Sphingolipid Requirement for Generation of a Functional V₁ Component of the Vacuolar ATPase*

Ji-Hyun Chung, Robert L. Lester, and Robert C. Dickson‡

From the Department of Molecular and Cellular Biochemistry and the Lucille P. Markey Cancer Center, University of Kentucky College of Medicine, Lexington, Kentucky 40536

Received for publication, January 28, 2003, and in revised form, April 25, 2003
Published, JBC Papers in Press, May 13, 2003, DOI 10.1074/jbc.M300943200

Vacuolar ATPases (V-ATPases) are found in all eukaryotes where they are required for receptor-mediated endocytosis, renal acidification, bone resorption, neurotransmitter accumulation, and activation of acid hydrolases (reviewed in Refs. 1 and 2). The Saccharomyces cerevisiae V-ATPase, the current paradigm for V-ATPases, moves protons from the cytosol to the lumen of the vacuole to establish a proton gradient that is necessary for vacuolar transport proteins to drive ions and small molecules, amino acids, and metabolites into the vacuole (reviewed in Ref. 3). Knowledge of the S. cerevisiae V-ATPase has grown rapidly but remains incomplete. One area where little is known is the influence of membrane lipids on V-ATPase activity. Here we show that sphingolipids are necessary for V-ATPase activity in S. cerevisiae.

S. cerevisiae V-ATPase contains two components or domains, V₁ and V₀, which associate to form an active V₁V₀ complex on the vacuolar membrane (reviewed in Refs. 3–5). The V₁ domain has ATPase activity and is composed of 8 different proteins. It can exist free in the cytoplasm or complexed with V₀ on the vacuolar membrane. The V₀ domain contains 5 protein subunits and is imbedded in the vacuolar membrane where it serves as a proton pore. The V₁ and V₀ domains are assembled and associate in the ER to form functional V₁V₀ complexes, which are then transported from the Golgi to the vacuole (reviewed in Refs. 3 and 5). Alternatively, the two domains assemble independently and then associate once V₀ reaches the vacuole.

The ER is also where sphingolipid synthesis begins (reviewed in Ref. 6) with generation of ceramide. Ceramide is transported to the Golgi where it is converted sequentially into the complex sphingolipids inositol-phosphoceramide, mannoseinositol-phosphoceramide, and finally to mannose-(inositol-phospho)₂-ceramide. Complex sphingolipids are delivered to cellular compartments, particularly the plasma membrane (7) and to a lesser extent the vacuole (8).

One of the distinguishing features of sphingolipids in S. cerevisiae is the C₂₆ acyl group. Fatty acids with 20 or more carbons, very long chain fatty acids (VLCFAs), are ubiquitous in nature, but little is known about their functions. VLCFAs are mostly found in the ceramide portion of sphingolipids. The importance of the C₂₆ acyl component of S. cerevisiae sphingolipids was demonstrated by the isolation of mutant strains that do not make sphingolipids (9), but instead make a set of novel sphingolipid mimics in which ceramide is replaced by diacylglycerol (10). The presence of a C₂₆ acyl group is the unique feature of these novel glycerolipids, which enables them to mimic some sphingolipid functions.

Further evidence for the essentiality of the C₂₆ acyl group in sphingolipids comes from studies of the FEN1 (ELO2) and SUR4 (ELO3) genes. In fen₁Δ cells only 29% of the sphingolipids contain a C₂₆ acyl group, the rest contain C₂₂ and C₂₄ acyl groups. The sphingolipids in sur₄Δ cells contain only C₂₂ and C₂₄ acyl groups and no C₂₆s (11, 12). Fen₁p and Sur₄p are components of the enzyme system that elongates C₁₆ and C₁₈ fatty acids to form VLCFAs. The exact function of Fen₁p and Sur₄p is unclear because the elongation system has not been fully characterized in any organism. sur₄Δ and fen₁Δ cells are viable, although they have many mutant phenotypes (reviewed in Ref. 13) and deletion of both genes is lethal (14).

A fraction of sphingolipids in higher eukaryotes also contain VLCFAs (15) and the mouse genes Sce₁ and Cig₃₀ complement a sur₄Δ and a fen₁Δ mutant, respectively (16). Interestingly, the mice mutants Quaking and Jimpy, which develop intense
tremors at the age of about 2 weeks as a result of severe demyelination of the central nervous system, have reduced levels of Scc1 mRNA and reduced fatty acid elongation activity (17). Cig30 mRNA has the interesting property of being induced in brown adipose tissue when animals are exposed to cold temperature. These results suggest that VLCFAs are performing important, but unknown functions in mammals, and that some of these functions may be evolutionarily conserved. Recently Kohlwein et al. (12) reported that sur4Δ and fen1Δ cells contain small vacuoles called fragmented vacuoles that fail to properly fuse to form larger vacuoles. This observation suggested to us that sphingolipids with a C26 acyl group are required for V-ATPase activity (49). Cells supplemented with 100 mM CaCl2,4 mM CaCl2, or buffered to pH 7.5 with 100 mM Hepes were prepared as described previously (21). YPD medium contained 1% yeast extract, 2% Bacto-peptone, and 2% glucose.

Quinacrine Staining and Semiquantitative Quinacrine Assay—Vacuolar accumulation of quinacrine was assessed by fluorescence microscopy as described by Roberts et al. (22) or by a semiquantitative quinacrine assay (21). Fluorescence measurements of cell suspensions were done in a Beckman spectrofluorometer (excitation = 419 nm, emission = 425 nm) and the OD at 600 nm was monitored in a spectrophotometer.

