The signaling lipid PI(3,5)P₂ stabilizes V₁–Vo sector interactions and activates the V-ATPase

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ABSTRACT Vacuum proton-translocating ATPases (V-ATPases) are highly conserved, ATP-driven proton pumps regulated by reversible dissociation of its cytosolic, peripheral V₁ domain from the integral membrane Vo domain. Multiple stresses induce changes in V₁–Vo assembly, but the signaling mechanisms behind these changes are not understood. Here we show that certain stress-responsive changes in V-ATPase activity and assembly require the signaling lipid phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂). V-ATPase activation through V₁–Vo assembly in response to salt stress is strongly dependent on PI(3,5)P₂ synthesis. Purified Vo complexes preferentially bind to PI(3,5)P₂ on lipid arrays, suggesting direct binding between the lipid and the membrane sector of the V-ATPase. Increasing PI(3,5)P₂ levels in vivo recruits the N-terminal domain of V₁-sector subunit Vph1p from cytosol to membranes, independent of other subunits. This Vph1p domain is critical for V₁–Vo interaction, suggesting that interaction of Vph1p with PI(3,5)P₂-containing membranes stabilizes V₁–Vo assembly and thus increases V-ATPase activity. These results help explain the previously described vacuolar acidification defect in yeast fab1Δ and vac14Δ mutants and suggest that human disease phenotypes associated with PI(3,5)P₂ loss may arise from compromised V-ATPase stability and regulation.

INTRODUCTION Vacuum proton-translocating ATPases (V-ATPases) are highly conserved proton pumps that acidify the Golgi apparatus, endosomes, and lysosomes of all eukaryotic cells (Kane, 2006; Forgac, 2007). The yeast V-ATPase comprises 14 different subunits arranged into a peripheral complex (V₁) containing the sites of ATP hydrolysis attached to an integral membrane complex (Vo) containing the proton pore (Kane, 2006; Forgac, 2007). Modulation of V₁–Vo assembly levels is a major mechanism of V-ATPase regulation (Kane, 2006; Forac, 2007). Glucose-responsive reversible disassembly of the V-ATPase is the best-characterized example of this type of regulation, but it has become clear that V₁–Vo assembly responds to other signals as well, including osmotic stress and elevated extracellular pH (Voss et al., 2007; Diakov and Kane, 2010; Li et al., 2012; Lin et al., 2012). Subunits at the interface of the V₁ and Vo sectors, particularly V₁ subunit C (encoded by VMAS) and Vo subunit a (encoded by VPH1 and its isofrom STV1 in yeast) are critical for V₁–Vo interactions and believed to play important roles in V-ATPase regulation by different stimuli (Kawasaki-Nishi et al., 2001; Voss et al., 2007; Oot and Wilkens, 2012; Rahman et al., 2013). However, the signaling mechanisms governing V-ATPase assembly are not completely understood.

Phosphoinositides are a critical class of signaling molecules. They are transiently generated at specific organelles and membrane sub-domains, where they can recruit effectors from the cytosol and regulate the assembly and/or activation of resident proteins (De Camilli et al., 1996; Di Paolo and De Camilli, 2006; Roth, 2004; Strahl and Thorner, 2007). The inositol headgroup can be singly or multiply phosphorylated through the activity of lipid kinases, and the localized action of these kinases and the opposing phosphatases creates an organelle-specific distribution of different phosphoinositides. In
addition, the activity of lipid kinases and phosphatases is controlled by various signals and stresses, and changes in the levels of these lipids are key events in multiple signal transduction pathways (De Camilli et al., 1996; Roth, 2004; Strahl and Thorner, 2007). Protein recognition of specific phosphoinositide headgroups mediates the functions of inositol phospholipids (Lemmon, 2008; Suh and Hille, 2008). Membrane proteins, and particularly transporters, are among the critical regulatory targets of these lipids. For example, plasma membrane ion channels and transporters such as the inward rectifier K+ channel, the KCNQ K+ channel, and a number of TRP Ca2+ channels require phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), a phosphoinositide residing primarily in the plasma membrane, for full function and are inhibited by signaling pathways that reduce PI(4,5)P2 levels (Hilgemann, 2007; Suh and Hille, 2008; Young et al., 2010). In these cases, binding of the lipid headgroup to a cytosolic domain of the membrane protein leads to a conformational change that modulates activity (Suh and Hille, 2008; Young et al., 2010).

Phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) is a low-abundance phosphoinositide located in the endosomal and lysosomal membranes of fungi and higher eukaryotes (reviewed in Ho et al., 2012; Shisheva, 2012). Phosphorylation of phosphatidylinositol 3-phosphate (PI(3)P) produces PI(3,5)P2 through the action of the conserved PI 5-kinase Fab1p, also known as PIKfyve (Cooke et al., 1998; Gary et al., 1998). Fab1p associates with a large complex containing the scaffolding protein Vac14p and the Fig4p phosphatase, which converts PI(3,5)P2 back to PI(3)P (Gary et al., 2002; Botelho et al., 2008; Jin et al., 2008). Curiously, both Vac14p and Fig4p must be present for Fab1p function (Duex et al., 2006b; Botelho et al., 2008), suggesting that PI(3,5)P2 levels are tightly regulated by coordinated activity of lipid kinases and phosphatases. Although basal levels of PI(3,5)P2 are very low, they change dramatically and transiently in response to specific extracellular stresses (Dove et al., 1997, 1999; Bonangelino et al., 2002). In Saccharomyces cerevisiae, salt shock transiently increases PI(3,5)P2 levels 20-fold above basal levels (Dove et al., 1997; Duex et al., 2006a). Elevated PI(3,5)P2 levels lead to vacuolar fission, by which vacuoles increase in number and decrease in size (Cooke et al., 1998; Bonangelino et al., 2002; Dong et al., 2010). Conversely, abolishing PI(3,5)P2 production by deleting yeast Fab1, Vac14, or Fig4 genes results in grossly enlarged vacuoles that appear to be poorly acidified (Cooke et al., 1998; Gary et al., 1998; Dove et al., 2002; Rudge et al., 2004). Loss of Vac14 or Fig4 function in mice leads to neurodegeneration and cellular vacuolation (Chow et al., 2007; Zhang et al., 2007; Jin et al., 2008), and homozygous deletion of Fab1 in mice is lethal (Ikonomov et al., 2011; Takasuga et al., 2013).

