Role of the V-ATPase in Regulation of the Vacuolar Fission–Fusion Equilibrium

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Like numerous other eukaryotic organelles, the vacuole of the yeast *Saccharomyces cerevisiae* undergoes coordinated cycles of membrane fission and fusion in the course of the cell cycle and in adaptation to environmental conditions. Organelle fission and fusion processes must be balanced to ensure organelle integrity. Coordination of vacuole fission and fusion depends on the interactions of vacuolar SNARE proteins and the dynamin-like GTPase Vps1p. Here, we identify a novel factor that impinges on the fusion–fission equilibrium: the vacuolar H⁺-ATPase (V-ATPase) performs two distinct roles in vacuole fission and fusion. Fusion requires the physical presence of the membrane sector of the vacuolar H⁺-ATPase sector, but not its pump activity. Vacuole fission, in contrast, depends on proton translocation by the V-ATPase. Eliminating proton pumping by the V-ATPase either pharmacologically or by conditional or constitutive V-ATPase mutations blocked salt-induced vacuole fragmentation *in vivo*. In living cells, fission defects are epistatic to fusion defects. Therefore, mutants lacking the V-ATPase display large single vacuoles instead of multiple smaller vacuoles, the phenotype that is generally seen in mutants having defects only in vacuolar fusion. Its dual involvement in vacuole fission and fusion suggests the V-ATPase as a potential regulator of vacuolar morphology and membrane dynamics.

INTRODUCTION

Cellular compartments are very dynamic structures that adapt their shape, size, and number to cellular metabolism, differentiation state, and environmental conditions. Changes in mitochondrial shape during animal development (Chan, 2006), proliferation and degradation of peroxisomes in response to nutrient availability (Yan *et al.*, 2005), and extensive fragmentation of the mammalian Golgi during mitosis (Shorter and Warren, 2002) are only a few examples highlighting organellar dynamics. Organelle homeostasis in the face of permanent membrane remodeling requires an equilibration and coordination of the fundamental processes of membrane fission and fusion.

Important insights into membrane dynamics have come from studies of the vacuole of the budding yeast *Saccharomyces cerevisiae*. Vacuoles are highly dynamic structures that undergo regulated cycles of membrane fission and fusion in the course of the cell cycle and in adaptation to changing environmental conditions. When a yeast cell buds and initiates growth of a daughter cell, the vacuole pinches off

vesicles that migrate into the growing daughter cell where they fuse to form the new vacuolar compartment (Weisman, 2003). Vacuolar rearrangements also occur when yeast cells are faced with nutrient limitation or osmotic stress. Exposure of yeast cells to hypertonic medium induces fragmentation of the vacuole into numerous small vacuolar vesicles, a process involving Fab1p-mediated phosphatidylinositol-3,5-bisphosphate synthesis (Efe *et al.*, 2005). Conversely, in fast adaptation to hypotonic shock, vacuoles fuse to give rise to a more voluminous vacuole. These rapid changes of the surface-to-volume ratio of the vacuole via fission or fusion allow uptake or release of water to restore the osmotic balance of the cell.

Vacuolar membrane dynamics can be readily analyzed in living yeast cells by fluorescence microscopy (Vida and Emr, 1995). Moreover, homotypic vacuole fusion can be assayed cell free on isolated organelles (Conradt *et al.*, 1992; Haas *et al.*, 1994). Detailed studies of vacuolar fusion have allowed to dissect the process into different stages and to categorize the molecular players accordingly (Wickner, 2002). In the first stage of vacuole fusion, priming, the ATPase Sec18p/NSF and its cofactor Sec17p/ α -SNAP disassemble *cis*-soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes, thereby activating them for fusion. Vacuoles are then tethered by the action of the Rab-GTPase Ypt7p, the homotypic fusion and vacuole protein sorting (HOPS) complex, and the Ccz1p–Mon1p complex (Wang *et al.*, 2003). This allows *trans*-SNARE complexes to form and fusion partners to associate more tightly (docking). Addition of recombinant Vam7p to the *in vitro* fusion reaction promotes docking of vacuoles while bypassing the requirement for priming and reducing the level of Ypt7p needed to drive fusion (Thorngren *et al.*, 2004). The final membrane fusion step involves further factors, such as the armadillo repeat protein Vac8p and the membrane sector of

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Abbreviations used: HOPS, homotypic fusion and vacuole protein sorting; PtdIns(3,5)P₂, phosphatidylinositol-3,5-bisphosphate; V_o, membrane sector of the vacuolar H⁺-ATPase; V₁, peripheral sector of the vacuolar H⁺-ATPase; V-ATPase, vacuolar-type ATPase; Vma, vacuolar membrane H⁺-ATPase.