SDS-PAGE, Immunoblotting, and Antibodies—SDS-PAGE and immunoblotting were performed according to procedures recommended for the Bio-Rad Tris-Blot S.D. Semi-Dry transfer cell (Bio-Rad Inc.). Monoclonal antibodies against Vph1p, Vma1p, Vma2p, CPY, and ALP were from Molecular Probes. Anti-Myc antibodies were from Roche Monoclonal antibodies against Vph1p, Vma1p, Vma2p, CPY, and ALP were from Molecular Probes. Anti-Myc antibodies were from Roche. Anit-Myc antibodies were from Roach. Monoclonal antibodies against Vph1p, Vma1p, Vma2p, CPY, and ALP were from Molecular Probes. Anit-Myc antibodies were from Roach. Monoclonal antibodies against Vph1p, Vma1p, Vma2p, CPY, and ALP were from Molecular Probes. Anit-Myc antibodies were from Roach.

HPLC Analysis of LCβs and LCBβs—Lipids were extracted from whole cells, converted to fluorescent derivatives and analyzed by HPLC as described previously (23).

Treatment of Purified Vacuoles with PHS—A 100× stock of PHS (10 mm PHS in 95% EtOH) was diluted into a suspension of purified vacuolar membranes suspended in buffer (10 mM Tris, 10 mM MES, pH 6.9, 5.5 mM MgCl2, 25 mM KCl) to give a final concentration of 100 μM. Samples were incubated on ice for 0, 30, 60, 90, and 120 min followed by centrifugation at 13,000 rpm for 10 min in a microcentrifuge (4 °C). Proteins in the supernatant fluid were precipitated by incubating with 5% trichloroacetic acid (final concentration) for 2 h on ice. Pellets and trichloroacetic acid-precipitated proteins were resuspended in 50 μl of cracking buffer (8 mM urea, 5% SDS, 1 mM ethylenediamine tetroacetate, 50 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, Ref. 24) and equal volumes were analyzed by SDS-PAGE and immunoblotting.

Miscellaneous Procedures—Vacuolar membrane vesicles were purified by centrifugation on Ficoll gradients and Mg-ATPase activity was measured at 23 °C as described previously (22) except that membranes were not homogenized before centrifugation on the second Ficoll gradient. Vacuolar membrane vesicles were also purified by sucrose density gradient centrifugation as previously described (25), except that the concentration of sucrose was increased to prevent membranes from pelleting on the bottom of the centrifuge tube. For these experiments, 4 ml of the membrane fraction was overlaid onto a 32-ml gradient composed of equal volumes of 10, 30, 50, and 60% (w/v) sucrose. The gradient was centrifuged for 55 min at 100,000 × g in a Sorvall AH629 rotor at 4 °C and then fractionated starting from the top into 9 fractions of 4, 7, 2, 6, 2, 6, 2, 7 ml and the resuspended pellet. Fractions were frozen in liquid nitrogen and stored at −80 °C. V V0 complexes on vacuolar membranes prepared by sucrose gradient centrifugation were dissociated by treatment with KI as described previously (26, 27).

Cell-free protein extracts used for analysis of Vph1p (Fig. 3), were prepared as described by Kunz et al. (28). Cell-free protein extracts used for the analysis Vma1p, Vma2p, and Vma5p were prepared as described (29).

To measure the calcium-dependent ATPase activity of cytosolic V1 domains, cells were grown, lysed, and a high speed supernatant fraction was prepared as previously described (5). Proteins were precipitated by treatment with 5% trichloroacetic acid as described above, resuspended in and dialyzed against buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM p-methylsulfonylfluoride, 10% glycerol), and centrifuged on a glycerol gradient (30). Fractions (750 μl) were collected from the top of the gradient. Fractions (9–12) containing V1 domains were located by immunoblotting for Vma1p, Vma2p, Vma5p, and Vph1p. The pooled V1-containing fractions were assayed for calcium-dependent ATPase activity as described previously (5). Values are expressed as the difference between assays performed with and without 1.6 mM CaCl2.

The subunit composition (Fig. 7) of V0 and the V V0 complex were analyzed by using a published procedure to cross-link proteins before immunoprecipitation and SDS-PAGE analysis (31). The procedure was modified so that 1 OD of 600-nm units of spheroplasts were incubated with 50 μCi of Trans[35S]label (ICN Inc., 1175 Ci/mmol, 5100607). Resuspension of the sample with protein A-Sepharose or its beads (Sigma Inc.), 400 μl a solution of 5% bovine serum albumin/phosphate-buffered saline containing 5 μl of antibody solution was added, and the sample was incubated overnight on ice with mixing. Protein A-Sepharose (40 μl of a 40% (w/v) suspension) was added to each sample and incubated for 2 h on ice with mixing. Immunoprecipitates were collected by centrifugation at 5,000 rpm for 5 min in a microcentrifuge. Pellets were washed four times in buffer (1% Triton X-100, 1% deoxycholic acid, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl), and precipitated proteins were eluted from the beads by incubation for 10 min at 95 °C in 50 μl of 4× SDS-loading buffer (50 mM Tris base, pH 6.8, 8% glycerol, 1.6% SDS, 4% β-mercaptoethanol, 0.04% bromphenol blue). Half of each precipitate
was subjected to SDS-PAGE and phosphorimage analysis.

Cells were prepared for indirect immunofluorescent microscopy by using a published procedure (22). The secondary antibody was goat anti-mouse IgG labeled with FluoroLink™ Cy3™ (Amersham Biosciences). Fluorescent images were obtained with a Nikon Eclipse E800 fluorescence microscope equipped with a Nikon 100X/1.3 plan fluor oil-immersion objective and a Diagnostic Instruments Spot camera controlled by Adobe Photoshop software. For Cy3 fluorescence, samples were excited at 510–560 nm and viewed with a barrier filter of 570–650 nm. Adobe Photoshop software was used to process images. Protein concentrations were determined with the Bio-Rad DC protein assay kit with bovine serum albumin as a standard.