The source of the organelle acidification defect in PI(3,5)P2-deficient cells is unknown. This defect and the presence of PI(3,5)P2 in vacuolar and endosomal compartments rich in V-ATPases raise the tantalizing possibility that PI(3,5)P2 might regulate the V-ATPase in these compartments. In this study, we test the hypothesis that V-ATPase activity and assembly are regulated by PI(3,5)P2 levels and suggest that loss of organelle acidification in fab1Δ and vac14Δ mutants arises from loss of V-ATPase regulation.

**RESULTS**

**V-ATPase disassembly in response to glucose deprivation is not controlled by PI(3,5)P2**

We assessed V-ATPase function in deletion mutants that lacked the Fab1p PI 5-kinase and Vac14p, a scaffold protein necessary for Fab1p function. We isolated vacuolar vesicles from wild-type, fab1Δ, and vac14Δ cells grown in rich medium and assayed V-ATPase-specific activity and proton pumping. Rapid disassembly of the V-ATPase after glucose deprivation is a major regulatory mechanism for V-ATPases, so we also compared ATPase and proton pumping activities after 20 min of glucose deprivation before cell lysis. As shown in Figure 1, V-ATPase activity in vacuolar vesicles isolated from glucose-deprived wild-type cells is ~40% lower than the activity from cells maintained in glucose, and the initial rate of proton pumping shows a comparable decrease. In fab1Δ and vac14Δ mutants, V-ATPase and proton pumping activity are significantly reduced in vacuolar vesicles isolated from both glucose-deprived and glucose-maintained cells. Of note, V-ATPase regulation in response to glucose is retained in fab1Δ and vac14Δ mutants even though V-ATPase activity in both glucose-maintained and glucose-deprived cells is significantly lower. Moreover, ATPase activity and proton pumping between the fab1Δ and vac14Δ mutants was not significantly different, even though PI(3,5)P2 production is completely abolished in the fab1Δ strain, whereas vac14Δ mutants retain ~5% of the wild-type levels of this lipid (Duex et al., 2006b).

We compared the protein levels of V-ATPase subunits in the isolated vacuolar vesicles by immunoblot (Figure 1C). The peripheral (V1) sector is reversibly released from the integral membrane (V0) sector upon glucose deprivation. Levels of the integral membrane V0-a subunit (Vph1p) and the vacuolar marker alkaline phosphatase (ALP) are comparable in the presence and absence of glucose in all of the strains. (The appearance of the lower-mobility, unprocessed form of ALP in fab1Δ and vac14Δ is characteristic of cells with reduced V-ATPase activity because lower vacuolar protease activity reduces processing at the vacuole; Sambade et al., 2005.) In contrast to the V0 subunit, a, levels of V1 subunits, particularly V1 subunit C, decrease upon glucose deprivation (Figure 1, C and D). This reflects disassembly of V1 from V0 at the vacuolar membrane. In fab1Δ and vac14Δ mutants, a reduction of V1 subunits also occurs upon glucose deprivation, reflecting normal V-ATPase regulation by glucose. However, in both glucose-deprived and glucose-replete conditions, the levels of V1 subunits in the mutants appear to be reduced relative to wild type. These results indicate that reduced V-ATPase activity and proton pumping in PI(3,5)P2 mutants are at least partially accounted for by reduced assembly of V1 subunits at the vacuolar membrane but that disassembly upon glucose deprivation is independent of PI(3,5)P2 level.

**V-ATPase activity and assembly increase under conditions that raise PI(3,5)P2 level**

PI(3,5)P2 is one of the least abundant inositol phospholipids, but its level increases dramatically in response to several extracellular stresses (Bonangelino et al., 2002; Duex et al., 2006b; Mollapour et al., 2006). The response to salt stress is best described. Addition of NaCl to the extracellular medium can induce a transient, ~20-fold increase in cellular PI(3,5)P2 level (Bonangelino et al., 2002; Duex et al., 2006a). We previously found that V-ATPase activity and assembly can be increased by salt shock (Li et al., 2012), and we tested whether this response was dependent on PI(3,5)P2 by exposing fab1Δ cells to 500 mM NaCl for 20 min before cell lysis and vacuole isolation. As shown in Figure 2A, salt shock results in an approximately twofold increase in ATPase activity in wild-type vacuoles. In contrast, there is very little salt activation of the V-ATPase in fab1Δ cells. Figure 2B demonstrates that V-ATPase activation in response to salt is accompanied by increased assembly of V1 subunits at the vacuolar membrane in wild-type cells. Of note, the increase in V1 subunit assembly in the presence of salt is almost completely absent in the fab1Δ mutant. The normalized ratio of the V1-C subunit to the V0-a subunit (Figure 2C) parallels the changes in V-ATPase activity in response to salt. Therefore salt activation of the V-ATPase occurs, in...
part, through increased V₁ assembly at the membrane, and this response requires PI(3,5)P₂ production.

