Yeast Lipin 1 Orthologue Pah1p Regulates Vacuole Homeostasis and Membrane Fusion*

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Received for publication, October 25, 2011, and in revised form, November 20, 2011 Published, JBC Papers in Press, November 25, 2011, DOI 10.1074/jbc.M111.317420

Vacuole homotypic fusion requires a group of regulatory lipids that includes diacylglycerol, a fusogenic lipid that is produced through multiple metabolic pathways including the dephosphorylation of phosphatidic acid (PA). Here we examined the relationship between membrane fusion and PA phosphatase activity. Pah1p is the single yeast homologue of the Lipin family of PA phosphatases. Deletion of PAH1 restored fusion, whereas the phosphatase dead mutant Pah1pΔD398E had no effect. We discovered that the lack of PAH1 alters the sorting of fusion factors to the vacuole and inhibits fusion.

Background: Pah1p is a phosphatidic acid phosphatase that generates diacylglycerol.

Results: Deletion of PAH1 alters the sorting of fusion factors to the vacuole and inhibits fusion.

Conclusion: Conversion of phosphatidic acid to diacylglycerol is integral to vacuole homeostasis.

Significance: This is the first report that links a Lipin 1 homologue to the recruitment and activation of a Rab GTPase or the sorting of SNARE proteins.

Eukaryotic cells are compartmentalized by membrane-bound organelles that communicate through the trafficking of transport vesicles. The transport of cargo between organelles as well as between the plasma membrane and organelles requires a series of trafficking events that culminates in the fusion of membranes and mixture of luminal contents. The mechanisms and machinery that drive membrane fusion are conserved from single cell eukaryotes to metazoans (1). We use vacuolar lysosomes from the yeast Saccharomyces cerevisiae to examine the regulation of vesicle fusion. Vacuole fusion requires many factors including three Q-SNAREs (Vam3p, Vti1p, and Vam7p), one R-SNARE (Nvy1p), and the SNARE priming machinery Sec17p (α-soluble NSF attachment protein) and Sec18p (NSF). Fusion also requires the Rab GTPase Ypt7p, the effector complex HOPS, and regulatory lipids that include phosphoinositides, ergosterol, and DAG (2–4).

Vacuole fusion occurs through a series of stages that begins with priming when cis-SNARE complexes bound to Sec17p are disassembled by Sec18p, causing the release of Sec17p and the soluble SNARE Vam7p from the membrane (5, 6). During Ypt7p-dependent tethering/docking, Vam7p rebinds the membrane through its Phox homology domain that interacts with HOPS and phosphatidylinositol 3-phosphate (PI3P) (5, 7–9). Next, SNAREs form trans-complexes across the docking junction (10), stimulating the release of luminal calcium stores (11). During the tethering/docking stages vacuoles form three distinct morphological features. Vacuoles become tightly associated and form two flattened membrane discs termed the “boundary membrane.” Proteins and lipids that mediate fusion become enriched at the boundary perimeter, termed the “vertex ring” (2, 12, 13).

Lipid metabolism is critical for the assembly and function of the vertex ring and is essential for membrane fusion (2–5, 14, 15). However, little has been reported on the role of individual metabolic reactions during fusion. Here we examined the role of phosphatidic acid (PA) dephosphorylation to form DAG. The importance of DAG and PA has been recently highlighted in several trafficking pathways. DAG is important for the formation of COP-I vesicles in retrograde traffic from the Golgi to the trans-Golgi network (16). The importance of DAG and PA has been recently highlighted in several trafficking pathways. DAG is important for the formation of COP-I vesicles in retrograde traffic from the Golgi to the trans-Golgi network (16).

The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; CBP, calmodulin-binding peptide; COR-VET, class C core vacuole/endosome tethering; DAG, diacylglycerol; HOPS, homotypic fusion and vacuole protein sorting complex; NSF, N-ethylmaleimide-sensitive factor; PA, phosphatidic acid; PI, phosphatidylinositol; PI3P, phosphatidylinositol 3-phosphate; PAP, PA phosphatase; YPD, yeast extract/peptone/dextrose; Rh-PE, rhodamine phosphatidylethanolamine.

* This work was supported in part by March of Dimes Birth Defects Foundation Basil O’Connor Starter Scholar Research Award Grant 5-FY09-117 (to R. A. F.) and startup funds provided by the University of Illinois at Urbana-Champaign (to R. A. F.).

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Pah1p and Membrane Fusion

TABLE 1

<table>
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<tr>
<th>Strain</th>
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<tr>
<td>BJ3505</td>
<td>MATa pep4::HIS3 prb1::Δ1.6R his3-200 trp1::Δ101 (gal3) ura3-52 gal2 can1</td>
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<td>DKY6281</td>
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<td>GFP Ypt7</td>
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<td>RFY18, PAH1::3XHA</td>
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<tr>
<td>RFY27</td>
<td>DKY6281, PAH1::GFP</td>
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the endoplasmic reticulum (16) as well as tubulation within the Golgi apparatus (17). PA also plays an important role in SNARE activation during sporulation (18, 19), mitochondrial fusion (20), and exocytosis (21). Formation of both PA and DAG occurs through multiple metabolic pathways. In many studies the generation of PA and DAG is studied in the context of phospholipase activities. PA is the product of phospholipase D activity on phosphatidylcholine, whereas phospholipase C vari-

ants hydrolyze either phosphatidylcholine or phosphatidyli-

nositol 4,5-bisphosphate to produce DAG. Furthermore, PA
and DAG can be interconverted by DAG kinase and PA phos-

phatases. Although many studies have focused on phospho-

lipase C and phospholipase D activity in the context of mem-

brane trafficking, little is known about the importance of

PA phosphatase (PAP) activity in vacuole fusion.

The importance of PA phosphatase (PAP) activity in vacuole fusion has not been thoroughly explored. S. cerevisiae

contains three PAPs. Lpp1p is a polytopic PAP localized to

Golgi puncta and dephosphorylates PA, lysophosphatidic acid, and DAG pyrophosphate via a Mg2+-dependent mechanism (22). Dpp1p is a Zn2+-regulated seven-transmembrane domain PAP localized to the vacuole and acts on DAG pyrophosphate and PA (23). The soluble PAP Pah1p only hydrolyzes PA in a Mg2+-dependent manner (24). Although all three PAPs can hydrolyze PA, Pah1p is the principal producer of DAG from PA (24). Mutations in the PAH1 homologue LPIN1 have been identified as the cause of fatty liver dystrophy and termed an “obesity” gene (25). WT Lipin 1 functions in glucose metabolism (26) and in the regulation of insulin levels (26, 27). Furthermore, mutations in Lipin 1 have been shown to lead to obesity by reducing fatty acid oxidation and energy expenditure (28) and may lead to severe consequences such as the onset of insulin resistance, a hallmark of adult obesity and type 2 diabetes.

