Vma9p (Subunit e) Is an Integral Membrane Vₒ Subunit of the Yeast V-ATPase*

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The Saccharomyces cerevisiae vacuolar proton-translocating ATPase (V-ATPase) is composed of 14 subunits distributed between a peripheral V₁ subcomplex and an integral membrane Vₒ subcomplex. Genome-wide screens have led to the identification of the newest yeast V-ATPase subunit, Vma9p. Vma9p (subunit e) is a small hydrophobic protein that is conserved from fungi to animals. We demonstrate that disruption of yeast VMA9 results in the failure of V₁ and Vₒ V-ATPase subunits to assemble onto the vacuole and in decreased levels of the subunit a isoforms Vph1p and Stv1p. We also show that Vma9p is an integral membrane protein, synthesized and inserted into the endoplasmic reticulum (ER), which then localizes to the limiting membrane of the vacuole. All Vₒ subunits and V-ATPase assembly factors are required for Vma9p to efficiently exit the ER. In the ER, Vma9p and the Vₒ subunits interact with the V-ATPase assembly factor Vma21p. Interestingly, the association of Vma9p with the Vₒ Vma21p assembly complex is disrupted with the loss of any single Vₒ subunit. Similarly, Vma9p is required for Vₒ subunits Vph1p and Vma6p to associate with the Vₒ Vma21p complex. In contrast, the proteolipids associate with Vma21p even in the absence of Vma9p. These results demonstrate that Vma9p is an integral membrane subunit of the yeast V-ATPase Vₒ subcomplex and suggest a model for the arrangement of polypeptides within the Vₒ subcomplex.

The vacuolar proton-translocating ATPase (V-ATPase) is a multisubunit complex common to all eukaryotic organisms (1–3). The V-ATPase is found on the intracellular organelles of all eukaryotes and also on the plasma membrane of specialized cell types. Hydrolysis of ATP by the V-ATPase drives the transport of protons into the lumen of organelles that this macromolecular complex decorates. Similar to the F-type ATPases, V-ATPase coupling of ATP hydrolysis and proton transport involves a rotary mechanism (4–6). Organelle acidification by the V-ATPase is crucial to many cellular and physiological processes, such as receptor-mediated endocytosis (2), intracellular trafficking (1), neurotransmitter synaptic vesicle loading (1), synaptic vesicle exocytosis (7), repressing of cell–cell fusion (8), bone resorption (9), renal acidification (9), and perhaps tumor metastasis (10). Defects in subunits of the V-ATPase suggest that Vma6p encoding gene display characteristic Vma⁻ phenotypes as follows: nonacidified vacuoles and the failure to grow on media buffered to pH 7.5 or containing elevated CaCl₂. The V-ATPase can be divided into two subcomplexes designated V₁ and Vₒ. The V₁ subcomplex is composed of eight hydrophilic subunits ranging in size from 13 to 69 kDa and contains the ATP binding and hydrolysis domains. The Vₒ subcomplex is hydrophobic, composed of six subunits ranging in size from 10 to 100 kDa, and is responsible for proton translocation. The Vₒ subcomplex can be subdivided into two parts that move relative to each other, the stator and the proton-translocating ring. The Vₒ portion of the stator is composed of the 100-kDa subunit a (Vph1p or Stv1p). The proton-translocating ring is composed of subunits Vma3p, Vma11p, and Vma16p (subunits c, c', and c'' respectively; see Ref. 14). Vma3p, Vma11p, and Vma16p have multiple transmembrane domains and are termed proteolipids because of their very hydrophobic nature (15). The 5th Vₒ subunit Vma6p, subunit d, is a hydrophilic peripheral membrane protein. Cross-linking studies with the Thermus thermophilus V-ATPase suggest that Vma6p interacts with the Vₒ subcomplex through the proteolipids (16).