We previously showed that high extracellular pH stabilizes the V-ATPase (Diakov and Kane, 2010). In cells grown at high pH, there is both increased V₁-Vo assembly and activity and less sensitivity to the glucose disassembly signal. Combining glucose deprivation with high extracellular pH yields less V₁-Vo disassembly than in normal, acidic pH. PI(3,5)P₂ level showed a sustained sixfold increase during high-pH stress (pH 7.6; Mollapour et al., 2006), so we studied the potential role of PI(3,5)P₂ kinase activity in V-ATPase stabilization at high extracellular pH. Although fab1Δ and vac14Δ mutants do not exhibit the full Vma– phenotype of mutants lacking all V-ATPase activity (Sambade et al., 2005), we found that both mutants grew poorly in minimal medium buffered to pH 7. The growth of the fab1Δ mutant was so poor that we could not obtain sufficient cells for vacuole preparation, but we were able to obtain enough vac14Δ cells despite its slow growth. Wild-type and vac14Δ cells were grown in glucose-containing minimal medium buffered to pH 5 or 7 and then exposed to media with or without glucose for 20 min before lysis. As reported previously (Diakov and Kane, 2010), wild-type vacuoles have significantly higher V-ATPase activity and less starvation-induced disassembly of the enzyme after growth in high-pH media (Figure 3A). Of interest, V-ATPase activity is also higher in vesicles isolated from the vac14Δ mutant grown at pH 7 compared with pH 5 (Figure 3A), but stabilization of V-ATPase during glucose deprivation is compromised. In vac14Δ mutants, more disassembly of V₁ sector from the vacuole membrane occurs upon glucose deprivation at high pH than in wild type (Figure 3B). Quantitation of the V₁/Vo ratio showed that there was a <15% reduction in assembly when wild-type cells grown at pH 7 were deprived of glucose. In contrast, vac14Δ cells showed a 48% drop in assembly with glucose deprivation at pH 7 (Figure 3C).

Taken together, these results suggest that PI(3,5)P₂ has a stabilizing effect on the V-ATPase. The low basal level of this lipid is necessary for full V-ATPase activity. Both activity and assembly are reduced in the fab1Δ and vac14Δ mutants, although disassembly of the enzyme in response to glucose deprivation still occurs. Increased level of PI(3,5)P₂ in response to extracellular salt or alkaline stress is accompanied by increased V-ATPase activity and assembly, and these changes depend on PI(3,5)P₂ synthesis. These data suggest an intimate connection between level of PI(3,5)P₂ and V-ATPase assembly but do not indicate whether this connection is direct or indirect.

Elevated PI(3,5)P₂ levels in a hyperactive FAB1 mutant recruit V₁ subunits from the cytosol to the vacuolar membrane

Stabilization of V₁ assembly with Vo sectors at the vacuole by endogenous levels of PI(3,5)P₂ suggests that increasing PI(3,5)P₂ levels might help to actively recruit V-ATPase subunits to the vacuolar membrane. To test this, we expressed an extra copy of a DsRed-tagged V₁-C subunit (Vma5-DsRed) in cells containing the constitutively active FAB1-VLA, as well as in wild-type cells, a fab1Δ mutant, and a kinase-dead fab1-EEE mutant. The FAB1-VLA

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**FIGURE 1:** fab1Δ and vac14Δ mutants reduce ATPase activity and assembly. Vacuolar vesicles were isolated from wild-type (wt) and mutant yeast cells after growth in YEPA, pH 5, medium. (A) Concanamycin A-sensitive ATPase activity in vacuolar vesicles isolated with (+) or without (–) incubation in glucose for 20 min just before spheroplast lysis and vacuole isolation. The mean specific activity for at least three independent vacuole preparations is shown, before spheroplast lysis and vacuole isolation. (B) The same samples described in A were tested for ATP-driven proton pumping using the ACMA quenching assay (see Materials and Methods). The initial rate of pumping after addition of MgATP to the vacuolar vesicles was determined, and the mean ± SEM is shown. (C) Representative immunoblot showing V-ATPase subunit levels. Vacuolar vesicles were solubilized, and the same amount of total vacuolar protein was loaded for each strain. Blots were probed with monoclonal antibodies against the indicated V-ATPase subunits or the vacuolar protein ALP. (D) Relative levels of V₁/Vo assembly in the indicated strains were quantitated by measuring the ratio of the V₁-C and Vo-a subunit signals on immunoblots of the vacuolar preparations used in A and B and then normalizing to a wild-type + glucose sample run in parallel. Mean assembly level ± SEM.
mutant causes a constitutive, 17-fold increase in the cellular steady-state level of PI(3,5)P$_2$ dependent. Vacular vesicles were prepared from wild-type and fab1∆ cells grown to log phase in fully supplemented minimal medium buffered to pH 5 with MES, converted to spheroplasts, and then incubated with (+) or without (−) 500 mM NaCl for 20 min before lysis. (A) Concanamycin A–sensitive ATPase activity from three independent vacuolar vesicle preparations for wt cells and two independent preparations of fab1∆ cells was determined and normalized to the level in wild-type cells incubated without salt. (B) Representative immunoblot showing levels of V1 and V0 subunits. (C) Relative levels of V1/V0 assembly determined from ratio of V1-C and V0-a subunit signals as described in Figure 1D and normalized to a wild-type sample without salt run in parallel.