Table 1. Yeast strains used in this study

Strain	Genotype	Source or reference
BJ3505	<i>MATa pep4::HIS3 prb1-Δ1.6R lys2-208 trp1-Δ101 ura3-52 gal2 can</i>	Jones <i>et al.</i> (1982)
BJ3505 <i>ccz1Δ</i>	BJ3505; <i>ccz1::kanMX</i>	This study
BJ3505 <i>nyo1Δ</i>	BJ3505; <i>nyo1::TRP1</i>	Nichols <i>et al.</i> (1997)
BJ3505 <i>nyo1Δ vph1Δ</i>	BJ3505 <i>nyo1Δ</i> ; <i>vph1::kanMX</i>	This study
BJ3505 <i>stv1Δ</i>	BJ3505; <i>stv1::TRP1</i>	This study
BJ3505 <i>stv1Δ vph1Δ</i>	BJ3505 <i>vph1Δ</i> ; <i>stv1::TRP1</i>	This study
CUY369a (<i>vac8 cys4,5,7ala</i>)	BJ3505; <i>vac8::TRP1</i> , <i>VAC8pr::pRS406-VAC8pr-vac8cys4,5,7ala</i>	Subramanian <i>et al.</i> (2006)
BJ3505 <i>vam3Δ</i>	BJ3505; <i>vam3::TRP1</i>	Nichols <i>et al.</i> (1997)
BJ3505 <i>vam3Δ vma3Δ</i>	BJ3505 <i>vma3Δ</i> ; <i>vam3::kanMX</i>	This study
BJ3505 <i>vam6Δ</i>	BJ3505; <i>vam6::kanMX</i>	This study
BJ3505 <i>vma3Δ</i>	BJ3505; <i>vma3::TRP1</i>	This study
BJ3505 <i>vma6Δ</i>	BJ3505; <i>vma6::TRP1</i>	This study
BJ3505 <i>vph1Δ</i>	BJ3505; <i>vph1::kanMX</i>	Bayer <i>et al.</i> (2003)
BJ3505 <i>vph1Δ</i> GFP-Pho8p	BJ3505; <i>vph1::kanMX</i> ; <i>pRS316-GFP-PHO8</i>	This study
BJ3505 <i>ypt7Δ</i>	BJ3505; <i>ypt7::URA3</i>	Haas <i>et al.</i> (1995)
BY4732	<i>MATα his3Δ200 met15Δ0 trp1Δ63 ura3Δ0</i>	Brachmann <i>et al.</i> (1998)
BY4732 <i>pep4Δ</i>	BY4732; <i>pep4::URA3</i>	This study
BY4732 <i>pep4Δ vam2Δ</i>	BY4732 <i>pep4Δ</i> ; <i>vam2::HIS3</i>	This study
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Brachmann <i>et al.</i> (1998)
BY4741 <i>vma1Δ</i>	BY4741; <i>YDL185w::kanMX4</i>	Euroscarf
BY4741 <i>vma2Δ</i>	BY4741; <i>YBR127c::kanMX4</i>	Euroscarf
BY4741 <i>vma3Δ</i>	BY4741; <i>YEL027w::kanMX4</i>	Euroscarf
BY4741 <i>vma5Δ</i>	BY4741; <i>YKL080w::kanMX4</i>	Euroscarf
BY4741 <i>vma6Δ</i>	BY4741; <i>YLR447c::kanMX4</i>	Euroscarf
BY4741 <i>vph1Δ</i>	BY4741; <i>YOR270c::kanMX4</i>	Euroscarf
DKY6281	<i>MATα leu2-3 leu2-112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pho8::TRP1</i>	Haas <i>et al.</i> (1994)
DKY6281 <i>vma6Δ</i>	DKY6281; <i>vma6::kanMX</i>	This study
DKY6281 <i>vph1Δ</i>	DKY6281; <i>vph1::kanMX</i>	Bayer <i>et al.</i> (2003)
FCRN044-04B (AL)	FY; <i>MATα ura3-52 his3Δ200 leu2Δ1 LYS2 TRP1 YGL124c (23, 1893)::kanMX4</i>	Euroscarf
FY1679 <i>pep4Δ mon1Δ</i>	FCRN044-04B (AL); <i>pep4::URA3</i>	This study
FY1679-2B	<i>MATα ura3-52 leu2Δ1 TRP1 his3Δ200 GAL2</i>	Winston <i>et al.</i> (1995)
FY1679-2B <i>pep4Δ</i>	FY1679-2B; <i>pep4::URA3</i>	This study
JWY1 (<i>vma4-1^{ts}</i>)	SF838-5A; <i>vma4-1</i>	Zhang <i>et al.</i> (1998)
OMY1	BY4732; <i>pep4::URA3 prb1::TRP1</i>	Muller <i>et al.</i> (2002)
OMY1 <i>vma6Δ</i>	OMY1; <i>vma6::HIS3</i>	This study
SF838-5A	<i>MATα ade6 leu2-3 leu2-112 ura3-52 gal2</i>	Stevens <i>et al.</i> (1986)
W303a	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</i>	John York (Duke University, Durham, NC)
W303a <i>vph1Δ</i>	W303a; <i>vph1::kanMX</i>	This study

the vacuolar H⁺-ATPase (V₀) (Peters *et al.*, 2001; Veit *et al.*, 2001; Wang *et al.*, 2001; Bayer *et al.*, 2003; Subramanian *et al.*, 2006).

Vacuolar-type ATPases (V-ATPases) are multisubunit enzymes mediating ATP-driven translocation of protons from the cytosol into intracellular compartments or extracellular space (Nishi and Forgac, 2002; Graham *et al.*, 2003; Kane, 2006). The V-ATPase complex is composed of the peripheral V₁ domain (subunits A–H) and the membrane integral V₀ domain (subunits a, c, c', c'', d, and e). ATP hydrolysis by the V₁ domain drives proton translocation through the V₀ sector. In budding yeast, two isoforms of subunit a, Stv1 and Vph1, are expressed. Stv1-containing complexes are targeted to the late Golgi, whereas Vph1-containing complexes are found on vacuoles (Manolson *et al.*, 1994). Both isoforms can partially substitute for each other. Besides their crucial role in intracellular pH regulation, V-ATPases have been implied in vacuole fusion in yeasts (Peters *et al.*, 2001; Bayer *et al.*, 2003), in exocytosis of multivesicular bodies in worms (Liegeois *et al.*, 2006), in synaptic exocytosis in flies (Hiesinger *et al.*, 2005), and in insulin secretion in mammalian cells (Sun-Wada *et al.*, 2006). In all these systems, fusion requires physical presence of the V-ATPase complex, but not its proton translocation activity.

MATERIALS AND METHODS

General Methods

Yeasts were cultured in YPD. For growth of V-ATPase mutants, YPD was buffered to pH 5.5 with 50 mM 2-(N-morpholino)ethanesulfonic acid.

Reagents

Concanamycin A was purchased from MP Biomedicals (Irvine, CA), N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl)hexatrienyl-pyridinium dibromide (FM4-64; SynaptoRed C2) was from Biotium (Richmond, CA), and G-418 sulfate was from Calbiochem (San Diego, CA). Vam7p was recombinantly expressed and purified from *Escherichia coli* (Merz and Wickner, 2004).