Pah1p is a Mg2+-dependent soluble 95-kDa protein with a central catalytic motif and an N-terminal amphipathic helix (29). The PA phosphatase activity of Pah1p is constitutive; however, its association with membranes and subsequent enzymatic activity is controlled through its state of phosphorylation. Phosphorylated Pah1p is soluble and present in the cytoplasm, whereas dephosphorylated Pah1p localizes to membranes and functions as a PA phosphatase (30). Pah1p is phosphorylated by the cyclin-dependent kinase Cdc28p and dephosphorylated by the membrane-anchored Nem1p-Spo7p phosphatase complex (31, 32). The association of Pah1p with membranes is mediated by an N-terminal amphipathic helix (33) that once dephosphorylated can bind to membranes, and deletion of the helix prevents catalytically active Pah1p from acting on its substrate. Although most of the membrane-bound Pah1p is found on the endoplasmic reticulum, active Pah1p can bind to other membranes, including lipid droplets, or artificial liposomes (30, 33, 34).

Pah1p plays a critical role in regulating the overall synthesis of lipid. When Pah1p is phosphorylated it indirectly regulates the expression of phospholipid synthesis genes (35). Phosphorylated Pah1p translocates to the nucleus where it regulates INO1 for the production of lipids. This is critical during mitosis when the nuclear envelope and endoplasmic reticulum have to expand to replicate the organelles for daughter cells. Unphosphorylated Pah1p generates DAG, which can feed into the synthesis of triacylglycerol, phosphatidylcholine, and phosphati-
dylethanolamine (24). Deletion of PAH1 leads to an accumulation PA and depletion of DAG and triacylglycerol.

EXPERIMENTAL PROCEDURES

Reagents—Reagents were dissolved in PS buffer (20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol). Anti-Vam3p (36), anti-Sec18p (6), anti-Ypt7p (8), anti-Vps33p (37), GST-FYVE (38), His6-Gyp1–46p (13), Gdi1p (39), MARCKS effector domain (2), and GST-Vam7p (WT, Q283R and Y42A) (40, 41) were described previously. Propranolol, atenolol, and acebutolol were from Sigma and dissolved in PS buffer.

Strains—BJ3505 and DKY6281 were used for fusion assays (42, 43) (Table 1). BJ3505 modulin binding peptide (CBP)-Vam3p nvy1Δ was used for trans-SNARE complex isolation (44). BJ3505 CBP-Vam3p harboring Nyv1p was used for Sec18p-SNARE complex isolation. PAH1 was deleted by homologous recombination with the kanMX6 cassette using PCR products amplified from pFA6a-kanMX6 (45) with homology flanking the PAH1 coding sequences with the primers 5'-PAH1-KO and 3'-PAH1-KO (Table 2). The PCR product was transformed by standard lithium acetate methods into BJ3505 and DKY6281 to generate RFY17 and RFY18, respec-
TABLE 2

<table>
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<tr>
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<td>3′-PAH1-GFP</td>
<td>5′-ATTGATGACAGATGATCCAGGAAGATTAGAATTCG AAATAGTGGACGCTCGTTTAAAC-3′</td>
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</table>

Transformants were selected using YPD media containing G418. For complementation experiments, WT PAH1 and PAH1D398E, were subcloned from pGH312 and pGH312-D398E plasmids (46) (a gift from G. Carman, Rutgers University) into YEp352 using XbaI and SpeI. RY17 and RY18 were transformed with pPAH1 or pPAH1D398E to generate RY19– 22. Transformants were selected using complete synthetic media lacking uracil. DPP1 was deleted with the LIRA3 cassette using PCR products amplified form pRS406 with homology flanking the DPP1 open reading frame with the primers 5′-DPP1-KO and 3′-DPP1-KO. The PCR products were transformed into BJ3505 and DKY6281 to generate RY23 and RY24. LPP1 was deleted using the hphMX4 or LIRA3 cassettes using PCR products amplified form pAG32 or pRS406, respectively, with homology flanking the LPP1 open reading frame with the primers 5′-LPP1-KO-BJ3505 and 3′-LPP1-KO-BJ3505 or 5′-LPP1-KO-DKY6281 and 3′-LPP1-KO-DKY6281 (45, 47). The PCR products were transformed into BJ3505 and DKY6281 to generate RY25 and RY26. For vacuole localization studies, PAH1 was fused in-frame to GFP by homologous recombination. DKY6281 was transformed with a PCR product amplified from pFA6a-GFP(S65T)-kanMX6 with the primers 5′-PAH1-GFP and 3′-PAH1-GFP with homology flanking the stop codon of the PAH1 gene to make RY27 (48).

Sec18p-SNARE Complex Detection—Analysis of Sec18p binding to cis-SNARE complexes was conducted using a modified trans-SNARE complex isolation assay (44, 50). For this assay we used vacuoles from BJ3505-CBP-Vam3p (CBP-VAM3 NYV1). The isolation of cis-SNARE-bound Sec18p was performed using 16× large-scale reactions (480 μl) containing 96 μm of vacuoles and incubated at 27 °C for 5 or 10 min and treated with buffer, propranolol, or anti-Sec18p to inhibit priming. Separate reactions were kept on ice as 0 min samples. After incubating, reactions were placed on ice for 5 min, and 30-μl samples were withdrawn from each reaction to assay Pho8p activity. The remaining 450-μl samples were centrifuged (13,000 × g, 15 min, 4 °C), and the supernatants were decanted. Vacuole pellets were overlaid with 200 μl of ice-cold solubilization buffer (20 mm Tris-Cl, pH 7.5, 150 mm NaCl, 1 mm MgCl2, 0.5% Nonidet P-40 alternative, 10% glycerol) with protease inhibitors (0.4 μg/ml leupeptin, 3.5 μg/ml pepstatin A, 2.4 μg/ml Pefabloc-SC, 1 mm PMSF) and gently resuspended. Solubilization buffer was added to a final volume of 600 μl, and extracts were mixed (20 min, 4 °C, nutating). Detergent-insoluble debris was removed by centrifugation (16,000 g, 20 min, 4 °C). Supernatants were transferred to fresh tubes, and 10% of (100,000 × g, 1 h, 4 °C) and resuspended in buffer (20 mM HEPES-KOH, pH 7.4, with protease inhibitors).