FIGURE 2: Salt activation of V-ATPase activity and assembly is PI(3,5)P$_2$ dependent. Vacular vesicles were prepared from wild-type and fab1∆ cells grown to log phase in fully supplemented minimal medium buffered to pH 5 with MES, converted to spheroplasts, and then incubated with (+) or without (−) 500 mM NaCl for 20 min before lysis. (A) Concanamycin A–sensitive ATPase activity from three independent vacuolar vesicle preparations for wt cells and two independent preparations of fab1∆ cells was determined and normalized to the level in wild-type cells incubated without salt. (B) Representative immunoblot showing levels of V1 and V0 subunits. (C) Relative levels of V1/V0 assembly determined from ratio of V1-C and V0-a subunit signals as described in Figure 1D and normalized to a wild-type sample without salt run in parallel.

FIGURE 3: PI(3,5)P$_2$ deficiency reduces stabilization of the V-ATPase to glucose deprivation at high pH. Wild-type and vac14∆ cells were grown to log phase in fully supplemented minimal medium buffered to either pH 5 or 7 with 50 mM MES. Vacular vesicles were isolated from spheroplasts maintained in glucose (+) or deprived of glucose (−) for 20 min before lysis. (A) Mean concanamycin A–sensitive ATPase activities normalized to the activity in vesicles from wild-type cells grown at pH 5 and maintained in glucose. All are mean ± SEM for at least three independent vacuole preparations, except for the wild-type, pH 5, samples, which represent only two samples. (B) Representative immunoblot of subunit levels in vacuolar vesicles isolated from wt and vac14∆ mutant cells grown at pH 7 and then incubated in the presence or absence of glucose as described in Figure 1C. The lower band in the V0-a blot is a proteolytic fragment that was observed previously but is not specific to these growth conditions. (C) Relative levels of V1/V0 assembly determined from the ratio of V1-C and V0-a subunit signals as described in Figure 1D and normalized to a wild-type sample + glucose run in parallel.
colocalization with Vph1-GFP (Figure 4A). Note that the grossly enlarged vacuoles in the fab1Δ mutant are characteristic of loss of Fab1p function (Gary et al., 1998). Addition of an empty plasmid (pRS416) or a kinase-dead fab1 mutant (fab1-EEE) on a plasmid did not improve colocalization (Figure 4B), but wild-type FAB1 expressed from a plasmid partially restored localization of Vma5-DsRed subunit to vacuoles, as well as restoring wild-type vacuolar morphology. Of note, a significant amount of cytosolic Vma5-DsRed subunit is still observed in the presence of the wild-type FAB1-VLA mutant when other V1 subunits (vma2Δ, vma8Δ, vma10Δ, and vma13Δ) or V0 subunit c (vma3Δ) are missing. These results show that the Vma5-DsRed subunit is not recruited directly to PI(3,5)P2 in the FAB1-VLA mutant but instead binds as part of the V-ATPase complex, requiring both intact V1 and V0 subcomplexes.

Purified yeast V0 sectors show preferential binding to PI(3,5)P2

Membrane recruitment of V1 subunit C in response to elevated PI(3,5)P2 requires the integral membrane V0 subcomplex, and portions of this subcomplex are in close proximity to phospholipid headgroups (Bendek et al., 2012; Oot and Wilkens, 2012). This supports a model in which the effects of PI(3,5)P2 on V-ATPase assembly are mediated through lipid interactions in the V0 complex. To address this, we first tested for direct binding of PI(3,5)P2 to the V0 complex in vitro. Assembly of V0 complexes were isolated from a solubilized membrane fraction of the tagged C subunit is possible in the presence of higher PI(3,5)P2 level. These results indicate that more Vma5-DsRed subunit could be recruited to the vacuolar membrane when PI(3,5)P2 level is constitutively increased.

Recruitment of Vma5-DsRed to the vacuole under conditions of elevated PI(3,5)P2 is consistent with the increased V1-V0 assembly shown in Figures 2 and 3, but this experiment does not distinguish direct binding of Vma5-DsRed to the membrane from recruitment as part of the V1 complex or a partially assembled subcomplex. To determine whether recruitment of the C subunit in response to elevated PI(3,5)P2 level depends on the presence of other V-ATPase subunits, we expressed the Vma5-DsRed construct in the context of individual V-ATPase subunit deletions in both wild-type FAB1 and FAB1-VLA mutant cells. As shown in Figure 5, the Vma5-DsRed subunit is not recruited to the membrane in the FAB1-VLA mutant when other V1 subunits (vma2Δ, vma8Δ, vma10Δ, and vma13Δ) or V0 subunit c (vma3Δ) are missing. These results show that the Vma5-DsRed subunit is not recruited directly to PI(3,5)P2 in the FAB1-VLA mutant but instead binds as part of the V-ATPase complex, requiring both intact V1 and V0 subcomplexes.
lipid to the membrane-bound V₀ sector. However, association with PIP blots is an in vitro method to assess physical interactions with lipid species that has a number of limitations, and so we conducted further in vivo assays to look at membrane binding of integral V₀-sector subunits in conditions that change P(3,5)P₂ levels.