Strains and Genetic Modifications

Yeast strains used are listed in Table 1. BJ3505 and DKY6281 are standard fusion strains (Haas *et al.*, 1994). OMY1 is a *pep4-* and *prb1*-deficient strain (Muller *et al.*, 2002). Deletion mutants for *vam3* and *nyo1* (Nichols *et al.*, 1997), for *ypt7* (Haas *et al.*, 1995), and for *vph1* (Bayer *et al.*, 2003) have been described previously. BJ3505 *vph1Δ* GFP-Pho8p was generated by transformation of BJ3505 *vph1Δ* with pRS316 GFP-PHO8 expressing green fluorescent protein (GFP)-Pho8p under its endogenous promoter. The plasmid was a kind gift from Robert Piper (Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA). *vma4-1* (JWY1) and the corresponding wild-type (WT) strain (SF838-5A) were generously provided by Patricia Kane (Department of Biochemistry and Molecular Biology, SUNY Upstate Medical University, Syracuse, NY) (Stevens *et al.*, 1986; Zhang *et al.*, 1998). CUY369a (*vac8 cys4,5,7ala*) was a kind gift from Christian Ungermann (Department of Biochemistry, University of Osnabrück, Osnabrück, Germany) (Subramanian

et al., 2006). BY4741 and its *vma1::kanMX4*, *vma2::kanMX4*, *vma5::kanMX4*, and *vph1::kanMX4* derivatives as well as FY1679-13A and its *mon1::kanMX4* derivative were purchased from Euroscarf (Frankfurt, Germany). BY4732 was obtained from the American Type Culture Collection (Manassas, VA). *pep4::URA3* derivatives of FY1679, FY1679 *mon1Δ*, and BY4732 were constructed by one-step gene disruption with pTS15 (from Tom Stevens, Institute of Molecular Biology, University of Oregon, Eugene, OR). *VAM2* was deleted in BY4732 *pep4Δ* by disruption with the *HIS3* cassette by using pYVQ213 (from Yoh Wada, Division of Biological Sciences, Institute of Scientific and Industrial Research, Osaka University, Osaka, Japan) as described previously (Nakamura *et al.*, 1997).

All other deletion mutants were generated by polymerase chain reaction (PCR)-mediated gene disruption by using either the *loxP-kanMX-loxP* module from pUG6 (Guldener *et al.*, 1996) or the auxotrophic marker genes *HIS3* or *TRP1* from pRS403 and pRS404, respectively (Brachmann *et al.*, 1998). To generate the double knockout of *nyo1* and *vph1*, the *VPH1* open reading frame (ORF) was deleted in BJ3505 *nyo1Δ* by one-step gene replacement with the *kanMX* cassette from plasmid pUG6 (Guldener *et al.*, 1996). Primers used have been described previously (Bayer *et al.*, 2003). For generation of W303a *vph1Δ*, the *VPH1* ORF was replaced by the *kanMX* module analogously. Parental strain W303a was kindly provided by John York (Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC). *CCZ1* was disrupted in BJ3505 with the same strategy by using primers Ccz1_Kan_fwd: 5'-CTG ATT CAA TAC CCT CTT TAA ACG AAA AAC TGT CCA TCA TAG CAG CTG AAG CTT CGT ACG C-3' and Ccz1_Kan_rev: 5'-ATT TCC CTG GAT TCC CAG CAG TCA GTC ACG TCA CGA CCT AAA CGC ATA GGC CAC TGA TGG ATT TG-3'. For the generation of deletion mutants of *VMA3*, *VMA6*, or *STV1* in BJ3505 or in BJ3505 *vph1Δ*, the respective ORF was replaced by the *TRP1* cassette from pRS404 (Brachmann *et al.*, 1998) using the following primers: *Vma3_Del_pRS_fwd*: 5'-GAC TGA CCC TTG ATA GTT TTG TAC AAT TAT ACA CTC GTT CTG AGA TTG TAC TGA GAG TGC AC-3', *Vma3_Del_pRS_rev*: 5'-CTC TAT TCC TGC TTT AGT GAT TCA GAA GCT GCC TTA ACA GAC CTG TGC GGT ATT TCA CAC CG-3', *Vma6_Del_pRS_fwd*: 5'-CGC TAA GAG CTA GCA AAA GAG TAT ACC ATT CGG ATC GTG TTG CAG ACG CAG ATT GTA CTG AGA GTG CAC-3', *Vma6_Del_pRS_rev*: 5'-CAC TAA CAC CAC CAC ACG CTT GTA ACA AAC TAA GGA TGT CCT GAC CCT GTG CGG TAT TTC ACA CCG-3', *Stv1_Del_pRS_fwd*: 5'-CGA GGC TTA CAT TAA GGT GCA TAT CAT AAT TCG CAA CAG ACG CAG CAT ATT GTA CTG AGA GTG CAC-3' and *Stv1_Del_pRS_rev*: 5'-CCA CTT GAC CGA CCC AGA CAT ATA TAT ACC GTA AAC TAT GTG CTG TGC GGT ATT TCA CAC CG-3'. *VAM6* was replaced in BJ3505 by the *kanMX* module from pUG6 by using primers *VAM6* Kan fw: 5'-GTC TTA TAT TGA TCA GCA AAA ACC CTT CAA AAT ATC AAT TCA GCT GAA GCT TCG TAC GC-3' and *VAM6* Kan rev: 5'-GAA ATA CTA ACA ACA ATA ACA GCA GCT GTT AAG GGA TCT TGC ATA GGC CAC TAG TGG ATC TG-3'.

FM4-64 Staining

To visualize vacuolar membranes *in vivo*, cells were stained with the vital, lipophilic dye FM4-64 as described previously (Vida and Emr, 1995) with the following modifications: Cells were grown overnight at 25°C to logarithmic phase ($OD_{600} < 1$) in YPD, YPD, pH 5.5, or selective medium. Cultures were adjusted to $OD_{600} = 0.4$, and 10 μ M FM4-64 was added from a 10 mM stock solution in H₂O. Cells were incubated for 1 h at 25°C, harvested (1 min; 3000 $\times$ g), washed twice in fresh medium, resuspended in YPD at $OD_{600} = 0.4$, and shaken for 2–3 h at 25°C. For microscopy, cells were pelleted (15 s; 8000 $\times$ g) and resuspended in YPD at $OD_{600} = 10$, and then they were immediately analyzed by spinning disk confocal microscopy by using an excitation laser at 488 nm and a 100 $\times$ objective. To quantify vacuole morphology, photos of at least 10 random fields were taken. The number of visible vacuolar vesicles in 100 cells per experiment and condition was determined, and cells were accordingly grouped into one of four categories: one to two, three to four, five to eight, or more than eight vacuoles per cell. Data from three or more independent experiments were averaged, and the corresponding standard deviations were calculated.