In yeast there are three ER-localized proteins that function as V-ATPase assembly factors, which are required for the function of the V-ATPase but are not components of the final assembled complex (17). The V-ATPase assembly factors Vma12p, Vma21p, and Vma22p are found in the ER where they function to assemble the Vₒ subcomplex (18). Vma12p is a transmembrane protein that anchors the peripheral membrane protein Vma22p in the ER (19). This Vma12p–Vma22p complex has been shown to interact with the Vₒ subunit a early in Vₒ biosynthesis. Vma21p is an integral membrane protein that has been found to associate with the Vₒ subcomplex in the ER and may escort it to the Golgi body (20).

An additional small hydrophobic V-ATPase Vₒ subunit (subunit e) has been identified in Manduca midgut (21) and bovine chromaffin granule (22) V-ATPase preparations. Recently such a polypeptide has been identified as a likely component of the yeast V-ATPase (23, 24). Two different genome-wide yeast mutant screens identified the subunit e encoding gene VMA9 (previously CWH36), which when disrupted resulted in the full spectrum of Vma⁻ phenotypes (23, 25). Subunit e is conserved from yeast to humans, and Caenorhabditis elegans fus-1 (subunit e) mutants show cellular hyperfusion (8). Yeast Vma9p has been found to co-purify with the V-ATPase and is required for V-ATPase function (24).

We also identified the VMA9 gene in a yeast genome-wide screen for proteins involved in V-ATPase function (3). We report here that cells lacking Vma9p do not assemble a Vₒ subcomplex, and that the subunit a isoform Vph1p is rapidly degraded. Biochemical and localization studies reveal that Vma9p is an integral membrane vacuolar protein that fails to be transported out of the ER in cells lacking any Vₒ subunit or V-ATPase assembly factor. Vma9p associates with the Vₒ Vma21p assembly complex in the ER, and Vma9p is required for the association...
of two other \( V_0 \) subunits with the \( V_0 \)-Vma21p complex. These data indicate that Vma9p is a critical component of the \( V_0 \) subcomplex, and based on the results we propose a model for the position of Vma9p relative to the proteolipid ring.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Enzymes utilized for DNA manipulations were purchased from New England Biolabs, Fermentas, and Stratagene; standard protocols for molecular biology with modifications were followed (26). The plasmids utilized in this study are shown in Table 1. Plasmid pLG42 was created by HindIII/SacI digestion of genomic DNA was utilized in PCR with inward-facing oligonucleotides from the stop codon. The resulting PCR product was transformed into SF838-1D yeast with a high efficiency lithium acetate protocol (29). Transformants were suspended in YEPD (yeast extract/powder/dextrose), pH 5.0 (50 mm sucinate/phosphate), and selected on YEPD plates containing 200 \( \mu \)g/ml geneticin sulfate (G418). Colonies from G418 selection plates were screened on YEPD + 100 mm CaCl\(_2\) plates to confirm a Vma\(^-\) phenotype (lack of growth). Western blot analysis confirmed the lack of Vma9p in strain MACY10 (vma9\( \Delta \)-kan\( ^\text{R} \)). The same method (see MACY10 above) was utilized with strain LGY70 and pMAC200 to generate MACY13, strain SF838-1D\( \Delta \)a, and pLG128 to generate MACY11, strain SF838-1D\( \Delta \)a, and pLG130 to generate LGY115. TASY006 was also constructed by a similar means with starting strain SF838-1D\( \Delta \)a, but the initial vma21\( \Delta \)-kan\( ^\text{R} \) PCR template was genomic DNA of the yeast genome deletion collection vma21\( \Delta \)-kan\( ^\text{R} \), the loop into loop out method with EcoR1-digested pDJ4 was utilized to integrate STV1:3xHA into the VMA21 promoter. For strains LSY70 and MACY11 starting with strains KHY31, LGY113, LGY115, LGY117, LGY127, LGY128, LGY129, LGY135, MACY10, MACY11, MACY12, MACY13, and TASY006, the PCR product was ligated into the plasmid pCR4 Blunt-TOPO vector (Invitrogen) generating pMAC200. The same method utilized to create pMAC200 was also used to generate pLG128 (vma9\( \Delta \)-kan\( ^\text{R} \), pLG129 (vma11\( \Delta \)-kan\( ^\text{R} \), and pLG130 (vma16\( \Delta \)-kan\( ^\text{R} \)) from the appropriate yeast genome deletion collection diploid strains.