The Vph1NT domain is recruited to intracellular membranes under conditions that increase P(3,5)P₂ levels

Vph1p contains a large cytosolic N-terminal domain (Vph1NT) along with a C-terminal domain containing multiple transmembrane helices. Structural information places the N-terminal region of Vph1p at the interface of the V₁ and V₀ sectors in the holoenzyme, where it is a potential site for enzyme regulation (Diepholz et al., 2008; Zhang et al., 2008b; Muench et al., 2009; Benlekbir et al., 2012; Oot and Wilkens, 2013). This domain also establishes stabilizing interactions between the V₁ and V₀ sectors, congruent with a role in controlling V₁-V₀ stability and V-ATPase function (Oot and Wilkens, 2010; Rahman et al., 2013). We created a fluorescently tagged version of Vph1NT by replacing the C-terminal region of Vph1p, including the transmembrane helices, with GFP. This construct does not complement the phenotype of a VPH1 deletion, and both V₁ and V₀ subunits are absent from the vacuolar membrane when this construct is expressed (Manolson et al., 1992). However, if a binding site for P(3,5)P₂ exists in the Vph1NT domain, then the tagged construct might be recruited to membranes that have increased P(3,5)P₂ concentration in the absence of the other subunits.

We assessed recruitment of the Vph1NT-GFP construct to intracellular membranes by microscopy. We used two strategies to increase cellular P(3,5)P₂ level. In the first approach, we exposed cells to salt shock (0.5 M NaCl) and monitored changes in Vph1NT-GFP localization over time. Previous work suggests that salt shock induces a rapid, transient increase in cellular level of P(3,5)P₂ (Dux et al., 2006a). Vph1NT-GFP diffusely stains the cytosol of cells grown in low-salt media. On salt exposure, it is transiently recruited to intracellular compartments (Figure 7A). Localization to membranes is first observed ~2 min after salt shock, peaks at 6–7 min of salt exposure, and then is lost by 16 min. This recruitment depends on P(3,5)P₂—it does not occur in a vac14Δ mutant (Figure 7A). A time course of Vph1NT-GFP recruitment in a single salt-treated cell is shown in Supplemental Figure S1.

In the second approach, we increased P(3,5)P₂ level with the constitutively active FAB1-VLA mutant described earlier. Consistent with Figure 7A, cells containing Vph1NT-GFP and only the wild-type FAB1 allele show little colocalization of the GFP signal with the...
PI(3,5)P2 binds and activates V-ATPase

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<th>B. wild-type FAB1</th>
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<tr>
<td>no salt</td>
<td>Wild-type</td>
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<td>7 min. salt</td>
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FAB1 wild-type cells and 0.14 for FAB1-VLA cells. This indicates that there was very little overlap of the signals, despite the extensive recruitment of Vph1NT-GFP to membranes in the FAB1-VLA samples. Salt treatment of cells after staining with FM4-64 also indicated that recruited Vph1NT-GFP localized adjacent to FM4-64-stained vacuoles (unpublished data). This staining pattern may be consistent with recruitment of Vph1NT-GFP to late endosomes, perivacuolar membranes that also contain PI(3,5)P2, rather than vacuoles.

The phenotypes described in Figures 1–3 indicate stabilization of the V-ATPase in vacuolar membranes, but the Vph1NT-GFP construct did not appear to reach the vacuolar membrane. It is possible that Vph1NT-GFP is initially recruited to late endosomes because they contain the highest PI(3,5)P2 levels, but that this interaction cannot be sustained in the absence of other interactions available to the intact enzyme. Several PI(3)P-binding FYVE domains support binding to PI(3)P in the context of intact proteins but cannot localize to PI(3)P-containing membranes as isolated as FYVE-GFP fusions (Hayakawa et al., 2004). Dimerization of these FYVE domains can increase avidity for the membrane and allow PI(3)P-dependent recruitment. To address this possibility, we constructed a Vph1NTNT-GFP containing a tandem fusion of two Vph1NT domains followed by GFP. The Vph1NTNT-GFP protein is also largely cytosolic when cells are grown in low-salt medium but is recruited to FM4-64-stained vacuoles in response to salt (Figure 8). Recruitment to membranes is sustained for longer periods than recruitment of the single Vph1NT-GFP, as shown by the extensive recruitment after 14 min of salt exposure. These results provide in vivo evidence that Vph1NT is able to independently bind to vacuoles under conditions that increase PI(3,5)P2, suggesting that there is a binding site for the lipid on this subunit.

DISCUSSION

V-ATPase assembly and stability can be regulated by PI(3,5)P2 levels

V-ATPase assembly and activity are responsive to multiple extracellular stimuli (Kane, 1995; Diakov and Kane, 2010; Batelli et al., 2007; Li et al., 2012), but the signaling pathways responsible for this response have remained elusive. These results highlight one signaling pathway that regulates V-ATPase assembly and activity. Remarkably, the V-ATPase both requires the very low basal level of PI(3,5)P2 present during growth for full activity (Figure 1) and responds to changes in PI(3,5)P2 levels in the presence of specific extracellular stresses (Figures 2 and 3). V-ATPase activation in response to hyperosmotic stress is highly dependent on PI(3,5)P2 synthesis, and stabilization of the V-ATPase at high extracellular pH appears to
have both PI(3,5)P₂-dependent and -independent components. In contrast, loss of PI(3,5)P₂ has little effect on reversible disassembly of the V-ATPase in response to glucose (Figure 1D). This suggests that a distinct glucose-responsive signaling pathway continues to operate in PI(3,5)P₂-deficient mutants. Taken together, the results indicate that PI(3,5)P₂ is a significant regulator of V-ATPase assembly and activity, although not the exclusive regulator.

How could PI(3,5)P₂ regulate V-ATPase activity?