Lipid Mixing Fusion Assay—Lipid mixing assays were conducted using rhodamine phosphatidylethanolamine (Rh-PE; Invitrogen) as described with minor modifications (50). Briefly, BJ3505 background vacuoles (300 μg) were incubated in 400 μl of PS buffer containing 150 μm Rh-PE (10 min, 4 °C, nutating). Samples were mixed with 15% Ficoll in PS buffer (w/v) and transferred to a polyalumirultracentrifuge tube. Samples were overlaid with 1.0 ml each of 8, 4, and 0% Ficoll. Labeled vacuoles were re-isolated by centrifugation using a Beckman SW60 Ti rotor (105,200 g, 30 min, 4 °C) and recovered from the 0–4–Ficoll interface. Lipid mixing assays (90 μl) contained 2 μg of Rh-PE-labeled vacuoles and 16 μg of unlabeled vacuoles in fusion buffer. Reaction mixtures were transferred to a black, half-volume 96-well flat-bottom microtiter plate with nonbinding surface (Corning). Rhodamine fluorescence (λex = 544 nm; λem = 590 nm) was measured using a POLARstar Omega fluorescent plate reader (BMG Labtech) at 27 °C. Measurements were taken every min for 75 min, yielding fluorescence values at the onset (F0) and during the reaction (F). The final 10 measurements of a sample were taken after adding 0.33% (v/v) Triton X-100, averaged, and used as a value for the fluorescence after infinite dilution (F0×100). The relative total fluorescence change ΔF/F0×100 = (F – F0)/F0×100 was calculated.
the extract was removed for input samples. The remaining extracts were brought to 2 mM CaCl₂. CBP-Vam3p complexes were incubated with 50 μl of equilibrated calmodulin-Sepharose 4B (GE Healthcare) (4 °C, 12 h, nutating). Beads were collected by centrifugation (4,000 g, 2 min, 4 °C) and suspended 4 times with the solubilization buffer followed by bead sedimentation. Bound proteins were eluted with SDS sample buffer containing 5 mM EGTA and heated at 95 °C for 5 min. The samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed by immunoblotting. Goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Pierce) was used with enhanced chemiluminescence substrate (GE Healthcare). Images were acquired using a Storm PhosphorImager 8400 (Molecular Dynamics).

**Trans-SNARE Complex Assay**—The method for detecting trans-SNARE complexes is essentially identical to that of Sec18p-SNARE complex isolation with one modification. In the trans-SNARE assembly assay, we used vacuoles that lack NYV1 and contain CBP-Vam3p on the same membrane (CBP-VAM3 NYV1). These vacuoles were incubated with WT DKY6281 (VAM3 NYV1) for 60 min and processed for CBP-Vam3p isolation as described above.

**GST-Vam7 Pulldown**—GST-Vam7p-protein complex isolation was performed as described (40, 41). Briefly, large-scale 6× fusion reactions (180 μl) were incubated with 85 μg/ml anti-Sec17p IgG to block priming. After 15 min, anti-Vam3p IgG was added to selected reactions and incubated for an additional 5 min, adding buffer or 400 nm GST-Vam7p. After a total of 90 min, reactions were placed on ice for 5 min, and 30–μl aliquots were removed to measure fusion activity. The remaining vacuoles were re-isolated by centrifugation (11,000 × g, 10 min, 4 °C), and the supernatants were decanted before extracting vacuoles with solubilization buffer (20 mM HEPES-KOH, pH 7.4, 100 mM NaCl, 2 mM EDTA, 20% glycerol, 0.5% Triton X-100, 1 mM DTT) with protease inhibitors (1 mM PMSF, 10 mM Pefabloc-SC, 5 μM pepstatin A, and 1 μM leupeptin). Vacuole pellets were gently resuspended with 200 μl of solubilization buffer and incubation for 20 min on ice. Insoluble debris was sedimented by centrifugation (16,000 g, 10 min, 4 °C), and 176 μl of supernatants were transferred to new chilled tubes. Next, 16 μl was removed from each reaction as 10% total samples, mixed with SDS loading buffer, and heated (95 °C, 5 min). The remaining extracts were incubated with 30 μl of equilibrated glutathione-Sepharose 4B beads (GE Healthcare) (12 h, 4 °C, nutating). Beads were sedimented (3,000 rpm, 2 min, 4 °C) and washed with 1 ml solubilization buffer five times, and bound material was eluted with SDS loading buffer. Protein complexes were examined by Western blotting.

**PA Phosphatase Activity**—To examine native PAP activity on isolated vacuoles, we used thin layer chromatography (TLC). Isolated vacuoles were incubated with PA that was covalently tagged with the fluorescent molecule NBD. In this assay short chain Di-C6-NBD PA (0.4 μg; Avanti Polar lipids) was incubated with 3 μg of isolated vacuoles and treated with propa- nolol. This was carried out in reaction buffer (50 mM HEPES-KOH, pH 7.4, 200 mM sorbitol 100 mM NaCl, and 0.5 mg/ml BSA) and the ATP regenerating system. Reactions were incubated for 30 min at 30 °C. Lipids were extracted as described with some modification (51). Before extractions, three freeze-thaw cycles were performed using a 95 °C dry-bath and −80 °C freezer. Reactions were phase-extracted with a monophase of chloroform/methanol/water (2:1:0.8). Next, 1 ml each of chloroform and 1 x HCl were added to form two phases. The lower organic phase was removed and dried under an N₂ stream. Dried reactions were resuspended in 10 μl chloroform and spotted on to glass backed TLC plates (K₆ silica gel; Whatman). Lipids were resolved in chloroform/methanol/acetic acid/water (53:10.5:21:10:5.3) and air-dried. Fluorescence was visualized using a Storm PhosphorImager 8400 with an excitation of 450 nm and emission of 520 nm.

**Vacuole Docking**—Vacuoles were purified from Pah1p-GFP, pah1Δ (RFY18), or DKY8281 strains and used in docking assays as previously described (2). Docking reactions contained 6 μg of vacuoles in 30 μl of docking reaction buffer (100 mM KCl, 0.5 mM MgCl₂, 20 mM Pipes-KOH, pH 6.8, 200 mM sorbitol), ATP regenerating system (0.3 mM ATP, 6 mM creatine phosphate, 0.7 mg/ml creatine kinase), 20 μM coenzyme A, and 283 nM IB₂ (inhibitor of protease B). After incubation (30 min, 27 °C), reactions were placed on ice, and vacuoles were stained with either 3 μM FM4-64 or 1 μM PSS-380. Where indicated, PI3P was detected with 0.2 μM Cy5-FYVE domain. Vacuoles were then mixed with 50 μl of 0.6% agarose in PS buffer and vortexed (3 s, medium setting), and 15–μl samples were mounted on slides and observed by fluorescence microscopy.

**Ratiometric Quantitation**—For ratiometric measurements of Cy5-FYVE localization, the maximum pixel values for vertex and outer edge membrane were measured for specific and non-specific probes in each fluorescence channel as described (2). All vertices and outer edge membranes were measured within a vacuole cluster. In each experiment 15–20 clusters containing 100–300 vertices were analyzed for each treatment in multiple experiments. The data are presented in a representative experiment. First, pixel ratios of vertex-to-outer edge membrane were taken for each fluorescent channel. Pixel ratios for non-specific labeling were normalized to 1. Next, ratios of specific-to-non-specific labels were taken at each vertex and outer edge membranes, and enrichment was expressed relative to outer edge membrane intensities.