Concanamycin A Treatment

Cells were grown and stained with FM4-64 as described above. Concanamycin A at 1 μ M (MP Biomedicals) was added to the cultures from a 250 $\times$ stock in dimethyl sulfoxide (DMSO) or ethanol. For kinetic analysis, cells were incubated for 10, 20, or 40 min at 25°C with shaking. For all other experiments, incubation was 2–3 h at 25°C with shaking. Control samples were treated with the corresponding solvent.

In Vivo Fragmentation Test

Yeast cells were grown to logarithmic phase, stained with FM4-64, and treated with concanamycin A if applicable. The culture medium was supplemented with 0.4 M NaCl from a 5 M stock solution in H₂O. After 10-min incubation at 25°C, 200 μ l of cells was centrifuged (15 s; 8000 $\times$ g), resuspended in 10 μ l of their supernatant, and then immediately analyzed by fluorescence microscopy.

Heat Inactivation of the Conditional *vma4* Allele

Cells carrying the conditional *vma4-1* allele were grown logarithmically overnight at 25°C. Vacuolar membranes were labeled with FM4-64 as described above. For inactivation of *vma4-1*, an aliquot of the culture was shifted to 37°C for 40 min. Isogenic wild-type cells were treated in the same way as the mutant cells.

Vacuole Isolation and Fusion

Yeast was precultured in YPD medium (6–8 h; 30°C). Overnight cultures were inoculated (30°C; 14–16 h; 225 rpm) in baffled 2-l Erlenmeyer flasks with 1 l of YPD medium. At an OD_{600} of 1–1.5, cells were centrifuged (3 min; 4000 $\times$ g; 23°C; JA10 rotor), resuspended in 50 ml of 0.1 M Tris-Cl, pH 8.9, with 10 mM dithiothreitol, incubated (5 min; 30°C), centrifuged (3 min; 4000 $\times$ g; 2°C; JA10 rotor), resuspended in 15 ml of SB (50 mM K-phosphate, pH 7.5, 600 mM sorbitol in YPD with 0.2% dextrose and 3600 U ml⁻¹ lyticase for BJ3505 or OMY1 and 1800 U ml⁻¹ for DKY6281), and transferred into 30-ml Corex tubes. Cells were incubated (20 min; 30°C), reisolated (1 min, 800 $\times$ g; then 1 min, 1500 $\times$ g, 2°C; JA20 rotor), and resuspended in 2.5 ml of 15% Ficoll 400 in PS buffer [10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) PIPES/KOH, pH 6.8, and 200 mM sorbitol] by gentle stirring with a glass rod. DEAE-dextran (200 μ l for BJ3505 or OMY1 and 100 μ l for DKY6281) was added from a frozen stock (0.4 g l⁻¹) in 15% Ficoll in PS buffer. The cells were incubated (2 min at 0°C; 75 s at 30°C), chilled again, transferred to a SW41 tube, and overlaid with 3 ml of 8% Ficoll, 3 ml of 4% Ficoll, and 1.5 ml of 0% Ficoll (all in PS buffer). After centrifugation (90 min; 150,000 $\times$ g), the vacuoles were harvested from the 0%/4% interphase. A standard fusion reaction contained 3 μ g of each vacuole type (isolated from strains BJ3505 or OMY1 and DKY6281) in a total volume of 30–35 μ l of reaction buffer (20 mM PIPES/KOH, pH 6.8, 200 mM sorbitol, 150 mM KCl, 0.5 mM MgCl₂, 0.5 mM MnCl₂, 0.5 mM ATP, 7.5 μ M Pefablock SC (Roche, Basel, Switzerland), 7.5 μ g l⁻¹ leupeptin, 3.75 μ M *o*-phenanthroline, 37.5 μ g l⁻¹ pepstatin A, 20 mM creatine phosphate, and 35 U ml⁻¹ creatine kinase). The ATP regenerating system, containing MgCl₂, ATP, creatine phosphate, and creatine kinase, was added from a frozen 20 $\times$ stock solution in PS buffer with 150 mM KCl. After 60 min at 27°C, alkaline phosphatase activity was determined as described previously (Wickner and Haas, 2000). One unit of fusion activity is defined as 1 μ mol of *p*-nitrophenol developed per minute and micrograms of BJ3505 vacuoles at 27°C.

RESULTS

In previous reports, we have addressed the involvement of the vacuolar H⁺-ATPase in homotypic fusion of the yeast vacuole (Peters *et al.*, 2001; Bayer *et al.*, 2003; Muller *et al.*, 2003). Using a cell-free reaction of vacuole fusion, we have shown that the V₀ sector of the V-ATPase plays a direct role in vacuole fusion that is distinct from its function in proton translocation. Mutation of fusion-relevant components often results in fragmentation of vacuoles into multiple small vesicles *in vivo* (Raymond *et al.*, 1992; Wada *et al.*, 1992). However, most deletion mutants of the V-ATPase do not display fragmented vacuoles *in vivo* (Seeley *et al.*, 2002; Graham *et al.*, 2003). This poses the question whether V-ATPase is either not necessary for fusion of vacuoles *in vivo* or whether the reverse process, vacuole fragmentation, could be affected by it. Vacuolar integrity in a given strain should be determined by the differential rates of vacuole fusion and fission. If fusion prevails over fission, only a few larger vacuoles should be observed. If, in contrast, fusion is outweighed by fission, vacuoles should be more numerous and smaller. We sought to understand the seemingly contradictory phenotype of the V-ATPase mutants; hence, we began to characterize the role of V-ATPase in the reaction reversing vacuole fusion, i.e., the fission of vacuoles into smaller fragments.