We provide evidence that PI(3,5)P₂ binds in the membrane V₀ sector of the V-ATPase—specifically, the cytosolic N-terminal domain of the largest membrane subunit, Vph1NT. Vph1NT occupies a key position at the interface of the V₁ and V₀ sectors in the V-ATPase and provides critical contacts to several subunits of the peripheral V₁ sector (Benlekbir et al., 2012; Oot and Wilkens, 2012). It is thus positioned to stabilize the overall V₁-V₀ interaction, and in fact, has been implicated in regulating V₁-V₀ assembly (Kawasaki-Nishi et al., 2001). An emerging body of work reveals that the activity of many transmembrane channels and transporters is modulated by phosphoinositides. In this context, lipids often induce conformational changes by binding to a cytosolically exposed domain (Hilgemann, 2007; Suh and Hille, 2008). V-ATPase activation by PI(3,5)P₂ could occur by a similar mechanism as depicted in Figure 9. In this mechanism, the binding of Vph1NT to PI(3,5)P₂ headgroup (shown in dark gray), is portrayed as giving stronger V₁-V₀ binding and higher ATPase activity and H⁺-transport. Complete loss of PI(3,5)P₂ in the fab1Δ mutant precludes access to this conformation, resulting in lower ATPase activity and lower levels of assembly. Wild-type cells in which PI(3,5)P₂ synthesis has not been activated will have both PI(3,5)P₂-bound and unbound fractions. Both PI(3,5)P₂-bound and unbound V-ATPases are susceptible to disassembly in response to glucose deprivation; this suggests that glucose signaling does not occur through PI(3,5)P₂.
not bound to V1 (Wilkens and Forgac, 2001; Qi and Forgac, 2008). This change is believed to silence proton translocation and block V1 interaction and might be susceptible to modulation by lipid interactions. Excess V1 and V6 sectors that are not assembled into active complexes are present even in wild-type cells (Kane, 1995; Parra et al., 2000) and could provide a pool of V6 sectors susceptible to PI(3,5)P2 intervention. The absence of a PI(3,5)P2-stabilized V6 conformation in a fab1Δ or vac14Δ mutant reduces assembly of V1 subunits at the membrane with no reduction in the total cellular levels of V1 subunits, suggesting a shift toward disassembled V1 and V6 complexes. In contrast, increasing PI(3,5)P2 levels, through hyperosmotic stress, would increase the population of Vph1NT in the stabilizing conformation, resulting in higher levels of V1 binding and activity.

We do not yet know the binding motif in Vph1NT responsible for PI(3,5)P2 interaction. Specific lipid-binding peptide motifs for PI(4,5)P2 and PI(3)P have been determined, but it is also clear that a number of lipid-binding sites are three dimensional and require protein folding (Lemmon, 2008; Baskaran et al., 2012). A limited number of PI(3,5)P2-binding proteins are known, and there are no well-defined binding motifs. In addition, there is no high-resolution structure of yeast Vph1NT, but the related yeast Stv1NTI isoform has been modeled based on the structure of an archaeal homologue (Srinivasan et al., 2011; Finnigan et al., 2012), allowing comparison to known PI(3,5)P2-binding proteins. Several PROPPIN proteins have been shown to bind PI(3,5)P2 (Baskaran et al., 2012; Tamura et al., 2013), but the β-propellers involved in binding by these proteins are not present in Vph1NT. The N-terminal 70 amino acids of the intracellular Ca2+-release channel TRPML1 contain a PI(3,5)P2-binding site that controls channel opening but has detectable homology to neither Vph1NT nor PROPPIN-like sequences. Also note that although the Vph1NTNT-GFP showed better vacuolar recruitment than Vph1NT, there is little evidence that Vph1NT is dimerized in the intact enzyme. In the intact V-ATPase, Vph1NT is anchored in proximity to the membrane by the rest of the complex, and this could easily provide the increased avidity required to bind Vph1NT to PI(3,5)P2 in the vacuolar membrane.

Potential implications of the V-ATPase as a PI(3,5)P2 target
Localized signaling has been proposed as one of the main advantages of phosphoinositides as signaling molecules (Suh and Hille, 2005, 2008) and could reinforce other V-ATPase regulatory mechanisms, such as isoform composition (Forgac, 2007). In plants, activation of V-ATPase activity in response to salt stress has been proposed to drive organellar salt sequestration by providing a pH gradient to drive Na+/H+ exchangers (Queiros et al., 2009; Silva and Geros, 2009). Because these exchangers reside in endosomes and lysosomes, where PI(3,5)P2 is enriched, activation via PI(3,5)P2 would localize V-ATPase activation to sites of salt uptake.

Vacularification defects, based on defective uptake of the lysosomotropic amine quinacrine, have been documented in yeast mutants compromised in PI(3,5)P2 biosynthesis (Cooke et al., 1998; Gary et al., 1998; Dove et al., 2002; Rudge et al., 2004). Reduced V-ATPase activity is likely to be directly responsible for these defects. Compromised V-ATPase function and organellar acidification defects could also account for certain PI(3,5)P2-associated phenotypes in other systems. Fibroblasts and neurons cultured from mouse mutants deficient in PI(3,5)P2 synthesis exhibit defects in membrane-trafficking pathways such as endosome-to-trans-Golgi network retrograde trafficking (Chow et al., 2007; Zhang et al., 2007). Similar phenotypes can also be seen in cultured mammalian cells that overexpress dominant-negative Fab1 (Ikonomov et al., 2003), as well as in cells from Caenorhabditis elegans and Drosophila with Fab1/PIKfyve mutations (Nicot et al., 2006; Rusten et al., 2006). V-ATPase activity is very important in endolysosomal trafficking and thus may well contribute to these phenotypes (Yan et al., 2009). The neurological disease Charcot–Marie–Tooth 4J maps to mutations in the human Fig4 gene (Chow et al., 2007), and distinct mutations in Fig4 have been linked to amyotrophic lateral sclerosis and primary lateral sclerosis (Chow et al., 2009). Mice lacking Vac14 or Fig4 function also exhibit profound neurodegeneration and cellular vacuolation (Chow et al., 2007; Zhang et al., 2007; Jin et al., 2008). In each of these cases, the underlying causes of the neurological defects have not yet been fully determined. Both defective trafficking and defective autophagy have been cited as possible roots of the degeneration (Chow et al., 2007; Zhang et al., 2008a; Ferguson et al., 2009). The V-ATPase plays a central role in autophagy as well as trafficking pathways (Forgac, 2007; Wolls et al., 2010). Thus, compromised V-ATPase function may well contribute to disease phenotypes associated with loss of PI(3,5)P2 homeostasis.