**Whole Cell Imaging**—Vacuole morphology was monitored by incubating yeast cells in YPD broth containing 5 μM FM4-64. Cultures (5 ml) were grown for 1 h in YPD with FM4-64 at 30 °C, washed with PBS, and chased for 3 h with fresh YPD alone. After incubation, the cells were concentrated by centrifugation, resuspended in 50 μl of PBS, mixed with 50 μl 0.6% agarose, and mounted (15 μl) on glass slides for observation. Images were acquired using a Zeiss Axio Observer Z1 inverted microscope equipped with a X-Cite 120XL light source, Plan Apochromat 63× oil objective (NA 1.4), and an AxioCam CCD camera.

**RESULTS**

**Phosphatidic Acid Phosphatase Activity Is Required for Vacuole Homeostasis and Fusion**—Vacuole fusion requires multiple regulatory lipids including DAG (2, 3). Although the generation of vacuolar DAG through the hydrolysis of phosphatidylinositol 4,5-bisphosphate by Plc1p has been demonstrated, the production of DAG through the dephosphoryl-
To test whether propranolol inhibited vacuole fusion, we used a content mixing assay as previously described (43). Fusion was measured by the maturation of pro-alkaline phosphatase (proPho8p) by proteinase A (Pep4p). Vacuoles were purified from the reciprocally deleted strains DKY6281 (PEP4 pep4Δ) and BJ3505 (pep4Δ PHO8) and used in vitro to measure fusion as described under “Experimental Procedures.” We found that propranolol potently inhibited vacuole fusion in a dose-dependent manner (Fig. 1E), suggesting that PAP activity was required for vacuole fusion. The concentrations required to inhibit fusion mirrored the IC50 values reported for purified mammalian Lipin 1 orthologues (54). To determine whether other β-adrenergic receptor antagonists would affect fusion, we also tested atenolol and acebutolol and found that neither compound affected fusion (not shown), suggesting that propranolol inhibited fusion by directly inhibiting PAP activity and not through nonspecific interactions with the membrane. To validate that the effects of propranolol were not due to the inhibition of the Pho8p detection system, we examined its effect on fusion using a real-time lipid-mixing assay (55, 56). Vacuoles were labeled with Rh-PE at concentrations that caused self-quenching dimerization of the fluorophore. Rh-PE-labeled vacuoles were incubated with an excess of unlabeled vacuoles and monitored for fluorescence dequenching upon fusion. Fusion reactions were incubated with buffer (Fig. 1F, closed circles) or propranolol (Fig. 1F, open circles). Rh-PE dequenching was inhibited by propranolol in a dose-dependent manner that recapitulated the inhibition curve seen in content mixing assays.

Deletion of Lipin 1 Orthologue Pah1p Disrupts Vacuolar Homeostasis—Because propranolol inhibited vacuole fusion, we next sought to determine which of the three yeast PA phosphatases regulated this process. We deleted DPP1, LPP1, and PAH1 from our fusion tester strains and examined their vacuolar phenotype using FM4-64. Relative to WT cells (Fig. 2, A and B) we found that the deletion of either DPP1 (Fig. 2, C and D) or LPP1 (Fig. 2, E and F) had no effect on vacuole morphology. Importantly, the vacuoles in pah1Δ cells (Fig. 2, G and H) were fragmented and deformed in a manner similar to the effect of propranolol, indicating that Pah1p activity was specifically involved in vacuolar homeostasis.

We next tested in vitro fusion using purified vacuoles from WT and pah1Δ cells and found that fusion was severely attenuated by the deletion of PAH1 (Fig. 3A). Because the lack of fusion was likely due to the absence of PAP activity, we complemented pah1Δ strains with plasmids that expressed either WT PAH1 or the phosphatase dead mutant PAH1D398E (46). Complementation of pah1Δ strains with WT pPAH1 fully rescued fusion, illustrating that the inhibition in fusion was directly due to the loss of Pah1p (Fig. 3B). In contrast, pPAH1D398E did not rescue the null phenotype, demonstrating that Pah1p phosphatase activity was required for vacuole fusion (Fig. 3C). We next labeled the vacuoles of WT, pah1Δ, and complemented strains with FM4-64. As seen in Fig. 2, WT cells contained 1–2 vacuoles per cell (Fig. 3D), whereas pah1Δ cells harbored fragmented and deformed vacuoles (Fig. 3E). When pah1Δ cells were complemented with pPAH1, we observed the restoration of WT vacuole morphology (Fig. 3F), whereas the expression of
pPAH1<sup>D398E</sup> did not affect the fragmentation phenotype (Fig. 3G). To verify that changes in fusion using pah1Δ strains were not due to the inactivation of the Pho8p/Pep4p reporter system, we also performed lipid-quenching experiments using pah1Δ vacuoles. As seen with the content mixing assay, pah1Δ vacuoles were blocked in fusion relative to WT vacuoles (Fig. 3H). We also deleted DPP1 and LPPI from our fusion tester strains and found that dpp1Δ and lpp1Δ vacuoles fused as well as WT vacuoles (Fig. 3, I and J, respectively). Together these data demonstrated that only Pah1p played a role in vacuole fusion.

**Pah1p Localizes to Vacuole**—Because Pah1p is a soluble phosphatase that mostly associates with the endoplasmic reticulum, which in whole cells masks its localization to other organelles, we examined purified vacuoles for the recruitment of this enzyme. To this end we probed for Pah1p on vacuoles by microscopy. Purified Pah1p-GFP vacuoles were incubated in docking reactions and observed by wide-field fluorescence microscopy. FM4-64 was used to stain the membrane. We found that Pah1p-GFP was present on the vacuole and evenly distributed across the membrane (Fig. 4B). This was not easily observed when examining whole cells by microscopy. The high concentration of Pah1p-GFP in the cytosol masked vacuole-associated protein (not shown). To confirm that Pah1p-GFP was indeed localized to the vacuolar membrane and not due to contaminating debris, we performed deconvolution fluorescence microscopy. Here, Pah1p-GFP vacuoles were co-stained with PSS-380 to label phosphatidylinositol (57). Z-series of images were collected of individual vacuoles and deconvolved using Axiovision software. Individual vacuoles were used instead of clusters to eliminate the possible fluorescence contributed by membranous debris. Fig. 4C shows central slice images of Pah1p-GFP and PSS-380. We used PSS-380 in lieu of FM4-64 due of its resistance to bleaching during multiple exposures in Z-series acquisition. This further demonstrated that Pah1p-GFP was truly recruited to the vacuolar membrane and not associated with contaminating membranes.

**PAP Activity Is Required for SNARE Priming**—To determine when PAP activity functions during the fusion pathway, we tested the temporal acquisition of resistance to propranolol (6, 7, 36). As a fusion reaction passes a specific stage, such as Sec18p-dependent priming, it gains resistance to inhibitors that target that particular stage (e.g. anti-Sec18p) (6). Here we compared the ability of propranolol or anti-Sec18p antibody to inhibit fusion. Inhibitors were added at fixed concentrations to individual reactions at the indicated times and incubated for a total of 60 min. We found that fusion became resistant to anti-Sec18p (Fig. 5A, open diamonds) with kinetics similar to previous reports (6, 58, 59). In addition, we found that the acquisition of resistance toward propranolol followed a kinetic curve similar to that of anti-Sec18p (Fig. 5A, open circles). This strongly suggested that PAP function was only required during the priming stage.