RESULTS

sur4Δ Cells Have a Unique Set of Vma− Phenotypes That Indicate Reduced V-ATPase Activity—The presence of fragmented vacuoles in sur4Δ cells suggests that the C26 acyl group of sphingolipids is necessary for some vacuolar function. Functional vacuoles require a proton pump or V-ATPase to acidify their lumen. When the V-ATPase is defective a set of phenotypes, referred to as Vma−, are produced. For example, vma mutants do not grow on YPD buffered to pH 7.5 (32, 33). We reasoned that sur4Δ cells might have Vma− phenotypes if sphingolipids containing a C26 acyl group are needed for V-ATPase activity. Indeed, we found that sur4Δ cells grow very poorly at pH 7.5 just like the authentic vmaΔ cells (Fig. 1) (34). In contrast, fen1Δ cells grow better at pH 7.5 (Fig. 1), indicating that they are able to acidify their vacuoles more effectively than sur4Δ cells.

Another Vma− phenotype is failure to grow on YPD plates containing 4 mM ZnCl2 (34). We found that sur4Δ cells have this phenotype whereas fen1Δ cells do not since they grow almost as well as wild-type cells (Fig. 1). Other Vma− phenotypes include failure to grow in the presence of 100 mM CaCl2 (35) or on medium containing a non-fermentable carbon source such as glycerol (36). We found that 100 mM CaCl2 inhibits growth of sur4Δ cells but does not inhibit growth of fen1Δ cells (Fig. 1), again supporting the idea that sur4Δ cells are less able to acidify their vacuoles than fen1Δ cells. In addition, sur4Δ cells grow slowly on YP-glycerol while fen1Δ cells grow slightly faster and both grow better than vmaΔ control cells (Table II).

These data show that the Vma− phenotypes of sur4Δ cells are nearly as pronounced as those in vmaΔ cells and that the phenotypes are less severe in fen1Δ cells. Based upon these phenotypes it appears that the V-ATPase is more impaired in sur4Δ cells than in fen1Δ cells.

To directly examine vacuolar acidification in vivo we used the lysosomotropic fluorescent dye quinacrine, which is taken up by cells and concentrated in acidified vacuoles; if vacuoles are not acidified the dye remains in the cytoplasm and gives a diffuse fluorescent signal (37). A strong fluorescent vacuolar signal was observed in wild-type cells (Fig. 2A). No fluorescent signal was seen in sur4Δ cells, which behaved like vmaΔ control cells that do not acidify their vacuole. The fluorescent signal in fen1Δ cells was lower than in wild-type cells but greater than in sur4Δ cells.

The fluorescent microscopy results were verified by a semi-quantitative spectrophotometric assay of the relative fluorescence of populations of cells stained with quinacrine. By this technique, wild-type cells fluoresced strongly whereas sur4Δ cells fluoresced near the background level measured in vmaΔ cells (Fig. 2 and Table II). The fluorescent signal in fen1Δ cells was 60% of the wild-type level indicating that they are able to acidify their vacuoles better than sur4Δ cells but not as well as wild-type cells. Taken together these data show that sur4Δ cells have a unique set of Vma− phenotypes and that they fail to acidify their vacuoles. These phenotypes are less severe in fen1Δ cells, which have some ability to acidify their vacuoles, although they do not acidify as well as wild-type cells.

V-ATPase Activity Is Reduced in sur4Δ Cells and Ficoll Dissociates the V1/V0 Complex—The data presented in Figs. 1 and 2 and Table II suggest that vacuolar membranes isolated from sur4Δ cells should have less V-ATPase activity than vacuolar membranes isolated from fen1Δ cells and both should have less activity than those isolated from wild-type cells. In agreement with this prediction, vacuolar membranes isolated by Ficoll density gradient centrifugation from sur4Δ cells had only 10% of the wild-type V-ATPase activity while those isolated from fen1Δ cells had 25% of the wild-type activity (Table III).

Reduced V-ATPase activity could be due to either a reduced specific activity or fewer molecules. To differentiate between these alternatives the concentration of Vph1p, a subunit of the V0 domain, and Vma1p, Vma2p, and Vma5p, subunits of the V1 domain, were measured by immunoblotting of whole cell protein extracts and of purified vacuolar membranes (Fig. 3). Whole cell protein extracts from wild-type, sur4Δ, and fen1Δ mutants contained a similar level of each of the four proteins, indicating that the steady-state level of the proteins is similar in mutant and wild-type cells. Likewise, Vph1p was present in similar amounts in vacuolar membranes purified from the three strains. In contrast, the level of Vma1p Vma2p, and Vma5p in vacuolar membranes isolated from sur4Δ and fen1Δ cells was greatly reduced (Fig. 3). These results suggest that there is a defect in the V1 domain in sur4Δ and fen1Δ cells or that the interaction between V0 and V1 is abnormal and V1 or some of its subunits dissociate during vacuolar purification.

The interaction of V1 with V0 has been examined by treating vacuolar membranes with a low salt buffer containing ethylenediamine tetraacetate and then determining if particular protein subunits remained in the vacuolar fraction (high speed pellet) or became soluble (38). Because Ficoll purification removed V1 subunits from vacuolar membranes, we used cell-free extracts in place of vacuolar membranes for these assays. Treatment with a low salt buffer did not reveal any difference in the level of Vma1p and Vma2p in the pellet and soluble fractions obtained with sur4Δ, fen1Δ, or wild-type cells (data not shown). We also determined if increasing concentrations of
the interaction between Vma1p and Vma2p and V0 in sur4Δ cells was further elucidated by determining whether V1 and V0 domains associate with V0 on the vacuolar membrane in sur4Δ cells but the association is abnormal because the Ficoll gradient procedure causes Vma1p, Vma2p, and Vma5p and possibly the entire V1 domain to dissociate from V0 domains. In addition, vacuolar membranes isolated from sur4Δ cells by either Ficoll or sucrose density gradients have reduced V-ATPase activity.