Vacuole Structure of V-ATPase Mutants Correlates to Their Ability to Acidify the Vacuole

Loss of V-ATPase function is associated with the vacuolar membrane H⁺-ATPase (*Vma*⁻) phenotype. This phenotype is characterized by complete loss of vacuolar acidification and ATPase activity, inability to grow on neutral media, and sensitivity to high Ca²⁺ concentrations (Kane, 2006). Deletion of any of the core V-ATPase subunits, except for subunit

Figure 1. Vacuolar morphology of V-ATPase mutants. (A) *vma1Δ*, *vma3Δ*, *vph1Δ*, and WT (BY4741) yeast cells were grown in YPD, pH 5.5, to logarithmic phase, stained with FM4-64, and analyzed by spinning disk confocal microscopy by using an excitation laser at 488 nm and a 100 $\times$ objective. As an alternative means to visualize vacuolar membranes, GFP-Pho8p was expressed in *vph1Δ* cells and revealed by fluorescence microscopy as described above. (B) Vacuolar membranes of wild-type and *vph1Δ* cells (in BJ3505 and W303a background) were labeled with FM4-64 and visualized by fluorescence microscopy.

a, results in the Vma^- phenotype. Subunit a is the only V-ATPase subunit in yeast that is expressed in two isoforms, the vacuolar isoform Vph1p and the Golgi/endosomal isoform Stv1p. They can substitute for one another in proton translocation (Manolson *et al.*, 1994). Deletion of Vph1p does not result in the full Vma^- phenotype (Manolson *et al.*, 1994) because the Stv1p-containing V-ATPase isoform remains. Vacuoles of *vph1Δ* cells still show basal vacuole acidification (Perzov *et al.*, 2002).

We assayed the vacuolar morphology of V-ATPase mutants by microscopy after labeling the vacuolar membranes with the red fluorescent vital dye FM4-64 (Vida and Emr, 1995). *vma* mutants deleted for the V_1 -subunit Vma1p or the V_0 subunits Vma3p or Vma6p typically showed one enlarged vacuole per cell (Figure 1A). In contrast to partial deletions of the V_0 subunit Vph1p in W303 background, for which no fragmentation was observed (Perzov *et al.*, 2002), complete knockouts of Vph1p displayed numerous small vesicle-like structures, independently of the strain background (BY4741, BJ3505, or W303a; Figure 1, A and B). Visualization of vacuolar membranes in *vph1Δ* by fluorescence microscopy of the vacuolar marker proteins GFP-Pho8p (Figure 1A) and Vtc1p-GFP (data not shown) identi-

Figure 2. Fusion activity of V_0 mutants. Vacuoles were prepared from WT and mutant cells (*vma6Δ* and *vph1Δ*). Their fusion activity was assayed in standard reactions run in the presence or absence of recombinant 0.1 g l⁻¹ Vam7p. After 60 min on ice or at 27°C, alkaline phosphatase (ALP) activities were determined. ALP activity of the wild-type control at 27°C was 3–5 U. Ice values varied from 0.2 to 0.35 U, and they were subtracted from the respective 27°C values.

fied these structures as vacuolar fragments. This link between vacuolar acidification and morphology suggested that proton translocation by the V-ATPase might have an impact on vacuole morphology, possibly by interfering with the balance between fusion and fission events at vacuolar membranes.

The V-ATPase Is Required for Both Vacuole Fusion and Vacuole Fragmentation

Vacuole fusion can be studied on isolated organelles in a cell-free assay. Vacuoles are purified from a strain that expresses the enzymatically inactive proform of the alkaline phosphatase (pro-Pho8p) and from a strain that lacks alkaline phosphatase but provides the vacuolar proteases (Pep4p and Prb1p) required for maturation of pro-Pho8p. Vacuoles from both strains are mixed and incubated in the presence of ATP and salt. When vacuoles fuse, their contents mix; hence, Pep4p and Prb1p gain access to proalkaline phosphatase, resulting in activation of Pho8p. Alkaline phosphatase activity serves as readout for fusion (Haas *et al.*, 1994).

We analyzed vacuole fusion activity of mutants deleted for the V_0 subunits Vph1p or Vma6p by using this system. Vacuoles from *vph1Δ* incubated either alone or in combination with wild-type vacuoles did not fuse efficiently (Figure 2). Addition of the recombinant target membrane-associated-SNARE subunit Vam7p to the fusion reaction of *vph1Δ* vacuoles enhanced fusion only slightly (from 13 to 18% of wild type). Similarly, vacuoles from *vma6Δ* showed only marginal fusion activity (18%), when incubated alone. Recombinant Vam7p increased fusion of *vma6Δ* to 34% of wild-type. If *vma6Δ* vacuoles were fused in combination with wild-type vacuoles; however, 64–68% of wild-type fusion activity was reached. In the presence of recombinant Vam7p, fusion was even stimulated to wild-type levels. For the moment, we cannot explain the differential fusion defects of vacuoles from *vph1Δ* and *vma6Δ* cells with certainty. We speculate that the severe Vma^- growth phenotype associated with the complete loss of the V-ATPase in *vma6Δ* cells (Graham *et al.*, 2003) might trigger adaptive changes in cellular traffic that could alleviate the vacuolar fusion defects resulting from disruption of *VMA6*. Because *vph1Δ* cells do not show the severe Vma^- phenotype, they might not develop similar compensation. Further studies will be necessary to analyze the differences in the vacuolar fusion machineries between the two strains.

Figure 3. In vivo test for vacuole fragmentation. (A) Vacuole fragmentation in response to hypertonic stress. Wild-type cells (BJ3505) were grown in YPD at 25°C to logarithmic phase. Cells were stained with FM4-64, incubated for 10 min in the presence or absence of 0.4 M NaCl, and analyzed by fluorescence microscopy as described in Figure 1. (B) Quantification of vacuole morphology. The number of vacuolar vesicles per cell was determined, and cells were accordingly grouped into the indicated categories. For each experiment, 100 cells per strain and condition were analyzed. Three independent experiments were averaged, and standard deviations were calculated.

We tested a potential role of the V-ATPase in vacuole fission by using mutations resulting in conditional and constitutive deficiency of V-ATPase function. Fragmentation of yeast vacuoles in response to high salt can be readily analyzed in living yeast cells (Bonangelino *et al.*, 2002). Vacuolar membranes were stained with the vital dye FM4-64. Subsequently, cells were subjected to a 10-min incubation with 0.4 M NaCl, and they were immediately analyzed by fluorescence microscopy. Wild-type cells typically displayed one to four large vacuoles that were readily split into several vacuolar fragments upon salt treatment (Figures 3, A and B, and 4; note that different wild-type strains may differ in steady-state vacuole size and number). In contrast to the wild-type, deletion mutants of the V-ATPase catalytic headgroup subunits Vma1p and Vma2p, of the V_1 -stalk-subunit Vma5p, of the proteolipid-ring constituent Vma3p, and of the V_0 rotor-subunit Vma6p maintained their single enlarged vacuole in high salt (Figure 4A). Although these mutations abolish all cellular V-ATPase activity, knockout of Stv1p removes only the Golgi/endosomal isoform of the V-ATPase, but leaves the vacuolar isoform intact. Deletion of Stv1 did not reduce fragmentation activity (Figure 4B). The observed fragmentation defects thus suggest that both the V_1 and the V_0 sectors are necessary for vacuolar fragmentation and that only the vacuole-localized isoform of the V-ATPase (containing Vph1p) is necessary for fission.