We next examined the release of Sec17p (α-soluble NSF attachment protein) from SNAREs after cis-complex disassembly by Sec18p (6). Fusion reactions were incubated with buffer or propranolol for the indicated times followed by centrifugation to collect the vacuole fraction and decanting of the supernatant. Fig. 5B shows that Sec17p was released from vacuoles when treated with buffer alone; however, this was blocked in the presence of propranolol, indicating that SNARE priming required PAP activity. As a control for the loss of membranes during re-isolation, we probed for Ypt7p, a membrane-anchored Rab GTPase. Neither treatment affected Ypt7p levels, demonstrating that the loss of Sec17p during priming was not due to a loss of membranes.
PA Phosphatase Activity Regulates Sec18p-SNARE Binding—Because propranolol inhibition was shown to occur during the priming stage, we next examined the specific binding of Sec18p to SNARE complexes during priming. For these experiments we used vacuoles that harbored Vam3p containing an internal CBP between its regulatory N terminus and SNARE domain (CBP-VAM3 NYV1) (44). We monitored the release of Sec18p from SNARE complexes after priming. In untreated reactions, Sec18p was released from SNARE complexes over time as priming occurred (Fig. 5C). The release of Sec18p was blocked when priming was inhibited with anti-Sec18p antibody. When reactions were treated with propranolol we observed that Sec18p was sharply reduced at the beginning of the reaction. Fig. 5D shows quantitation of Sec18p associated with CBP-Vam3p complexes, suggesting that PAP activity was required for Sec18p association with cis-SNARE complexes and that downstream effects were manifest in the slowed rate of priming.

Next, we determined whether propranolol caused a general loss of Sec18p from the entirety of the vacuolar membranes. Fusion reactions were treated with buffer or propranolol, after which membranes were re-isolated by centrifugation and examined for membrane-bound Sec18p by Western blot. We found that Sec18p levels were not affected by propranolol treatment (Fig. 5E), suggesting that PAP activity was required for the association of Sec18p with cis-SNARE complexes and that Sec18p also binds vacuolar membranes independent of SNARE proteins. Importantly, the mammalian Sec18p homologue NSF has been shown to specifically bind PA (60); thus, we posit that PA phosphatase activity may lead to the release of membrane-bound pools of NSF/Sec18p and recruitment to cis-SNARE complexes.

Propranolol Inhibits Endogenous Vacuole PAP Activity during Fusion—To determine whether propranolol inhibited PAP activity on vacuoles, we next examined native PAP activity during fusion. We used thin layer chromatography and NBD-tagged PA to monitor the production of NBD-DAG. Short chain Di-C6-NBD PA was incubated with vacuoles and treated with propranolol. Lipids were extracted and resolved by TLC as described under “Experimental Procedures.” A clear NBD-DAG spot was produced when vacuoles were incubated with NBD-PA, indicating that vacuoles exhibited PAP activity during membrane fusion (Fig. 5, F and G). Increasing concentrations of propranolol inhibited DAG production, illustrating a positive correlation between the inhibitions of fusion and PAP activity.

Vam7p Bypasses Propranolol Block and Rescues Fusion—Because propranolol inhibited SNARE priming, we hypothesized that the block could be bypassed with exogenous Vam7p, a soluble SNARE that can bypass an anti-Sec17p priming block by interacting with free SNAREs to form fusogenic trans-SNARE complexes (40, 41, 61). Accordingly, we found that fusion was restored when propranolol-treated vacuoles were incubated with WT Vam7p (Fig. 6A). Strikingly, we also found that fusion was restored with Vam7p containing a mutation in the ionic zero layer of the SNARE domain (Vam7pQ283R). Dis-
Vam7p Does Not Rescue pah1Δ Vacuole Fusion—Because the propranolol block was bypassed by the addition of exogenous

rupting the 3Q:1R paradigm with Vam7pQ283R (2Q:2R) abolishes vacuole fusion unless vacuoles are treated with agents that increase membrane fluidity (41). We theorized that increasing membrane fluidity lowers the energy threshold required for SNARE complexes to deform membranes and mediate fusion. Others have found that propranolol in addition to other β-blockers increase the fluidity of biomimetic liposomes (62); thus, the activity of Vam7pQ283R SNARE complexes in the presence of propranolol was in accord with our previous findings linking fluidity to the function of Vam7pQ283R. However, it must be noted that other β-blockers that alter membrane fluidity did not alter vacuole fusion. In addition, the effect of propranolol only altered fusion at the priming stage. The acquisition of resistance seen above was indicative that the drug did not cause nonspecific changes to the membrane. Unlike the SNARE domain mutation, a Phox homology domain mutation (Vam7pY42A) that blocks PI3P binding did not rescue fusion.

This was consistent with our prior finding showing that Vam7pY42A could not bypass priming blocked with anti-Sec17p IgG (40) and was likely due to the stringent requirement for PI3P-binding by Vam7p.

To verify that the Vam7p (WT and Q283R) bypass of propranolol-blocked fusion was dependent on the endogenous fusion machinery, we used inhibitors that block fusion upstream and downstream of Vam7p binding. We found that Vam7p bypass was sensitive to antibodies against the SNARE Vam3p and the HOPS subunit Vps33p (Fig. 6B). Both Vam3p and Vps33p are essential for Vam7p bypass activity (61). Antibodies against the Rab GTPase Ypt7p and Sec18p did not inhibit Vam7p bypass, which was in accord with the notion that Vam7p stimulates fusion at the docking stage, downstream of Sec18p-dependent priming and reversible Rab-mediated tethering.

Propranolol Inhibits trans-SNARE Complex Formation—To confirm that PAP activity was required early during the fusion pathway, we examined a downstream event that required cis-SNARE priming. Here we tested whether trans-SNARE complexes would form when PAP activity was inhibited. The formation of trans-SNARE pairs occurs at the docking stage and precedes Ca^{2+} efflux, hemifusion, and full content mixing. We examined the formation of trans-SNARE pairs by monitoring the formation of Vam3p-Nyv1p complexes (44, 50). To ensure that Vam3p and Nyv1p were in trans, we used vacuoles harvested from two different strains. Vacuoles from one strain lacking Nyv1p and harboring CBP-Vam3p (CBP-VAM3 nyy1Δ) were incubated with WT vacuoles (VAM3 NYV1) and allowed to form trans-SNARE pairs that contained CBP-Vam3p from one vacuole and Nyv1p from the partner vacuole. We treated the reactions with propranolol, Vam7p alone or inhibitors that block trans-SNARE pairing. MED was used to bind phosphoinositides (2), and Ypt7p function was inhibited with a mixture of the Rab GTPase activating protein Gyp1p and Rab GDP dissociation inhibitor (61). Treatment with propranolol blocked the formation of trans-SNARE pairing as shown by the absence of Nyv1p in isolated CBP-Vam3p complexes (44, 50) relative to untreated reactions (64, lane 7). CBP-Vam3p bound ~0.6% of the Nyv1p from the acceptor vacuoles when reactions were treated with buffer alone and was reduced to 0.2% when treated with propranolol (Fig. 6D, gray bars). It should be noted that WT vacuoles also fuse with themselves, leading to reduced levels of Nyv1p available to complex with CBP-Vam3p. The inhibition of trans-SNARE formation and fusion by propranolol was as robust as the characterized inhibitors MED and Gyp1p/Rab GDP dissociation inhibitor (GDI) (64, lanes 9 and 10, respectively, and Fig. 6D). The addition of recombinant GST-Vam7p enhanced the formation of trans-SNARE complexes in untreated reactions (Fig. 6C, lane 8) without affecting the extent of fusion (Fig. 6D, black bars). However, when GST-Vam7p was added to propranolol-treated reactions, it restored trans-SNARE pairing as well as fusion (Fig. 6C, lane 12, and D). These data are in agreement with the inhibition of priming by propranolol. Furthermore, this demonstrated that the fusion machinery itself was fully operational, as shown by the ability of Vam7p to bypass the propranolol block.