MATERIALS AND METHODS

Media
Yeast extract/peptone/2% dextrose (YPD) medium was buffered to pH 5.0 with 50 mM potassium phosphate and 50 mM potassium succinate as described (Yamashiro et al., 1990). Synthetic complete (SC) medium was prepared as in Amberg et al. (2005) and buffered to pH 5 or 7 with 50 mM morpholineethanesulfonic acid (MES) as described (Diakov and Kane, 2010). For vacuolar vesicle preparations, yeast were grown to log phase in either in YEPD, pH 5.0, or buffered SC media as described (Diakov and Kane, 2010).

Yeast strains and plasmids
Yeast fab1Δ, and vac14Δ mutants in the BY4741 strain background were purchased as part of a yeast deletion mutant array from Open Biosystems (Pittsburgh, PA). The fab1Δ::kanMX and vac14Δ::kanMX alleles were PCR amplified from the mutant strains with oligonucleotides flanking the deletion and then transformed into wild-type yeast strain SF838-5A (MAT α leu3-2, 112, ura3-52, ade6, gal2). All vacuolar vesicle preparations were from the SF838-5Aα strain background.

Purification of vacuoles and biochemical analysis
Cells were grown to log phase, converted to spheroplasts, and lysed, and vacuolar vesicles were isolated by Ficoll density gradient centrifugation (Roberts et al., 1991). ATP hydrolysis rates were determined on freshly prepared vacuolar vesicles by a coupled enzyme assay described previously (Liu et al., 2005); concanamycin A was added directly to the assay mixture to a final concentration of 100 nM to determine inhibitor-sensitive activity. Specific V-ATPase activity represents the rate of concanamycin A-sensitive ATPase hydrolysis, expressed as micromoles of ATP consumed/minute per milligram of vacuolar protein. Proton pumping was observed using the 9-amino-6-chloro-2-methoxyacridine (ACMA) quenching assay described previously (Liu et al., 2005). A 10-μg amount of vacuolar vesicles was used for each assay. Pumping was initiated by adding 0.5 mM ATP and 1.0 mM MgSO4. The rate of proton pumping is represented by the initial rate (first 15 s after MgATP addition) of ACMA fluorescence quenching in the presence or absence of 100 nM concanamycin A and is normalized to the amount of vacuolar protein added.

For determination of salt-responsive V-ATPase activity, wild-type and mutant cells were converted to spheroplasts, resuspended in synthetic complete medium containing 1.2 M sorbitol with or...
with 500 mM NaCl, and incubated at 30°C for 20 min before cell lysis and isolation of vesicles (Li et al., 2012). For examination of extracellular pH dependence of V-ATPase activity, wild-type and vac14Δ mutant cells were grown in synthetic complete medium buffered to pH 5 or 7 with 50 mM MES and then incubated with or without glucose addition after spheroplasting as described (Diakov and Kane, 2010).

For Western blot analysis, vacuolar vesicles were solubilized in cracking buffer, separated by SDS–PAGE, and transferred to nitrocellulose as described (Smardon and Kane, 2007). V-ATPase subunits were detected with mouse monoclonal antibodies 10D7 (anti-Vph1p), BB1 (anti-V1-A subunit), 13D11 (anti-V1-B subunit), and 7A2 (anti-V1-C subunit; Kane et al., 1992). The vacuolar marker was detected with monoclonal antibody 1D3A10 (Life Technologies, Grand Island, NY). Quantitation was done using ImageJ 1.48g (National Institutes of Health, Bethesda, MD). For each independent vacuole preparation, signals from the V1-C and Vo-a immunoblots were quantitated (for loads determined to be in the linear range of detection), and the ratio of the two signals, representative of the level of V1/Vo assembly, was normalized to the ratio of a wild-type sample run in parallel. The variation in ratios for wild-type samples was determined by comparing multiple wild-type samples on the same immunoblot.

Colocalization of DsRed-tagged Yma5p with Vph1-GFP
In both fab1Δ and wild-type (LYW7235 MATA ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, sucl2-Δ9) yeast, VPH1 fused with green fluorescent protein at its C-terminus was integrated into the VPH1 locus and expressed from its endogenous promoter. The integrated allele was generated using the GFPkanMX6 cassette and standard protocols. To generate pRS416-VMA5-DSRed, PCR was used to amplify the open reading frame of VMA5 without the stop codon, along with 1 kb of upstream genomic DNA, and introduced into pDONR221 by recombination-based cloning (Gateway system; Invitrogen, Grand Island, NY). The VMA5 entry clone was introduced into the pAG416-ccdB-DsRED destination vector (Addgene, Cambridge, MA).