To ensure that the observed fragmentation defects were due to a direct role of the V-ATPase in fission rather than to potential secondary effects resulting from the Vma⁻ phenotype, we analyzed the fragmentation activity of a temperature-conditional V-ATPase mutant. The *vma4-1^{ts}* allele provides normal V-ATPase function at 25°C, but it results in loss of vacuolar acidification upon shift to the restrictive temperature of 37°C (Zhang *et al.*, 1998). *vma4-1^{ts}* mutants grown at 25°C displayed wild-type-like vacuole morphology and fragmentation activity (Figure 4C). If cells were

shifted to the restrictive temperature of 37°C 40 min before salt treatment, vacuole fragmentation was strongly impaired. A partial inhibition of fragmentation was apparent also in the wild-type at 37°C, however, to a significantly lesser degree than in the *vma4-1^{ts}* cells.

Proton Translocation by the V-ATPase Is Necessary for Fission

To further analyze the function of the V-ATPase in fission, we tested the effects of the V-ATPase inhibitor concanamycin A on salt-induced fragmentation of wild-type cells. Concanamycin A is a macrolide antibiotic that blocks V-ATPase-dependent proton translocation at nanomolar concentrations (Drose and Altendorf, 1997). We treated yeast cultures for 10, 20, or 40 min in the presence of 1 μ M concanamycin A before testing fragmentation activity. Wild-type cells treated only with the solvent of concanamycin A fragmented their vacuoles in response to high salt. However, cells that had received concanamycin A showed significantly reduced fragmentation activity (Figure 5) already after 10 min of concanamycin A treatment. A small fraction of cells (5%) displayed a single large vacuole surrounded by multiple smaller vacuolar fragments. This phenotype is reminiscent of vacuole structures seen in the deletion mutant of the yeast dynamin-like GTPase Vps1p, which is defective in vacuole fission (Peters *et al.*, 2004).

The apparent suppression of fragmentation by concanamycin A likely results from reduced fission. An alternative explanation would be enhanced fusion activity that immediately reverses and thus hides the fragmentation event. If enhancement of fusion underlies the fragmentation defect, inactivation of a fusion component by mutation should render vacuole fragmentation insensitive toward concanamycin A treatment. Hence, we tested the effect of concanamycin A on salt-induced fragmentation of a mutant lacking the fusion-relevant vacuolar vesicle (v)-SNARE Nyv1p (Nichols *et al.*, 1997). In this mutant, fragmentation activity was blocked by concanamycin A to the same extent as in wild-type cells (Figure 6, A and B). Hence, the abolishment of fragmentation by concanamycin A is unlikely to be due to enhanced fusion activity. We conclude that vacuole fragmentation requires proton translocation by the V-ATPase.

Fission Defects Are Epistatic to Fusion Defects

Because the V-ATPase fulfills a dual role in vacuolar fusion and fission, we sought to define whether one role might predominate over the other in vivo. Vacuole fusion requires the physical presence of the V-ATPase, but, in contrast to fission, it does not depend on the proton pump function of the V-ATPase (Bayer *et al.*, 2003; Muller *et al.*, 2003). The V-ATPase mutant *vph1 Δ* shows strongly reduced fusion activity (Figure 2), but it retains basal vacuole acidification and it shows vacuolar fragmentation in vivo. Hence, it offered a possibility to directly test the possibility of epistasis of fission over fusion. If the fission defect caused by elimination of vacuolar acidification was epistatic to a fusion defect caused by the physical absence of the V_0 complex from the vacuole, it should be possible to cure vacuolar fragmentation in a *vph1 Δ* mutant by abolishing V-ATPase pump activity. To test this hypothesis, we prevented vacuole acidification in *vph1 Δ* both genetically and pharmacologically. We deleted *STV1*, the gene encoding for the Golgi/endosomal isoform of the yeast a subunit, in the *vph1 Δ* background. The resulting *vph1 Δ stv1 Δ* double mutants show a complete loss of vacuole acidification (Manolson *et al.*, 1994). The additional deletion of Stv1p restored morphology of the fragmented *vph1 Δ* vacuoles and yielded cells with only few

Figure 4. Fragmentation defects of V-ATPase mutants. Salt-induced vacuole fragmentation in V_0 and V_1 mutants. Yeasts were logarithmically grown in YPD, pH 5.5, at 25°C, stained with FM4-64, and subjected to a 10-min salt shock. Vacuolar morphology was quantified as described in Figure 1. (A) Wild-type, *vma1Δ*, *vma2Δ*, *vma5Δ*, *vma3Δ*, *vma6Δ* (in BY4741). (B) Wild-type and *stv1Δ* (in BJ3505). (C) *vma4-1ts* and its isogenic wild-type SF838-5A were treated and analyzed as described above, but the cells had been transferred to 37°C, or they were kept at 25°C for 40 min before the salt treatment.

enlarged vacuoles (Figure 7A). As an independent means to block proton translocation by the V-ATPase, we incubated wild-type and *vph1Δ* cells with 1 μ M of the pump inhibitor concanamycin A before microscopic analysis. Concanamycin A treatment drastically reduced the fraction of *vph1Δ* cells displaying highly fragmented vacuoles (>8 vacuoles/cell) from 55 to 11% while increasing the number of cells showing one to two vacuoles from 14 to 62%. Concanamycin A only had small effects on vacuole structure of wild-type cells (Figure 7, B and C). The fraction of cells having one to two vacuoles per cell increased from 81 to 90%, whereas the percentage of cells with three to four vacuoles dropped from 18 to 8%. Cells generally displayed large spherical vacuoles. These results indicate that the defects of fission caused by the lack of vacuolar proton pump activity dominate over the defects in vacuole fusion caused by physical absence of the V_0 sector of the V-ATPase.