FIGURE 4. Pah1p localizes to the vacuole membrane. A, samples were isolated from WT DKY6281 and Pah1p-HA strains. Western blots were performed on whole cell lysate, cytosol, a microsomal fraction, and purified vacuoles. Each lane contained 10 μg of protein for lysate, cytosol, and microsome samples or 30 μg of vacuoles. Microsomes and lysates were prepared as described previously (49). Antibody against HA was used to detect Pah1p-HA, and anti-Ypt7p was used to detect the vacuolar Rab GTPase. B, vacuoles harboring Pah1p-GFP were used for fluorescence microscopy. Vacuoles were incubated for 30 min under docking conditions, co-stained with FM4-64, and mounted for wide-field fluorescence microscopy examination. Bar, 10 μm. C, Z-stacks of images were taken of individual Pah1p-GFP vacuoles incubated under docking conditions for 30 min. Vacuoles were co-stained with 1 μM PSS-380 to label phosphatidylserine. Images were deconvolved using AxioVision 3D software. Shown are the center slices of two separate Z-stacks of independent vacuoles.
The role of Vam7p in membrane fusion was studied to understand its function in the context of WT vacuoles and how it interacts with other components. Vam7p is known to bypass the priming block of WT vacuoles treated with anti-Sec17p. Interestingly, Vam7p did not rescue the fusion defect when used in place of Vam3p in the presence of NBD-labeled PA. This suggests that Vam7p is less able to associate with the membrane in the absence of Sec18p.

To further investigate the inability of Vam7p to rescue the fusion block of pah1Δ vacuoles, we performed GST-Vam3p pulldown experiments to examine protein interactions. As a control, we used WT vacuoles to test the binding of GST-Vam3p to other SNAREs and the HOPS complex. In WT vacuoles, we observed the binding of Sec18p to isolated CBP-Vam3p complexes. Vacuoles harboring CBP-Vam3p (NYV1 CBP-VAM3) were incubated at 27 °C for the indicated times in the presence of NBD-labeled PA. After 30 min, lipids were extracted and resolved by TLC. Generation of NBD-DAG was detected using phosphorimaging.

To further characterize the role of Vam7p in membrane fusion, we used GST-Vam7p pulldown experiments. Interestingly, Vam7p did not rescue the fusion of pah1Δ vacuoles, which suggested that deleting the Pah1p might block fusion via a mechanism other than priming.

To further investigate the inability of Vam7p to rescue the fusion block of pah1Δ vacuoles, we performed GST-Vam7p pulldown experiments to examine protein interactions. As a control, we used WT vacuoles to test the binding of GST-Vam3p to other SNAREs and the HOPS complex. In WT vacuoles, we observed the binding of Sec18p to isolated CBP-Vam3p complexes. Vacuoles harboring CBP-Vam3p (NYV1 CBP-VAM3) were incubated at 27 °C for the indicated times in the presence of NBD-labeled PA. After 30 min, lipids were extracted and resolved by TLC. Generation of NBD-DAG was detected using phosphorimaging.
Pah1p and Membrane Fusion

When pah1Δ strains were complemented with plasmid-encoded WT PAH1, the vacuolar concentration of all the tested proteins was restored to WT levels, whereas complementation with the phosphatase dead mutant PAH1D398E had no effect on the null phenotype. This demonstrated that the phosphatase activity of Pah1p was required for trafficking/recruiting key proteins to the vacuole. It is important to note that HOPS, Vam7p, and Vps34p are soluble proteins that are recruited from the cytosol to vacuoles by membrane-anchored proteins and lipids. In addition, although Ypt7p is anchored in the vacuole membrane through its geranylgeranyl lipid group, it is recruited from the cytoplasm to maturing endocytic membranes and not sorted through a biosynthetic pathway. To determine whether the absence of soluble or lipid anchored proteins from pah1Δ vacuoles was due to defective recruitment or degradation, we examined the levels of Vps34p, Vps39p, and Ypt7p on total membrane fractions. We found that protein levels were not reduced as a whole in pah1Δ cells and were present on other membranes. This illustrated that the lack of fusion effectors on pah1Δ vacuoles was due to faulty recruitment and not degradation (Fig. 8B).

Prolonged Propranolol Treatment Causes Ypt7p Mislocalization—In these experiments we have found that deleting PAH1 caused a defect in the recruitment of fusion factors from the cytoplasm, including Ypt7p, whereas treatment with propranolol had no great effect on the protein profile of vacuoles. In both cases PA phosphatase activity was targeted in the cytoplasm, including Ypt7p, whereas treatment with propranolol had no effect on the protein profile of vacuoles.
the factors necessary for fusion before exposure to propranolol. On the other hand, vacuoles isolated from \( pah1 \) cells reflected the effects of a chronic lack of Pah1p activity.

We next determined whether an extended treatment of intact cells with propranolol would alter protein recruitment to the vacuole. Yeast cells expressing GFP-Ypt7p were treated with buffer or propranolol for 12 h. Next, the vacuoles were pulse-labeled with FM4-64 for 1 h, washed with PBS, and chased with fresh YPD with or without propranolol for 3 h. After the chase, the cells were washed with PBS and prepared for observation by fluorescence microscopy. GFP-Ypt7p, FM4-64, and differential interference contrast (DIC) images were acquired separately. The GFP and FM4-64 images were merged using Photoshop.

FIGURE 8. **Deletion of \( PAH1 \) alters vacuole homeostasis.** A, to analyze the effects of deleting Pah1p on the core fusion machinery, Western blots were performed on vacuoles isolated from WT BJ3505, RFY17 (\( pah1 \)), RFY19 (\( pah1 \ + \ pPAH1 \)), and RFY21 (\( pah1 \ + \ pPAH1^{d398e} \)) cells. Vacuoles were solubilized with 2× loading buffer, resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against the indicated proteins. Protein bands were detected using enhanced chemifluorescence. *, to examine the effect of deleting \( PAH1 \) on Pep4p trafficking, vacuoles were isolated from DKY6281 and RFY18, RFY20, and RFY22. \( B \), samples were isolated from WT and \( pah1 \) strains, and Western blots were performed on microsomes and purified vacuoles. Microsomes were prepared as previously described (49).