**Strain Construction**—Yeast strains utilized in this study are shown in Table 2. To generate MACY10, PCR was performed with plasmid pMAC200 and inward-facing oligonucleotides that bind 500 bp 5’ of the VMA9 start codon and 500 bp 3’ of the stop codon. The resulting vma9\( \Delta \)-kan\( ^\text{R} \) PCR product was transformed into SF838-1D\( \Delta \)a yeast with a high efficiency lithium acetate protocol (29). Transformants were suspended in YEPD and plated on 5-bromo-4-chloro-3-indolyl-\( \beta \)-D-galactoside (Brdg) plates to confirm a Vma\(^-\) phenotype (lack of growth). Western blot analysis confirmed the lack of Vma9p in strain MACY10 (vma9\( \Delta \)-kan\( ^\text{R} \)). The same method (see MACY10 above) was utilized with strain LGY70 and pMAC200 to generate MACY13, strain SF838-1D\( \Delta \)a, and pLG128 to generate MACY11, strain SF838-1D\( \Delta \)a, and pLG130 to generate LGY115. TASY006 was also constructed by similar means with starting strain SF838-1D\( \Delta \)a, but the initial vma21\( \Delta \)-kan\( ^\text{R} \) PCR template was genomic DNA of the yeast genome deletion collection vma21\( \Delta \)-kan\( ^\text{R} \), the loop in loop out method with EcoR1-digested pDJ4 was utilized to integrate STV1:3xHA into the VMA21 promoter. For strains LSY70 and MACY11 starting with strains KHY31, LGY113, LGY115, LGY117, LGY127, LGY128, LGY129, LGY135, MACY10, MACY11, MACY12, MACY13, and TASY006, the PCR product was ligated into the plasmid pCR4 Blunt-TOPO vector (Invitrogen) generating pMAC200. The same method utilized to create pMAC200 was also used to generate pLG128 (vma9\( \Delta \)-kan\( ^\text{R} \), pLG129 (vma11\( \Delta \)-kan\( ^\text{R} \), and pLG130 (vma16\( \Delta \)-kan\( ^\text{R} \)) from the appropriate yeast genome deletion collection diploid strains.

**Isolation of Vacuolar Membrane Vesicles**—Vacuolar membrane vesicles were isolated as described previously, but with a second 8% Ficoll centrifugation (30, 31). Yeast were grown to log phase in YEPD buffered to pH 5.0, and 4000 \( A_{600} \) units of cells were resuspended in
spheroplast buffer (1.2 mM sorbitol, 50 mM KPO₄, pH 7.4, 1 mM MgCl₂) + 40 μg/ml Zymolyase 100T, and lysed with a Dounce homogenizer in Buffer A (10 mM (MES/Tris), pH 6.9, 0.1 mM MgCl₂, 12% Ficoll-400). Lysates were centrifuged at 60,000 × g for 35 min at 4 °C in an SW28.1 rotor (Beckman Instruments). After centrifugation, the white floating layer, composed predominantly of vacuoles, was removed with a spatula and resuspended in Buffer C (10 mM MOPS/MES, pH 6.9, 5 mM MgCl₂, 25 mM KCl) to fragment vacuoles into vacuolar vesicles. Vacuolar vesicles were washed in TE (10 mM Tris, pH 7.4, 1 mM EDTA) 10% glycerol and centrifuged at 37,000 × g for 30 min 4 °C. The final pellet was resuspended in TE 10% glycerol and assayed for protein content by the modified Lowry protocol (32). Finally, vacuolar membrane vesicles were diluted in Thorner buffer (1.2M sorbitol, 50 mM KPO₄, pH 7.4, 1 mM MgCl₂) + 250 μg/ml Zymolyase 100T, and lysed in phosphate-buffered saline (PBS) + protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) with vigorous pipetting. Unbroken cells were separated from samples by centrifugation at 500 × g. Samples were centrifuged at 13,000 × g for 10 min to pellet membrane fractions; supernatants were removed, and membrane samples were resuspended in PBS. The protein concentration of the membrane samples was determined by the modified Lowry protocol (32). Membrane extractions were performed in a similar manner as outlined previously (33). Briefly, 100 μg of yeast membrane protein was washed in TE (10 mM Tris, pH 7.4, 1 mM EDTA) and resuspended in 100 μl of TE, 100 mM Na₂CO₃, or 0.5% Triton X-100. Membrane treatments were incubated on ice for 30 min and centrifuged at 100,000 × g for 30 min. 100 μl of supernatant were removed, and an equal volume of Thorner buffer was added. Pellet fractions were resuspended in 100 μl of PBS and 100 μl Thorner buffer. Equal volumes of pellet and supernatant samples were utilized for Western blot analysis as described above.

