacuoles to fuse in response to hypo-osmotic shift. Interestingly PtdIns(4,5)P$_2$ did not appear to accumulate on all vacuoles, this may relate to the sensitivity of the detection of PtdIns(4,5)P$_2$ biosensor, or alternatively, it is possible that PtdIns(4,5)P$_2$ accumulation on the vacuole of sac1 inp54 mutants may result from the fusion of plasma membrane-derived PtdIns(4,5)P$_2$-decorated endocytic vesicles with one another, rather than with other vacuole membranes, generating a subset of PtdIns(4,5)P$_2$-enriched vacuoles.

An interesting question that arises from these observations is that if PtdIns(4,5)P$_2$ promotes vacuole fusion, why does the PtdIns(4,5)P$_2$-enriched vacuole not fuse with other vacuoles in the cell? It is possible that high levels of PtdIns(4,5)P$_2$ may change the membrane topology of the vacuole. Membrane topology depends on the lipid composition of the membrane...
PtdIns(4,5)P₂ on the Vacuole Membrane

bilayer, and changes in lipid composition affect membrane curvature (for review see (55)). As remodeling of membrane curvature allows budding and fusion of transport vesicles, it is possible that vacuolar membrane fusion also requires this process. The high concentration of PtdIns(4,5)P₂ on some vacuole membranes in sac1 inp54 mutants may stabilize the membrane topology to an extent that prevents further changes in membrane curvature. Alternatively, the high concentration of PtdIns(4,5)P₂ may mask other lipids that recruit other proteins which generate membrane curvature (reviewed in (56)), or proteins that facilitate vacuole fusion, e.g. PtdIns(3)P recruitment of Vam7p (57). As yet, it is not clear what happens to PtdIns(4,5)P₂ after vacuole priming and docking. Does it remain on the vacuole or is it hydrolyzed or sequestered away prior to fusion? Studies in mice lacking the 5-phosphatase synaptojanin 1 have revealed increased PtdIns(4,5)P₂ levels and accumulation of clathrin-coated vesicles at nerve terminals (58), indicating the importance of PtdIns(4,5)P₂ hydrolysis in vesicle uncoating, which facilitates fusion. The amount of PtdIns(4,5)P₂ on the vacuole may be critical for correct vacuolar fusion. Evidence from our studies suggests that the bulk of cellular PtdIns(4,5)P₂ has to be sequestered away from the vacuole for fusion to proceed correctly, as shown by the nonfragmented vacuoles of sac1 inp54 mutants when PtdIns(4,5)P₂ accumulation on the vacuole is abolished by either latrunculin A treatment or inactivating Mss4p.

Docking of vacuoles, which precedes fusion, also requires vacuole acidification; this is mediated by the V-ATPase proton pump that is needed for trans-SNARE complex formation during docking (59). Even though the growth of ∆sac1 inp54 mutants was compromised in alkaline medium, their vacuoles were sufficiently acidic to accumulate quinacrine. Furthermore, the presence of the V-ATPase subunit Vph1p on the vacuole membrane suggests that the V-ATPase proton pump assembles and functions properly in these mutants. Therefore, any defect in vacuole docking/fusion is more likely to be caused by PtdIns(4,5)P₂ accumulation on the vacuole, rather than from compromised acidification.

We propose that PtdIns(4,5)P₂ hydrolysis by lipid phosphatases is important for regulating vacuole fusion. Indeed, a genomic screen of yeast deletion mutants with vacuolar fragmentation suggests regulation of PtdIns(4,5)P₂ by phospholipase C is essential for vacuole fusion (48). In the same study, an inp54 mutant was also found to display vacuolar fragmentation, in contrast to our findings that Δinp54 mutants have normal vacuole morphology. This may be due to strain-specific differences. Double deletions of INP51, -52, and -53 resulted in fragmented vacuoles, again highlighting the importance of PtdIns(4,5)P₂ turnover in regulating vacuole fusion (23, 24).

Possible Functional Interaction of Sac1p and Inp54p in Vacuolar Function/Homeostasis—There are four distinct lipid phosphatases in yeast that hydrolyze PtdIns(4,5)P₂, forming PtdIns(4)P, Inp51-4p. In addition to their 5-phosphatase domain, Inp52p and Inp53p also contain a SacI-like catalytic domain, which functions principally to regulate the levels of PtdIns(4)P, as does Sac1p itself. It has been proposed that Inp52p and/or Inp53p can each hydrolyze PtdIns(4,5)P₂ to PtdIns(4)P by the 5-phosphatase domain and then PtdIns(4)P to PtdIns by the SacI-like domain (10). In contrast, Inp51p and Inp54p do not have a functional SacI-like domain. Although there is no reason per se to anticipate that Sac1p and Inp54p should functionally interact and given Inp54p is one of two yeast 5-phosphatases that does not have a functional SacI domain, it was possible that Sac1p serves this role for Inp54p. Equally possible, although not explored here, Inp51p may interact with Sac1p, as this 5-phosphatase also lacks a functional SacI domain.

A critical question to address is whether Sac1p and Inp54p functionally interact to regulate vesicular trafficking and/or vacuole fusion. The major defects noted in the sac1 inp54 mutants were vacuolar fragmentation and fusion defects, delayed CPY sorting, a marker of ER-Golgi-to-vacuole trafficking, associated with specific cargo secretion defects, suggesting impaired coatamer I (COPI) function (38). The latter observation is consistent with recent studies that revealed members of the COPII complex functionally interact with human SAC1 (60).

Despite vacuole fusion defects, CPY and FM4-64, although delayed, and ALP can reach the vacuole in Δsac1 Δinp54 mutants. This could be explained by the different requirements for homotypic vacuole fusion and heterotypic fusion between other organelles and the vacuoles. A defect in homotypic vacuole fusion does not necessarily indicate a defect in cargo sorting to the vacuole. For example, the vacuolar v-SNARE Nyv1p is required for vacuole docking (61), which precedes vacuole-vacuole fusion, but it is not required in the biosynthetic pathways to the vacuole (62). A genomic screen of 4828 nonessential gene deletions in yeast revealed many mutants with vacuolar fragmentation but no vps phenotype (48), which indicates that there is an overlap as well as distinction between pathways of trafficking to the vacuole and vacuole-vacuole fusion.

In summary, this study has demonstrated the lipid phosphatases Sac1p and Inp54p regulate plasma membrane PtdIns(4,5)P₂ distribution. Although PtdIns(4,5)P₂ has been implicated in vacuolar function, this study shows that PtdIns(4,5)P₂ can accumulate on vacuole membranes in the absence of specific lipid phosphatases in intact yeast cells and is associated with vacuole fusion defects.

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