FIGURE 9. **Propranolol treatment caused the missorting of GFP-Ypt7p.** Yeast cells expressing GFP-Ypt7p were grown in YPD and treated with buffer or propranolol for 12 h. Next, the vacuoles were pulse-labeled with FM4-64 for 1 h, washed with PBS, and chased with fresh YPD with or without propranolol for 3 h. After the chase, the cells were washed with PBS and prepared for observation by fluorescence microscopy. GFP-Ypt7p, FM4-64, and differential interference contrast (DIC) images were acquired separately. The GFP and FM4-64 images were merged using Photoshop.

and retained to the prevacuolar compartment. These puncta were not representative of a Class E phenotype, as they did not retain FM4-64 in vacuole-adjacent puncta.

\( PI3P \) is Absent from \( pah1 \) Vacuoles—Because Vps34p was depleted on \( pah1 \) vacuoles, we predicted that PI3P would be absent or at very low levels in the absence of the kinase. PI3P is made during the vacuole fusion reaction and can be monitored by labeling the lipid with fluorescent FYVE domain (2, 3, 38). Depletion or ligation of PI3P on vacuoles with either high concentrations of FYVE or the PI3P 3-phosphatase MTM-1 blocks vacuole fusion (2, 5); thus, the lack of Vps34p and subsequent PI3P production on \( pah1 \) vacuoles may be a prime mechanism for its abolished fusion.

PI3P accumulates at the vertex ring microdomain and is central in the assembly of fusion factors at this domain (2). To probe for the presence of PI3P on WT or \( pah1 \) vacuoles, we used Cy5-labeled FYVE domain at non-inhibitory concentrations (2, 63). Vacuoles were incubated under docking conditions for 30 min and prepared for fluorescence microscopy examination. Vacuoles were co-stained with FM4-64. As previously reported, we found that WT vacuoles accumulated Cy5-FYVE and, hence, PI3P at vertex ring sites, indicating that Vps34p produced PI3P (Fig. 10, A and B). In contrast, Cy5-
FYVE labeling was severely reduced on \( pah1^\Delta \) vacuoles (Fig. 10, C and D), which was consistent with the lack of Vps34p on these vacuoles.

To quantitate the enrichment of PI3P (Cy5-FYVE) at vertices, we used ratiometric fluorescence microscopy that compared the fluorescence intensity of Cy5-FYVE to FM4-64. The clustering of vacuoles and formation of docking sites leads to a doubling of membrane thickness at contact points. Thus, the effect of membrane doubling at docking junctions must be normalized through the use of ratiometric microscopy. In Fig. 10E, we show the ratiometric data for WT and \( pah1^\Delta \) vacuoles. Each vertex ratio was plotted in a cumulative distribution plot depicting the percentile values of Cy5-FYVE:FM4–64 ratio for each of the strains. Each ratio in a dataset was ordered and plotted versus the percentile rank of the values. The ratios of Cy5-FYVE-enriched vertices on WT vacuoles ranged from 0.6 to a maximum of 20 (Fig. 10E, black circles), causing a right-shift in the curve relative to the outer edge (Fig. 10E, black squares). When \( pah1^\Delta \) vertices were quantitated, we found that the curve was left shifted (empty circles) relative to WT vacuoles, indicating that PI3P was markedly reduced on \( pah1^\Delta \) vacuoles. Fig. 10F shows the geometric means and 95% confidence intervals for the ratios in Fig. 10E. These are lower bounds estimates of vertex enrichment due to asynchronous reactions of individual vacuoles within a cluster.

**DISCUSSION**

Much is known about the role of proteins such as Rabs and SNAREs in the regulation of membrane fusion; however, the regulation of these proteins by their immediate lipid microenvironment remains unclear. Moreover, the continuous modification of lipids species during the fusion pathway is only beginning to be elucidated. The homotypic fusion of yeast vacuoles requires a set of regulatory lipids that are chemically minor yet have critical roles in promoting fusion. Among the growing list of regulatory lipids are phosphoinositides, ergosterol, and DAG, which are modified through the activity of phosphatases, kinases, and lipases. For instance, DAG can be produced by the hydrolysis of phosphatidylinositol 4,5-bisphosphate by Plc1p and deletion or inactivation of Plc1p inhibits fusion (3). DAG is additionally produced by the dephosphorylation of PA; however, the role of PA phosphatases in the production of DAG was not clear in the context of membrane fusion. PA itself has also been found to be essential for maximal fusion of reconstituted proteoliposomes containing yeast vacuolar SNAREs (15), suggesting that a critical balance between these lipid intermediates may be controlled in conjunction with the SNARE machinery. In this study we examined the role of yeast PA phosphatases in vacuole homeostasis and fusion. We used a pharmacological and genetic approach to determine the effect of PAP activity on fusion. Initially we found that inhibiting PAP activity with the small molecule propranolol inhibited fusion at the priming stage, which was bypassed with the soluble SNARE Vam7p, indicating that the inhibition in fusion was not due to irreversible adverse effects of the drug. In testing the effects of deleting each of the three yeast PA phosphatases, we found that only the Lipin 1 orthologue Pha1p played a role in fusion. Our studies found that the enzymatic activity of Pha1p was essential for fusion to occur. Deletion of Pha1p not only led to an inhibition of SNARE-mediated fusion; it also led to the defective trafficking and recruitment of key fusion regulators to the vacuole membrane including the recruitment and activation of the vacuolar Rab GTPase Ypt7p.
**PA Phosphatase Activity and SNARE Priming**—Our findings indicate that PA phosphatase activity is required during the priming stage. Inhibiting PAP activity with propranolol resulted in a loss of Sec18p from cis-SNARE complexes without affecting the total concentrations of membrane-bound Sec18p. Based on these findings we posit that PA phosphatase activity regulates Sec18p-mediated cis-SNARE priming. Because pah1Δ vacuoles lacked multiple fusion factors, we were unable to determine whether priming was specifically altered in the deletion strain; however, the results garnered by the use of propranolol is enticing and may shed light on the regulation of the prepriming stage of fusion. Based on these results we envision two models in which PAP activity could affect Sec18p recruitment to SNARE complexes and trigger priming. Recent structural analysis of full-length SNARE complexes showed that cis-SNARE complexes contain continuous α-helical structures that extend through the SNARE motif into the linker region and transmembrane domain (64). Thus, it is possible that changes in the lipid bilayer due to PAP activity lead to the allosteric regulation of SNARE complex conformation and subsequent Sec18p engagement of the cis-SNARE complex.