V1 Associates with the Vacuolar Membrane in sur4Δ and fen1Δ Cells—Both density gradient procedures for isolating vacuolar membranes subjects the sample to non-physiological conditions and could create artifacts such as by proteolysis. To try and avoid these potential complications, we examined the association of V1 with V0 on the vacuolar membrane of intact cells by using indirect immunofluorescence microscopy with anti-Vma1p or anti-Vma2p antibodies (33). In wild-type cells both antibodies localized to the vacuolar membrane as expected (Fig. 5). A similar localization is seen in sur4Δ and fen1Δ cells except that the vacuoles are fragmented and do not stain as uniformly as do vacuoles in wild-type cells (Fig. 5). Control cells lacking the vma2 gene show diffuse staining throughout the cytoplasm with anti-Vma1p antibody (data not shown) because V1 subunits are not formed and no staining with anti-Vma2p is observed because the protein is absent (Fig. 5). These data verify those obtained by sucrose density gradient centrifugation and together the two sets of data establish two critical points about the V-ATPase in sur4Δ and fen1Δ cells. First, V1 domains are assembled and, second, at least some of them do associate with V0 on the vacuolar membrane.

V0 Domains Are Functional in sur4Δ Cells but V1 Domains Lack ATPase Activity—The nature of the V-ATPase defect in sur4Δ cells was further elucidated by determining whether V1, V0 or both domains are defective. For these experiments vacuolar membranes were isolated by sucrose density gradient centrifugation then treated with KI to dissociate V1 (27). Samples were then centrifuged to give a vacuolar membrane pellet (P) containing V0 and a supernatant fraction (S) containing V1. It has been shown previously that upon mixing the P and S fractions and dialyzing away the KI, V1 and V0 associate, as determined by immunoblotting, and V-ATPase activity is partially restored (35–40%) (27). We observed very similar results using the P and S fractions derived from wild-type cells. By immunoblotting, Vma1p and Vma2p (V1 subunits) were more concentrated in the P fraction following dialysis compared with
Sphingolipid Requirement for V-ATPase Activity

Table III

| Strain      | V-ATPase activity in purified vacuolar membranes | Sucrose gradient |
|-------------|-------------------------------------------------|------------------|
|             | V-ATPase activity % of wild type | V-ATPase activity % of wild type |
| Wild type   | 0.69 ± 0.04                                   | 0.76 ± 0.03      |
| sur4Δ       | 0.07 ± 0.03                                   | 0.14 ± 0.01      |
| fen1Δ       | 0.17 ± 0.01                                   | N.D.*            |

* Values represent the ATPase activity that is sensitive to 200 nM Concanamycin A and are the means of two experiments ± the S.D.  
* N.D., not determined.

Fig. 3. Vacuoles isolated from sur4Δ cells by Ficoll density gradient centrifugation lack the V1 subunits Vma1p, Vma2, and Vma5p. Cell-free extracts and Ficoll-purified vacuolar membrane vesicles were assayed for the presence of the V1 subunit Vph1p and the V1 subunits Vma1p, Vma2p, and Vma5p. Samples (20 μg of cell-free protein extracts and 10 μg of purified vacuolar membrane vesicles) were incubated in cracking buffer for 10 min at 95 °C for analysis of Vph1p or in SDS sample buffer for 5 min at 95 °C for the analysis of other proteins.

Fig. 4. V1 associates with V0 on vacuolar membranes isolated from sur4Δ cells by sucrose density gradient centrifugation.

V0 and V1 domains associate in vivo with vacuoles in sur4Δ cells. The location of the V1 domain in cells was determined by indirect immunofluorescence staining with anti-Vma2p primary antibody followed by Cy3-tagged secondary antibody (red images). Images taken by differential interference contrast optics are shown in black and white.

Fig. 5. V1 domains from sur4Δ cells lack Ca-ATPase Activity—Free V1 domains do not show ATPase activity when assayed in the absence of Mg2+, but do show activity when assayed in the presence of Ca2+ (20). Thus, to verify that V1 domains in sur4Δ cells lack ATPase activity we partially purified V1 complexes that were not treated with KI showed that all of the Vma1p and Vma2p were in the pellet along with Vph1p and that dialysis reduced ATPase activity slightly from 18 to 13% (Fig. 6A, sample 3). Results from other control reactions are shown in samples 7 and 8 of Fig. 6.

Mixing the S (V1) fraction from wild-type cells with the P (V0) fraction from sur4Δ cells followed by dialysis resulted in association of Vma1p and Vma2p with the P fraction and restoration of 36% of the ATPase activity (Fig. 6A, sample 5). A reciprocal mixing experiment with the P (V0) fraction from wild-type cells showed that Vma1p and Vma2p associate with the P fraction but there was no restoration of ATPase activity (Fig. 6A, sample 6). These experiments show that V0 domains in sur4Δ cells are fully functional and can associate with wild-type V1 domains to generate ATPase activity whereas the V1 domains are capable of association with V0 on the vacuolar membrane but they do not have ATPase activity.

V1 Domains from sur4Δ Cells Lack Ca-ATPase Activity—Free V1 domains do not show ATPase activity when assayed in the presence of Mg2+, but do show activity when assayed in the presence of Ca2+. Thus, to verify that V1 domains in sur4Δ cells lack ATPase activity we partially purified V1 complexes by velocity centrifugation on glycerol gradients and assayed them for ATPase activity in the presence Ca2+. To remove background ATPase activity that was not stimulated by Ca2+, the assay was also done in the absence of Ca2+ and this value was subtracted from the value obtained in the presence of Ca2+ to give the Ca-stimulated activity. In preliminary experiments increasing concentrations of protein in the pooled V1-containing sucrose gradient fractions were assayed for Ca-stimulated ATPase activity (release of Pi). Activity was linear with increas-
containing V0 domains and a supernatant fraction containing V1 domains were mixed (4, reactions 2) or not treated (4, reactions 1) and 17 (Vma11p) kDa proteins were cross-linked with a reversible cross-linker. Samples were immunoprecipitated with monoclonal antibodies specific for Vph1p, Vma1p, or Vma2p and radioactive proteins in the immunoprecipitate were analyzed by SDS-PAGE and phosphorimaging.