**Immunofluorescence Microscopy**—Immunofluorescence microscopy was performed as described previously (38). Briefly, yeast was grown in YEPD, pH 5.0, to 1 A₆₀₀ₙl fixed with 4% formaldehyde for 1 h, and incubated overnight in 4% paraformaldehyde. Cells were treated with 1% β-mercaptoethanol, resuspended in spheroplast buffer (1.2 mM sorbitol, 50 mM KPO₄, pH 7.4, 1 mM MgCl₂) + 150 μg/ml Zymolyase 100T, and permeabilized with 5% SDS. Yeast was applied to poly(l-lysine)-treated slides and blocked for 1 h with 1% normal goat serum in PBS + 5 mg/ml BSA. The cells were incubated with the Vma9p antibody for 2 h, incubated with biotin-conjugated goat anti-rabbit (Jackson ImmunoResearch) for 1 h, incubated with streptavidin-conjugated fluorescent isothiocyanate (Jackson ImmunoResearch) for 1 h, and mounted in 100% glycerol with 4,6-diamidino-2-phenylindole (DAPI) + p-phenylenediamine. After each antibody incubation, yeast were washed several times with PBS + 5 mg/ml BSA. Images were collected with an Axioplan 2 fluorescence microscope and AxioCam HRm digital camera (Carl Zeiss, Thornwood, NY).

**Vma21p Native Immunoprecipitations**—Yeast cells bearing the appropriate VMA21 plasmids (see figure legends) were grown overnight in selective media at 30°C. Overnight cultures were used to inoculate YEPD, pH 5.0, media, and cells were grown for two divisions to mid-log phase. To generate immunoprecipitation samples, 20 A₆₀₀₉ units of cells were made into spheroplasts and lysed as described above (see under “Extraction of Yeast Membranes with Chaotrophic Agents”). Lysed samples were solubilized for 30 min at 4°C with 1% C₂₃₋₄E₆ (nondenaturing) and simultaneously pre-cleared with protein A-Sepharose or protein G-Sepharose. Samples were centrifuged at 13,000 × g; supernatants were preserved, and monoclonal anti-HA prebound to protein A-Sepharose or goat anti-HA antibodies (for Vph1p final detection only) was added to the supernatants. Anti-HA protein A-Sepharose-treated supernatants were incubated for 3 h 4°C, centrifuged, and washed repeatedly with PBS, and finally resuspended in 100 μl of Thorner buffer (denaturing). Goat anti-HA antibody-treated supernatants were incubated for 3 h at 4°C, incubated for 2 h after addition of protein
G-Sepharose, centrifuged and washed repeatedly with PBS, and resuspended in 100 μl of Thorner buffer. To generate input samples, 4–8 A600 units of cells from the starting YEPD, pH 5.0, culture were prepared in the same manner as whole cell lysates (see above). However, 200 μl of Thorner buffer was added before vortexing with glass beads. To visualize proteins, samples were subjected to Western blot analysis (see above).