We tested whether the reversal of fragmentation occurred along the authentic pathway of SNARE-dependent fusion by measuring the effect of concanamycin A on the vacuole structure of a *vph1Δ* strain deleted for the vacuolar v-SNARE Nyv1p. Similar to *vph1Δ*, the double-knockout *vph1Δnyv1Δ* displayed highly fragmented vacuoles. Unlike the single knockout, however, *vph1Δnyv1Δ* cells maintained frag-

mented vacuoles in the presence of concanamycin A (Figure 7D). Quantification revealed only a moderate decrease in the fraction of cells with highly fragmented vacuoles (>8 vacuoles/cell) from 69 to 40%. The percentage of cells with five to eight vacuoles rose from 13 to 22%, of cells showing three to four vacuoles from 10 to 17% and of cells having one to two vacuoles from 8 to 20%. Thus, deletion of Nyv1p counteracted the reversion of vacuole structure in *vph1Δ*. This observation indicates that the phenotypic conversion is SNARE dependent and that it follows the normal fusion pathway.

Deletion of early-acting fusion factors that eliminate docking of the membranes completely prevents fusion. Deletion of late-acting factors can be slightly less severe, because spontaneous fusion of docked membranes can ensue if they are simply held in contact long enough. Consequently, we compared with which degree the vacuolar morphology of early-acting fusion mutants could be reverted by blocking proton translocation. Deletion of the V_0 subunit Vma3p in the SNARE knockout *vam3Δ* had no effect on vacuolar structure: vacuoles seemed highly fragmented in both strains (Figure 8A). Equally, blocking V-ATPase activity with concanamycin A in *vam3Δ* and in deletion mutants of other early-acting fusion factors such as the SNARE Vam7p, the

Figure 5. Proton translocation by the V-ATPase is indispensable for vacuole fragmentation. Effect of concanamycin A on in vivo vacuole fragmentation of wild-type cells (BJ3505). To block proton pumping by the V-ATPase, cells were incubated with 1 μ M concanamycin A for 10, 20, or 40 min before hyperosmotic shock in 0.4 M NaCl.

Rab-GTPase Ypt7p, and the HOPS complex components Vam2p and Vam6p as well as Ccz1p and Mon1p did not

Figure 6. Suppression of fragmentation by concanamycin A results from reduced fission and not from enhanced fusion. Effect of concanamycin A on in vivo vacuole fragmentation of wild-type (A) and fusion-defective *nyv1Δ* cells (B; both in BJ3505). To block proton pumping by the V-ATPase, cells were incubated with 1 μ M concanamycin A for 2 h before hyperosmotic shock in 0.4 M NaCl.

Figure 7. Epistasis of fragmentation and fusion defects. (A) Vacuole morphology of subunit A deletion mutants. Vacuolar membranes of wild-type, *vph1Δ*, *stv1Δ*, and *stv1Δvph1Δ* cells (all in BJ3505 background) were labeled with FM4-64 and visualized by fluorescence microscopy. (B) Effect of concanamycin A on vacuolar morphology of wild-type and *vph1Δ* cells. Cells were stained with FM4-64, and subsequently they were incubated for 2 h with 1 μ M concanamycin A or control buffer (DMSO). Microscopy was as described in A. (C) Quantification of the experiment in B. (D) Vacuole morphology of *nyv1Δvph1Δ* cells after 2-h concanamycin A treatment as described in B.

influence vacuole morphology (Figure 8, B-E; data not shown). However, in a mutant of Vac8p, a factor that is required in a late stage of the fusion reaction (Wang *et al.*, 2001), a reduction in the fraction of cells having five and more vacuoles from 18 to 4% (>8 vacuoles/cell) and from 19 to 10% (5–8 vacuoles/cell) was observed after concanamycin A treatment (Figure 8F). Concurrently, the percentage of cells with one to two and three to four vacuoles increased from 42 to 60% and from 20 to 25%, respectively. These experiments argue for an essential role of early-acting fusion factors in phenotypic conversion, and they suggest that only the effect of late-acting factors, such as Vac8p and Vph1p, can be suppressed.

DISCUSSION

Here, we have identified a requirement of V-ATPase proton pump activity for vacuole fission. This requirement of V-ATPase activity for vacuole fragmentation may relate to previous findings on this process: Vacuole fragmentation in response to hyperosmotic shock is initiated by Fab1p-depen-

Figure 8. Rescue of fusion-defective mutants by blocking proton pumping. (A) Vacuole structure in wild-type, *vam3Δ*, *vma3Δ*, and *vam3Δvma3Δ* cells (all in BJ3505). Labeling and microscopy are as described in Figure 7A. (B–F) Effect of concanamycin A on the morphology of the indicated fusion-defective mutants (all in BJ3505 background). Labeling, concanamycin A treatment, and microscopy are as described in Figure 7B.

dent synthesis of phosphatidylinositol-3,5-bisphosphate [PtdIns(3,5)P₂]. PtdIns(3,5)P₂ may promote fission through recruitment of effectors such as Svp1p/Atg18p to the vacuole (Dove *et al.*, 2004). However, in addition, *fab1* mutants show a reduction of vacuolar acidification, which led to the suggestion that PtdIns(3,5)P₂ could modulate V-ATPase activity (Weisman, 2003). Because we have now found that vacuolar acidification itself is crucial for fission, future studies should address the possibility that PtdIns(3,5)P₂ supports vacuolar fission by regulating V-ATPase activity.