The anti-Vph1p antibody used in these experiments only recognizes V0 domains that are not complexed with V1 (24) and is, therefore, useful for examining the subunit composition of V0 domains. Radio labeled proteins of 100 (Vph1p), 36 (Vma6p), 19 (identity unknown, Refs. 30 and 31) and 17 (Vma11p) kDa were immunoprecipitated by the Vph1p antibody in wild-type sur4/H9004, fen1/H9004, and vma2A cells (Fig. 7A). We observed small variations in the relative intensity of bands from experiment to experiment, and the samples shown in Fig. 7 were chosen to represent the average of the results of three independent experiments. The data shown in Fig. 7A are in agreement with published results (e.g. Refs. 30 and 31) and indicate that the subunit composition of the V0 domain is normal in sur4 cells and fen1A cells.

The anti-Vma1p and anti-Vma2p antibody used in these experiments recognize free V1 and V1V0 complexes (24, 30). Radioactive proteins immunoprecipitated by anti-Vma1p and anti-Vma2p are shown in Fig. 7, B and C, respectively. Again, there were small variations in the intensity of some radioactive bands from experiment to experiment, but overall our results indicate that the subunit composition of free V1 and the V1V0 complex in sur4 and fen1A cells is similar if not identical to that in wild-type cells and to published data. The vma2A cells served as a control for cells lacking V1 and V1V0 complexes (30).

A limitation of these data is that not all V1 subunits are readily detected including Vma7p, Vma10p, and Vma13p. Vma7p and Vma10p are probably present in the V1 domains we examined because if either protein were absent then V1 and V0 would not associate (39–42). Vma13p is not necessary for V1-V0 association and could be missing. To determine if it was present in vacuolar membranes isolated from sur4 cells, sur4 vma13A, and vma13A cells were transformed with a vector carrying a VMA13 allele having a Myc epitope inserted immediately downstream of the methionine start codon (20). Vacuolar membranes were isolated by sucrose density gradient centrifugation and fractions from the gradient were immunoblotted with anti-Myc antibody. The concentration of Myc-tagged V1 domains (detected by immunoblotting for Vma1p and Vma2p) V-ATPase values represent the average of three determinations ± S.D. Reactions 1, 3, 7, and 8 are controls. B, cytosolic V1 domains were isolated from RCD390 (wild-type, ○) and RCD410 (sur4, □) cells on sucrose density gradients as described in “Experimental Procedures” and 200 μg of protein was assayed for Ca-stimulated release of P, from ATP (i.e., ATPase activity). Values represent the average of three determinations ± S.D.
with Trans [35S] label, treating the samples with a protein cross-linker, it has been noted previously that Reduced V-ATPase Activity determined (11, 12, 43). Likewise, the concentration of long chain base phosphates (LCBPs) has not been measured.

We quantified LCBs and LCBPs by tagging them after extraction with a fluorescent reagent followed by HPLC (23). The analysis was done in two different strain backgrounds to see how similar or different they might be and if any differences correlated with mutant phenotypes. The five species of LCBs are at nearly identical levels in the two wild-type strains (Table IV). In the two sur4Δ strains all five species are elevated and their levels are similar in the two strain backgrounds except for C18-DHSP and C20-DHSP, which are less elevated in strain RCD410 (the W303 background). All five species are also elevated in the two fen1Δ strains but there is more variability between strains and only the C16-DHSP species have similar values.

LCBP values are also very similar in the two wild-type strains (Table V) and are quite low as we have reported for a wild-type strain related to RCD390 (44). All LCBPs show similar elevations in the two sur4Δ strains except for C18-DHSP and C20-DHSP. All LCBPs are also elevated in the two fen1Δ strains but the values vary between the strains.

The elevated levels of LCBs and LCBPs could be responsible for the lack of ATPase activity. However, if this were the case we would expect fen1Δ cells (RCD393), not sur4Δ cells (RCD389), to have a greater loss of V-ATPase activity because they have a higher total level of LCBs and LCBPs (Tables IV and V).

To determine directly if LCBs dissociate Vma1p and Vma2p from V0, vacuolar membranes purified from wild-type cells were incubated with increasing concentrations of PHS. After incubation, samples were centrifuged to give a pellet and a supernatant fraction, which were analyzed by immunoblotting for Vma1p and Vma2p. Because PHS has detergent-like properties and could release proteases from vacuoles, we also immunoblotted for CPY to control for proteolysis. In the presence, but not in the absence of PHS, the level of Vma1p and Vma2p in the pellet fraction gradually decreased over the 120-min incubation period (Fig. 8). However, the two proteins did not appear in the supernatant as would be expected if they were dissociating from V0. This result plus the fact that they disappeared (Fig. 8) at the same rate as CPY indicates that they are being digested by proteases. We conclude that LCBs do not dissociate Vma1p and Vma2p from purified vacuolar membranes.