In one model PAP activity may alter the influence of the membrane on cis-SNARE conformational changes needed to bind and Sec18p. The local lipid environment has been previously reported to regulate SNARE priming. Binding of either ergosterol or phosphatidylinositol 4,5-bisphosphate with small molecule ligands has been shown to inhibit Sec17p release during the priming stage (4, 14). Taken together, it is possible that the assembly of a lipid microdomain may be necessary for the allosteric regulation of the cis-SNARE complex and that in the absence of a favorable physical state, the SNAREs adopt a conformation that hinders Sec18p binding.

A second model proposes that Sec18p is bound to the membrane in a PA-dependent manner before priming. This may occur as a direct interaction with PA or indirectly through another factor. PA has been shown to physically bind mammalian NSF (Sec18p) (60); thus, it is possible that PA hydrolysis releases NSF/Sec18p from the membrane, allowing it to bind cis-SNARE complexes. This model is accordant with our data showing that propranolol reduced that amount of Sec18p bound to cis-SNARE complexes without affecting the total amount of Sec18p on vacuoles.

**Does Pah1p Play Role in Rab Conversion and Organelle Maturation?**—Deletion of PAH1 led to a sharp decrease in recruitment of the phosphatidylinositol 3-kinase Vps34p and the HOPS subunit Vps39p. Taken together with the absence of P13P and Ypt7p, we posit that Pah1p plays a role in vacuole maturation. Endosomal vesicles undergo a highly regulated conversion from early endosomes to late endosomes and lysosomes. This is characterized in part by the exchange of Rab GTPases and their cognate effectors. Although much remains unknown about these mechanisms, several groups have made seminal discoveries regarding the regulation of this transformation (65–68). Ungermann and co-workers (65) recently discovered the endosomal tethering complex CORVET that shares a core of Class C proteins (Vps11p, Vps16p, Vps18p, and Vps33p) with the vacuolar tethering complex HOPS. In addition to the Class C core, CORVET contains Vps8p and Vps3p, whereas HOPS contains Vps41p and Vps39p. Intermediate complexes exist that contain Vps41p and Vps3p (iCORVET) or Vps8p and Vps39p (i-HOPS). Because pah1Δ vacuoles lack Vps39p while retaining Vps41p, albeit at reduced levels, it is possible these vacuoles are trapped in an intermediate maturation stage containing iCORVET. The current model proposes that the exchange of CORVET for HOPS could be coupled with the exchange of Vps21p for Ypt7p, where CORVET-containing endosomes harbor Vps21p, and HOPS-containing vacuoles contain Ypt7p. However, this is only a model and needs to be tested. Because pah1Δ vacuoles lack Ypt7Δ, it is thus possible that these organelles are trapped at a Vps21p-containing stage. Rab exchange is linked to the acquisition of Mon1p and Ccz1p, a heterodimer that functions as the guanine nucleotide exchange factor for Ypt7p (69). Although previous works identified Vps39p as the guanine nucleotide exchange factor for Ypt7p (70), it is now proposed that the HOPS subunit is only indirectly involved through the initial binding of Ypt7p. Interestingly, although Ypt7p was absent from pah1Δ vacuoles, we found that it was relatively enriched in the total microsomal fraction and localized to vacuole-adjacent membranes, suggesting that in the absence of Pah1p Ypt7p was stably associated with another organelle. Similarly, Vps34p and Vps39p were enriched in the microsomal fraction independent of Pah1p. However, it is unclear whether Vps39p was in a complex with other HOPS subunits.

**Pah1p, PI3P, and Rab Conversion**—The role of Mon1p in Rab conversion has recently been the center of rigorous exploration (66, 71). Investigators have found that in Caenorhabditis elegans, the Mon1p homologue SAND-1 triggers the switch from early-to-late endosomes. The exchange of the early endosomal Rab5/Vps21p for the late endosomal Rab7/Ypt7p occurred through the recruitment of SAND-1/Mon1p and the inactivation of Rab5 (71), promoting the removal Rab5, whereas Rab7 was recruited to the transitioning endosome. In sand-1/mon1Δ cells, Rab5 remains active and blocks the recruitment of Rab7. Importantly, Mon1p recruitment requires the regulatory lipid PI3P. In our experiments we found that pah1Δ vacuoles lack the phosphatidylinositol 3-kinase Vps34p and its product PI3P. However, low levels of PI3P may still traffic to the vacuole from other membranes that harbor Vps34p. Thus, we hypothesize that Pah1p is required for Vps34p trafficking and PI3P production, thus promoting Mon1p binding and the recruitment of Ypt7p.

The mechanism by which Pah1p phosphatase activity leads to the recruitment of Vps34p remains unclear. In mammalian cells hVPS34 is recruited to early endosomes and activated in a Rab5-GTP dependent manner (72). Thus, PI3P is made before the recruitment of SAND-1 and Rab exchange. Unlike the mammalian homologue, yeast Vps34p activity appears to be independent of the early endosome Rab Vps21p. Instead, yeast Vps34p is activated via Gpa1p, the Ga subunit of a plasma membrane heterotrimeric GTPase (73). Monomeric Gpa1p localizes to the endosome and interacts with Vps34p and Vps15p, a myristoylated serine/threonine protein kinase and subunit of the endosomal phosphatidylinositol 3-kinase complex (74). However, it is unclear whether Gpa1p also recruits or activates Vps34p at the vacuole or if once recruited to the endo-
some Vps34p traffics to the vacuole in a Gpa1p-independent manner. Although Gpa1p does traffic to the vacuole, its sorting is dependent on its monoubiquitination by Rsp5p (75) that subsequently targets it for degradation. It is unclear whether Gpa1p monoubiquitination occurs before or after Vps34p activation at the endosome.

Although the link between Pah1p and PI3P production remains unclear at this time, it is quite certain that the lack of this lipid has deleterious effects on the vacuole fusion machinery. Aside from its role in Rab exchange, PI3P is essential for the formation of the vertex microdomain where fusion occurs (2, 12). Modification or ligation of PI3P inhibits the vertex enrichment of SNAREs, Ypt7p, and HOPS as well as ergosterol, DAG, and other phosphoinositides. Moreover, PI3P directly binds to the Phox homology domain of the soluble SNARE Vam7p and to the HOPS complex (5, 9). The formation of 3Q SNARE complexes as well as trans-SNARE complexes is also dependent on PI3P (44, 76, 77). Thus it is evident that recruitment of Vps34p is a major function of Pah1p during vacuole fusion, placing the Lipin 1 homologue and the conversion of PA to DAG at the top of a cascade that leads to the activation of SNARE priming and production of PI3P and Ypt7p recruitment.

Acknowledgments—We thank Dr. George Carman for strains and plasmids and Dr. William Wickner for generous gifts of antisera. We also thank Dr. Christian Ungermann, Margarita Cabrera, Dr. Joanna Shisler, and Jennifer Grant for critical reading of the manuscript and helpful discussions.

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Yeast Lipin 1 Orthologue Pah1p Regulates Vacuole Homeostasis and Membrane Fusion
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doi: 10.1074/jbc.M111.317420 originally published online November 25, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.317420

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