For colocalization studies, overnight cultures were diluted to 5 × 10^6 cells/ml in SC-Ura medium. After 24 h, cells were visualized with fluorescence and differential interference contrast (DIC) microscopy. Images were generated with a DeltaVision system (Applied Precision, Issaquah, WA).

Isolation of Vo sectors and analysis of binding to PIP arrays
Vo complexes were kindly provided by Sergio Couoh-Cardel and Stephan Wilkens (SUNY Upstate Medical University, Syracuse, NY). The Vo complexes were purified from a membrane fraction solubilized in dodecyl maltoside and then isolated via a TAP tag on VPH1 (S. Couoh-Cardel and S. Wilkens, unpublished data). The purified Vo was provided at 26 mg/ml protein and then diluted in blocking buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 3% fatty acid–free bovine serum albumin [BSA]) containing 0.02% dodecyl maltoside (Anatrace, Santa Clara, CA) to 80 μg/ml before probing the PIP Arrays (Echelon Research Laboratories, Salt Lake City, UT), which were blocked with blocking buffer for 1 h. All blocking, incubation, and wash steps were done with shaking at room temperature. Purified Vo sectors were incubated with the PIP Array at a final concentration of 80 μg/ml for 1 h. After this incubation, membranes were washed three times in Tris-buffered saline (TBS; blocking buffer without BSA) for 10 min each, incubated with anti-Vo-a subunit antibody (10D7; Kane et al., 1992) in blocking buffer for 1 h, washed another three times in TBS, and incubated with horseradish peroxidase–conjugated anti-mouse immunoglobulin G in blocking buffer for 1 h (Bio-Rad, Hercules, CA). After three washes in TBS, membranes were developed by enhanced chemiluminescence Western blotting detection reagent (GE Healthcare, Piscataway, NJ) and exposed to film for 1–3 s.

Construction and imaging of Vph1NT-GFP and Vph1NTNT-GFP
Vph1NT-GFP was constructed by genomic integration of GFP and a kanMX marker immediately after the sequence for amino acid 406 of Vph1p. The pFA6-GFPkanMX plasmid (Longtine et al., 1998) was used as a template for PCR amplification using oligonucleotides 5′-TCCCAAAGTAGTCTGTGACTGTTTAAGTCAGTACAGAA- AATACTATTAGTTACGTTACGTTACGTTGATTAAATG5′ and 5′-GCTTGAAA-GCGGGAGAGCTTGCACATGCAACACGACCTTCATGCTTTATTA- GAATCGAAGCTGGTTAAAAC3′, in which 51 bases of VPH1 sequence upstream and downstream of the insertion are italicized. The PCR product was then transformed into SF838-5Ax (wild type) or the congenic vac14Δ mutant cells, and transformants were selected by growth on YEPD containing μg/ml G418. Transformants were then screened for production of the ~75-kDa Vph1NT-GFP fusion protein by immunoblot. The construct removes the C-terminal transmembrane helices of Vph1p and replaces them with GFP. To construct the Vph1NTNT-GFP strain containing a tandem duplication of the first 406 amino acids of VPH1, genomic DNA was prepared from cells containing the Vph1NT-GFP-kanMX construct and used as a template for PCR amplification using oligonucleotides 5′-TCCCAAAGTAGTCTGTGACTTACTTAGTTACGTTACGTTACGTTGATTAAATG5′ (italicized nucleotides correspond to the sequence at the end of VPH1NT, and the final 20 nucleotides correspond to the beginning of VPH1 open reading frame) and 5′-AAGCTTTTCTAGATGATGAC- 3′ (complementary to a sequence 200 base pairs downstream from the VPH1 open reading frame). The 4-kb PCR product was isolated and transformed into SF838-5Ax cells, and transformants were selected by growth on G418 plates.

To increase PI(3,5)P2 levels by salt treatment, cells were resuspended in SC medium and NaCl added to a final concentration of 500 mM at time 0. The cells were then transferred to a slide and visualized over time. FM4-64 was purchased from Invitrogen. Vph1NT-GFP cells grown to log phase were suspended in YEPD, pH 5, at a density of 1 OD unit/ml and incubated with 8 μM FM4-64 for 60 min at 30°C. The cells were then pelleted by centrifugation, washed once in YEPD, pH 5, resuspended at the same density in YEPD, pH 5, and incubated for a 75-min chase period at 30°C. After the chase, cells were washed and resuspended in SC medium before visualization by fluorescence microscopy. GFP-tagged proteins and FM4-64 labeling were visualized on a Zeiss Imager Z1 fluorescence microscope using GFP and Texas red filter sets, respectively. Fluorescent and DIC images were captured with a Hamamatsu charge-coupled device camera and analyzed with Axiovision 4.8 software (Carl Zeiss Company, Peabody, MA). Figures were prepared using Photoshop 11.0.4 (Adobe, San Jose, CA).

Quantitation of overlap from micrographs
The extent of colocalization was quantitated using the Just Another Colocalization Plug-in (Bolte and Cordelieres, 2006) in ImageJ 1.48g. The same fields of cells labeled with GFP-tagged protein and either DsRed-labeled protein (Figure 4) or FM4-64 (Figures 7 and 8) were submitted, and the Manders coefficients M1 (fraction of red signal overlapping green) and M2 (fraction of green signal overlapping red) were calculated. Threshold values...
were generated by the plug-in. The M1 and M2 coefficients with automatic threshold were used in Figures 4 and 7. For Figure 8, the M2 coefficient with Costes' threshold was used because lower expression of the Vph1NTNT-GFP construct results in a low GFP signal relative to the FM4-64 signal (Bolte and Cordelieres, 2006).

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