**DISCUSSION**

Our analysis of sur4Δ and fen1Δ cells shows that sphingolipids containing a C26 acyl group are necessary for generation of a V0 domain that is capable of hydrolyzing ATP. This conclusion is based upon an analysis of sur4Δ cells, whose sphingolipids lack a C26 acyl group, and fen1Δ cells, whose sphingolipids have about 30% of the wild-type level of C26 acyl groups (11, 12). We predicted that cellular functions dependent upon sphingolipids with a C26 acyl group would be more disrupted in sur4Δ than in fen1Δ cells. Several results support this line of reasoning and our conclusion. First, we found that sur4Δ cells have some but not all Vma- phenotypes, indicating a defective V-ATPase, and that some of these phenotypes are more pronounced in sur4Δ than in fen1Δ cells. For example, growth is more inhibited in sur4Δ than in fen1Δ cells on YPD plates buffered to pH 7.5 or when the plates contain 100 mM CaCl2, 4 mM ZnCl2, or 2% glycerol as the carbon source (Fig. 1 and Table II). Second, V-ATPase activity in sur4Δ cells is as defective as that in vma2Δ cells, which lack V-ATPase activity, when measured by uptake of the vacuolar dye quinacrine (Fig. 2). In contrast, uptake in fen1Δ cells is about 4-fold higher than in sur4Δ cells or about 60% of the wild-type level. Finally, the ATPase activity of vacuoles purified from sur4Δ cells by Ficoll...
resent means 

ined by immunoblotting to determine if V0 and V1 domains were present. The results showed that vacuolar membranes from sur4Δ and fen1Δ cells had very low, barely detectable levels of Vma1p, Vma2p, and Vma5p, indicating that the V1 domain was not associated with the V0 domain (Fig. 3). Since these three V1 subunits are present in cell extracts (Fig. 3) their absence from purified vacuoles suggests that they mislocalize, do not assemble into a functional V1 domain or V1 associates abnormally with V0. The finding that V1 and V0 are found together in fractions of a sucrose density gradient where vacuolar membranes are located (Fig. 4, fractions 5 and 6), supports either of the latter two possibilities. In addition, indirect immunofluorescent microscopy on intact sur4Δ and fen1Δ cells showed that the V1 subunit Vma2p was bound to vacuoles (Fig. 5). Together these data show that V1 is able to associate with V0 on the cytoplasmic face of the vacuolar membrane to form V1V0 complexes in sur4Δ cells, but the complexes are not stable in Ficoll (Fig. 3), have low ATPase activity (Table III), and do not acidify vacuoles (Figs. 1 and 2).

Reduced ATPase activity in sur4Δ cells suggested that the V1 domain was defective. To test this hypothesis, V1 and V0 domains were isolated and tested in vitro for association and for restoration of ATPase activity. Wild-type V1 and sur4Δ V0 associated to produce V1V0 complexes with ATPase activity. Wild-type V0 and sur4Δ V1 also associated but the V1V0 complexes had no ATPase activity (Fig. 6). We also partially purified cytosolic V1 domains from wild-type and sur4Δ cells and assayed them for calcium-dependent ATPase activity. V1 domains from sur4Δ cells completely lacked activity (Fig. 6B). These results establish that V0 domains in sur4Δ cells are normal, but the V1 domains are defective and lack ATPase activity.

Reduced ATPase activity in sur4Δ cells suggested that the V1 domain was defective. To test this hypothesis, V1 and V0 domains were isolated and tested in vitro for association and for restoration of ATPase activity. Wild-type V1 and sur4Δ V0 associated to produce V1V0 complexes with ATPase activity. Wild-type V0 and sur4Δ V1 also associated but the V1V0 complexes had no ATPase activity (Fig. 6). We also partially purified cytosolic V1 domains from wild-type and sur4Δ cells and assayed them for calcium-dependent ATPase activity. V1 domains from sur4Δ cells completely lacked activity (Fig. 6B). These results establish that V0 domains in sur4Δ cells are normal, but the V1 domains are defective and lack ATPase activity.

To understand why V1 domains lack ATPase activity, we examined the subunit composition of V1 and V0 and found that they are the same in sur4Δ, fen1Δ, and wild-type cells (Fig. 7). Separate analysis of Myc-tagged Vma13p showed that it was present in V1 domains in sur4Δ cells (Fig. 7). The procedures used by us would not have detected the V1 subunits Vma7p or Vma10p, although we infer that they are present because if either protein was missing from cells, V1 domains would not associate with V0 (39–42). Thus, lack of a protein subunit is not likely to be the cause of the defect in ATPase activity in V1 domains present in sur4Δ cells.

Others have reported that sur4Δ and fen1Δ cells contain high levels of LCBs (11, 12, 43), but the levels have not been quantified nor have the species been identified. We were concerned that the high level of LCBs might impair ATPase activity. It

**Table IV**

| Strain   | C16-DHS | C18-PHS | C18-DHS | C20-PHS | C20-DHS | Total |
|----------|---------|---------|---------|---------|---------|-------|
| RCD408 (WT) | 2.6 ± 0.3* | 31 ± 4  | 20 ± 2 | 8.5 ± 0.9 | 2.3 ± 0.1 | 64 |
| RCD390 (WT) | 2.0 ± 0.1 | 31 ± 1  | 18 ± 2 | 8.9 ± 0.9 | 3 ± 0.7 | 64 |
| RCD410 (sur4Δ) | 64 ± 5 | 1131 ± 190 | 164 ± 9 | 591 ± 94 | 16 ± 1 | 1966 |
| RCD388 (sur4Δ) | 70 ± 3 | 1385 ± 2 | 388 ± 36 | 883 ± 24 | 58 ± 5 | 2794 |
| RCD412 (fen1Δ) | 69 ± 2 | 1271 ± 45 | 171 ± 24 | 644 ± 32 | 16 ± 5 | 2171 |
| RCD393 (fen1Δ) | 56 ± 0.8 | 838 ± 118 | 1049 ± 29 | 1584 ± 32 | 535 ± 71 | 4062 |

* Values are expressed as pmol/600 nm units and represent the average of two experiments ± S.D.