Because the V-ATPase has a dual role in vacuolar fission (as a pump) and in fusion (by physical presence of the V₀ sector and its interaction with SNAREs) (Peters *et al.*, 2001; Bayer *et al.*, 2003), disruption of the V-ATPase will affect both fission and fusion. Our data are consistent with the hypothesis that the apparent vacuole morphology results from a true equilibrium of the competing reactions of fusion and fission. The final outcome depends on the ratio of these reaction rates rather than on their absolute magnitude. Interfering with either one or both reactions by genetic manipulation or drug administration results in a shift of the fission–fusion equilibrium (Figure 9), which can explain the phenotype of V-ATPase mutants: The V-ATPase mutant *vph1Δ* retains basal vacuolar acidification (which may still support fission, even though perhaps at lower rate), and it

Figure 9. Schematic representation of the vacuolar fission-fusion equilibrium. Vacuolar morphology is determined by the equilibrium of the antagonistic processes of fission and fusion that constantly takes place with the specific rates k_{fis} and k_{fus} . Manipulations interfering with fission and/or fusion result in modified reaction rates. The relative changes in fission and fusion rates determine whether and to what extent the equilibrium point is shifted and hence the phenotypic outcome. Three cases can be distinguished: 1) Fission and fusion rates are balanced. Vacuole morphology is normal. 2) Fission is more strongly affected than fusion. As a result fusion prevails, leading to a cell with a single large vacuole. and 3) Fission is less strongly affected than fusion. As a consequence, fission outweighs fusion, resulting in a cell with fragmented vacuoles.

has, in addition, the most pronounced fusion defect. Therefore, fission still prevails and leads to a cell with fragmented vacuoles (Figure 9, type 3). Residual vacuolar acidification in *vph1Δ* could be eliminated either by additional deletion of Stv1p or by concanamycin A treatment. These manipulations may have reduced fission activity enough to allow it to be outweighed by fusion. Thus, the equilibrium of these two reduced rates now favors the restoration of a single large vacuole in *vph1Δ* cells (Figure 9, type 2). This produces the same vacuolar phenotype as seen in all other V-ATPase deletion mutants that completely lack vacuolar proton pump activity. The epistasis of fission over fusion provides a satisfactory explanation for the fact that, in contrast to numerous other vacuolar fusion mutants, V-ATPase mutants do not show a vacuole fragmentation phenotype.

Vacuole fusion proceeds through various steps. The Rab-GTPase Ypt7p, the HOPS complex of tethering factors, and a set of SNARE proteins are required for docking the membranes and for keeping them in close contact. Subsequently, further protein factors such as the V₀ sector and Vac8p are necessary to efficiently induce fusion of the apposed membranes. Disruption of proton pumping by the V-ATPase restored vacuolar morphology in both *vph1Δ* and in *vac8*

mutant cells. In contrast, mutants of early fusion factors such as SNAREs, the Rab-GTPase Ypt7p, the HOPS complex, Mon1p, and Ccz1p were not rescued to a similar extent. This can be explained by the observation that the early factors are needed to keep the membranes in tight apposition, which is an irreplaceable prerequisite for fusion. Bilayer fusion itself requires catalysis by late-acting factors, but it may also occur spontaneously, although at modest rates, if only the vacuoles are tethered and docked. Due to spontaneous fusion disruption of late-acting factors may lead to less profound blocks of fusion than defects in early-acting factors. Hence, fusion defects of early-acting factors may be less readily compensated by reductions of fission activity that can be produced by eliminating vacuolar proton pump activity (Figure 9, types 2 and 3).

Differential reduction of fusion rates may also account for the in vivo phenotype of other mutants in vacuolar fusion factors that do not show fragmented vacuoles, such as the Vtc proteins Vtc1p and Vtc4p, which are involved in SNARE priming (Muller *et al.*, 2002, 2003). The in vitro fusion activity of vacuoles from these strains is less severely impaired than that of *vam3Δ* vacuoles. This becomes especially evident if mutant vacuoles are fused in combination with wild-type vacuoles. A *vam3Δ*/WT combination is virtually nonfusogenic (<10%), whereas a *vtc1Δ*/WT or a *vac8Δ*/WT combination fuses well, i.e., with 50–70% of the activity of a WT/WT combination (Nichols *et al.*, 1997; Veit *et al.*, 2001) (Müller and Mayer, unpublished data). Consequently, the equilibrium of activities in *vtc* mutants might still favor the maintenance of large vacuoles.

The electrochemical gradient produced by proton translocation could influence fission in several ways. Association of peripheral membrane proteins with lipid bilayers often depends on the membrane potential. It is conceivable that cytosolic components of the fission machinery might interact with vacuolar membranes, depending on the pH at the membrane surface. Precedence for such a mode of interaction exists, as exemplified by the recruitment of the cytosolic small GTPase Arf6p and its cognate GDP/GTP exchange factor ADP-ribosylation factor nucleotide site opener (ARNO) to endosomal membranes (Hurtado-Lorenzo *et al.*, 2006). Here, increasing acidification of endosomes along the degradative pathway presumably triggers conformational rearrangements of the V-ATPase that permit subsequent association of Arf6p and ARNO with the V_0 sector. The electrochemical gradient could also influence transport processes across vacuolar membranes, e.g., the pH-dependent activity of vacuolar amino acid or ion transporters and exchangers. In this way, V-ATPase activity might modulate the flux of solutes and water across the vacuolar membrane. Such flux may be connected to vacuole fission, and it may even be essential to it because the fragmentation of a large vacuole into multiple small vesicles will alter the surface to volume ratio. If the available membrane surface stays constant multiple vacuolar fragments will provide much less volume than a single large vacuole. Fragmentation of a large vacuole will hence require an extrusion of water and perhaps also solutes from the organelle to adapt the surface-to-volume ratio.

Finally, the vacuolar proton gradient could also influence membrane structure directly. The transbilayer distribution of phospholipids in large unilamellar vesicles is highly pH sensitive (Hope *et al.*, 1989). And a redistribution of only a small fraction of phospholipids suffices to induce shape changes in giant liposomes (Farge and Devaux, 1992). At the yeast vacuole, elimination of the vacuolar pH gradient might similarly modulate the transmembrane distribution of

phospholipids, and in this way it may change the fission characteristics of the membrane. Moreover, the pH at the membrane surface could affect membrane curvature through modification of the charge of lipids and membrane proteins, which can change their conformation, shape, spontaneous curvature, and assembly state. In line with this, the pH-dependent self-assembly of lysobisphosphatidic acid is essential for the formation of multivesicular liposomes in vitro (Matsuo *et al.*, 2004). Also, other vacuolar processes that involve significant shape changes require a pH gradient, e.g., formation of autophagic tubes (large vacuolar invaginations forming during microautophagy) and scission of vesicles from their tip into the vacuole lumen (Kunz *et al.*, 2004).

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