**Table V**

| Strain   | C16-DHSP | C18-PHSP | C18-DHSP | C20-PHSP | C20-DHSP | Total |
|----------|----------|----------|----------|----------|----------|-------|
| RCE408 (WT) | 0.8 ± 0.2* | 3.2 ± 0.8 | 0.5 ± 0.04 | 0.7 ± 0.06 | 0.6 ± 0.05 | 5.8 |
| RCD390 (WT) | 0.7 ± 0.01 | 2.8 ± 0.4 | 0.5 ± 0.03 | 0.6 ± 0.06 | 0.8 ± 0.1 | 5.4 |
| RCD410 (sur4Δ) | 8 ± 1 | 118 ± 33 | 8 ± 1.5 | 47 ± 14 | 3 ± 0.1 | 184 |
| RCD388 (sur4Δ) | 9 ± 0.8 | 101 ± 3.2 | 22 ± 0.8 | 38 ± 2 | 12 ± 2 | 182 |
| RCD412 (fen1Δ) | 14 ± 0.3 | 178 ± 10 | 4 ± 0.9 | 63 ± 6 | 4 ± 0.6 | 263 |
| RCD393 (fen1Δ) | 1.3 ± 0.3 | 32 ± 1.5 | 30 ± 1.5 | 39 ± 2 | 13 ± 1 | 115 |

* Values are expressed as pmol/600 nm units and represent the average of two experiments ± S.D.

**Fig. 8.** Purified V-ATPase is resistant to PHS treatments. A, vacuolar membranes purified from wild-type cells (RCD390) were treated or not treated with 100 μM PHS various lengths of time and then separated by centrifugation into a pellet (P) and supernatant (S) fractions. Supernatants were precipitated by treatment with trichloroacetic acid. All pellets were suspended in the same volume of SDS sample buffer and equal volumes were subjected to Western blotting. B, data in A were quantified by phosphorimage analysis. The values represent means ± S.D. (n = 3).
was recently shown that the reduced level of glucan synthase activity in sur4Δ and fen1Δ cells is due to elevated levels of PHS and DHS (43). Our analysis of LCBS shows that all five species are elevated in both sur4Δ and fen1Δ mutants in two different strain backgrounds, W303 and J93-3d (Table IV). In the J93-3d strain background, which we used for the majority of our experiments, there is a 44-fold increase in total LCBS in sur4Δ cells and a 63-fold increase in fen1Δ cells. The same trends hold for the mutants in the W303 background but the increases are smaller. If elevated LCBS were responsible for disruption of V-ATPase activity, then we would expect the activity to be reduced more in fen1Δ than in sur4Δ cells because fen1Δ cells have a higher level of LCBS. Our results are just the opposite of this prediction and argue that elevated LCBS are not responsible for reduced ATPase activity. However, such arguments cannot eliminate the possibility that LCBS are interfering with V₁ function.

We also compared the total LCB content of fractions from a Ficol gradient to see if there was any correlation between reduced V-ATPase activity in sur4Δ and fen1Δ cells and the level of these compounds. The vacuolar membrane fraction of wild-type cells had a very low, barely detectable level of LCBS, as did the two fractions below the membrane fraction (data not shown). The pellet at the bottom of the gradient had the highest level of LCBS, but the concentration was still very low. The level of LCBS was higher in the gradient fractions obtained from sur4Δ and fen1Δ cells. As with the total LCB values (Table IV), the levels in the Ficol gradient fractions are higher in fen1Δ cells, suggesting that it is not the LCBS that are responsible for reduced V-ATPase activity. LCBS were not detectable in any of the Ficol gradient fractions nor in the cell-free extracts. Most likely they were degraded during the incubation period when cells were converted to spheroplasts.

We also determined if PHS added in vitro to purified vacuolar membranes could mimic what occurs in sur4Δ and fen1Δ cells and selectively release Vma1p and Vma2p from membranes. We found that neither protein was selectively released.

The results presented here are the first to indicate a role for C26 acyl groups in sphingolipid metabolism. The role of sphingolipids in vacuolar membrane activity was recently shown to be necessary for the activity of the V-ATPase (47) and some step in the action of RAVE may require sphingolipids with a C26 acyl group. Ficol, a polymer of sucrose, may dissociate V₁ from V₀ by interacting with one or more V₁ subunits that are not correctly folded or assembled in sur4Δ cells.

The results presented here are the first to indicate a role for C26 acyl groups and for sphingolipids in V-ATPase function. Their exact role will require further characterization of V₁ domains in sur4Δ cells. Our results suggest that sphingolipids may be important for the activity of V-ATPases and related ATPases in other organisms. S. cerevisiae contains another type of V-ATPase located in the Golgi/endoosomal compartments that is identical to the V-ATPase except that the V₀ domain contains Stv1p in place of Vhp1p (48). Our results suggest that the functionality of this V-ATPase may also require sphingolipids with a C26 acyl group.

Acknowledgments—We thank Patricia Kane for sharing reagents with us and Dr. Lois Weisman for advice on indirect immunofluorescence microscopy.

REFERENCES

1. Stevens, T. H., and Forgac, M. (1997) Annu. Rev. Cell Dev. Biol. 13, 779–808
2. Nishi, T., and Forgac, M. (2002) Nat. Rev. Mol. Cell Biol. 3, 94–103
3. Graham, L. A., Powell, B., and Stevens, T. H. (2000) J. Exp. Biol. 203 Pt 1, 61–70
4. Forgac, M. (2000) J. Exp. Biol. 203, 71–80
5. Kane, P. M., and Parra, K. (2000) J. Exp. Biol. 203 Pt 1, 81–87
6. Dickson, R. C., and Lester, R. L. (2002) Biochim. Biophys. Acta. 1583, 13–25
7. Patton, J. L., and Lester, R. L. (1991) J. Bacteriol. 173, 3101–3108
8. Hechtberger, P., Zasner, E., Saf, R., Hummel, K., Paltz, F., and Daum, G. (1994) Eur. J. Biochem. 225, 641–649
9. Nagiec, M. M., Wells, G. B., Lester, R. L., and Dickson, R. C. (1993) J. Biol. Chem. 268, 22156–22163
10. Lester, R. L., Wells, G. B., Oxford, G., and Dickson, R. C. (1993) J. Biol. Chem. 268, 845–856
11. Oh, C. S., Toke, D. A., Mandala, S., and Martin, C. E. (1997) J. Biol. Chem. 272, 17276–17284
12. Kohlwein, S. D., Eder, S., Oh, C. S., Martin, C. E., Gable, K., Bacikova, D., and Dunn, T. (2001) Mol. Cell. Biol. 21, 109–125
13. Dickson, R. C., and Lester, R. L. (1999) Biochim. Biophys. Acta. 1438, 305–321
14. Revardel, E., Bonnaud, M., Durren, P., and Aigle, M. (1995) Biochim. Biophys. Acta. 1263, 261–265
15. Lengeling, D. T. (1992) J. Lipid Res. 33, 301–313
16. Torkal, P., Westerberg, R., Silve, S., Asadi, A., Jacobsson, A., Cannon, B., Loison, G., and Jacobsson, A. (2000) J. Biol. Chem. 275, 767–718
17. Suna, S. K., Nago, M. N., Cook, L., and Cinti, D. L. (1991) J. Neurochem. 57, 140–146
18. Guldener, U., Heck, S., Fiedler, T., Beinhauer, J., and Hegemann, J. H. (1996) Nucleic Acids Res. 24, 2519–2534
19. Wada, A. (1996) Yeast 12, 265–269
20. Parra, K. J., Keenan, K. L., and Kane, P. M. (2000) J. Biol. Chem. 275, 21761–21767
21. Seel, J. H., Shevchenko, A., Shevchenko, A., and Deshaies, R. J. (2001) Nat. Cell Biol. 3, 384–391
22. Roberts, C. J., Raymond, C. K., Yamashiro, C. T., and Stevens, T. H. (1991) Methods Enzymol. 194, 71–81
23. Lester, R. L., and Dickson, R. C. (2001) Biochem. J. 361, 283–292
24. Kane, P. M., Kuehn, M. C., Howald-Stevenson, I., and Stevens, T. H. (1992) J. Biol. Chem. 267, 447–454
25. Leng, X. H., Manolson, M. F., Liu, Q., and Forgac, M. (1996) J. Biol. Chem. 271, 22487–22493
26. Psapo, K., and Forgac, M. (1990) J. Biol. Chem. 265, 14836–14841
27. Parra, K. J., and Kane, P. M. (1996) J. Biol. Chem. 271, 19982–19988
28. Kunz, J., Schneider, U., Howald, I., Schmidt, A., and Hall, M. N. (2000) J. Biol. Chem. 275, 37011–37020
29. Ragnat, M., Kerenas, S., Shevchenko, A., and Simons, K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3254–3259
30. Doherty, R. D., and Kane, P. M. (1995) J. Biol. Chem. 268, 61845–61851
31. Kane, P. M. (1995) J. Biol. Chem. 270, 17025–17032
32. Nelsen, H., and Nelson, N. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5025–5030
33. Yamashiro, C. T., Kane, P. M., Wolczyk, D. F., Preston, R. A., and Stevens, T. H. (1990) Mol. Cell. Biol. 19, 3757–3764
34. Oluwatosin, Y. E., and Kane, P. M. (1999) Mol. Biol. Cell. 18, 1534–1543
35. Ohya, Y., Uemoto, N., Tanida, I., Ohta, A., Iida, H., and Anraku, Y. (1991) J. Biol. Chem. 266, 13971–13977
36. Fouy, F. (1990) J. Biol. Chem. 265, 18554–18560
37. Weissman, L. S., Bacallao, R., and Wickner, W. (1987) J. Cell Biol. 105, 1539–1547
38. Ho, M. N., Hirata, R., Umemoto, N., Ohya, Y., Takatsu, A., Stevens, T. H., and Anraku, Y. (1993) J. Biol. Chem. 268, 18286–18292
39. Graham, L. A., Hill, K. J., and Stevens, T. H. (1994) J. Biol. Chem. 269, 25974–25977
40. Nelson, H., Mandiyan, S., and Nelson, N. (1994) J. Biol. Chem. 269, 24150–24155
41. Supekova, L., Supek, F., and Nelson, N. (1995) J. Biol. Chem. 270, 13726–13732
42. Tomashek, J. J., Graham, L. A., Hutchins, M. U., Stevens, T. H., and Klionsky, D. J. (1997) J. Biol. Chem. 272, 26787–26793
43. Abe, M., Nishida, I., Minemura, M., Qadota, H., Seyama, Y., Watanabe, T., and Ohya, Y. (2001) J. Biol. Chem. 276, 26923–26930
44. Ferguson-Yankey, S. R., Skrzypek, M. S., Lester, R. L., and Dickson, R. C. (2002) Yeast 19, 573–586
45. Bryant, N. J., and Stevens, T. H. (1996) Microbiol. Mol. Biol. Rev. 60, 230–247
46. Kane, P. M., Tarsio, M., and Kane, P. M. (2002) J. Biol. Chem. 277, 13831–13839
47. Smardon, A. M., Tarsio, M., and Kane, P. M. (2002) J. Biol. Chem. 277, 13831–13839
48. Manolson, M. F., Wu, B., Proteau, D., Taillon, B. E., Roberts, B. T., Hoyt, M. A., and Jones, E. W. (1994) J. Biol. Chem. 269, 14064–14074
49. Heitman, J., Movva, N. R., Hiestand, P. C., and Hall, M. N. (1991) Proc. Natl. Acad. Sci. USA 88, 1949–1952
50. Thomas, B. J., and Rothstein, R. (1989) Cell 56, 619–630
51. Brachmann, C. B., Davies, A., Coet, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Yeast 14, 115–132
Sphingolipid Requirement for Generation of a Functional V₁ Component of the Vacuolar ATPase
Ji-Hyun Chung, Robert L. Lester and Robert C. Dickson

J. Biol. Chem. 2003, 278:28872-28881.
doi: 10.1074/jbc.M300943200 originally published online May 13, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300943200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 51 references, 35 of which can be accessed free at http://www.jbc.org/content/278/31/28872.full.html#ref-list-1