RESULTS

Vma9p Is Required to Assemble the V-ATPase—To identify additional components required for yeast V-ATPase function, the yeast diploid deletion collection was screened for mutants that fail to grow on media buffered to pH 7.5 + 100 mM CaCl2. One new mutant was identified, and this mutant carried a deletion of the CWH36 gene.3 The CWH36 gene is now referred to as gene VMA9, because vma9Δ yeast cells exhibit characteristic Vma phenotypes: no growth at elevated pH, lack of vacuolar acidification in vivo, and a complete lack of V-ATPase activity in vacuole membranes (Refs. 23 and 24).3

To better understand the role of Vma9p in V-ATPase function, we examined the presence of V-ATPase subunits on the vacuoles of wild-type and vma9Δ cells. Blots comparing whole cell lysates and isolated vacuoles were probed with antibodies that recognize V0 and V1 subunits. The levels of Vma6p, Vma1p, and Vma5p in whole cell lysates were very similar between wild-type and vma9Δ cells (Fig. 1). The decreased Vph1p levels of vma9Δ cell extracts have been noted previously (23), although our results reveal a less severe effect. Both V-ATPase V0 subunits (Vph1p and Vma6p) and V1 subunits (Vma1p and Vma5p) were significantly dephosphorylated from the vacuoles of vma9Δ yeast. In addition, the V1 subunits Vma2p, Vma4p, Vma8p, Vma10p, and Vma13p were absent from the vacuoles of vma9Δ cells (data not shown). As expected, the non-V-ATPase vacuolar membrane protein ALP was present in isolated vacuoles of both wild-type and vma9Δ cells. These fractionation data are consistent with previous results (25) and indicate that V-ATPase subunits are not present on the vacuoles of vma9Δ yeast.

Subunit a of the V-ATPase is present in yeast as two isoforms, Vph1p and Stv1p (39). As shown above, the Vph1p vacular isoform is expressed at lower levels in vma9Δ strains as compared with wild type (Fig. 1). To further investigate the effects of Vma9p loss on the subunit isoforms, Western analysis was performed on yeast whole cell extracts. The level of Vph1p in vma9Δ cells was compared with yeast lacking a V1 subunit (vma1Δ), V0 subunit (vma3Δ), or V-ATPase assembly factor (vma21Δ). The lower Vph1p levels of vma9Δ yeast were similar to levels observed in vma3Δ and vma21Δ strains (Fig. 2A). Yeast lacking Vma1p had Vph1p levels that were indistinguishable from wild type. The V-ATPase of the late Golgi have Stv1p as their subunit a isoform (40). Like the Vph1p isoform, the level of Stv1p was reduced in vma9Δ, vma3Δ, and vma21Δ crude cell extracts as compared with wild-type and vma1Δ extracts (Fig. 2B). These results indicate that Vph1p and Stv1p require a competent V0 subcomplex, including Vma9p, to be expressed at wild-type levels.

Reduced Vph1p and Stv1p levels in vma9Δ cells could be due to decreased synthesis and/or stability of the subunit a isoforms. To investigate the stability of subunit a in vma9Δ cells, Vph1p was immunoprecipitated from radiolabeled wild-type, vma9Δ, and vma3Δ cells over a time course of 90 min. Immunoprecipitated Vph1p samples were separated on SDS-polyacrylamide gels and detected by autoradiography (Fig. 3A). In wild-type cells the levels of Vph1p remained essentially constant over the chase time, which is consistent with the previously reported Vph1p half-life of greater than 4–8 h (30, 41). In cells lacking a V0 subunit (vma3Δ), Vph1p was turned over quickly, and its half-life decreased to ~30 min. Vph1p was also degraded more rapidly in vma9Δ cells, exhibiting a half-life of ~45–60 min. We conclude from these data that Vph1p stability is decreased significantly in vma9Δ cells, as was observed previously when cells lack other V0 subunits (19).

Vma9p Is an Integral Membrane V0 Subunit That Requires a Fully Assembled V0 Subcomplex to Exit the ER—Yeast Vma9p is predicted to be a 73-amino acid protein with two transmembrane domains (Fig. 4A). To study Vma9p a polyclonal peptide antibody was raised against the N-terminal sequence of Vma9p, as was observed previously when cells lack other V0 subunits (19).

Hydropathy plots of Vma9p predict that it contains two transmembrane domains (Fig. 4A) (42), and Vma9p was found to be associated with the V-ATPase in isolated vacuolar membranes (24). To determine
Vma9p Analysis

Vma9p was extracted from membranes with both Na2CO3 and Triton X-100. The peripheral membrane protein Vma6p was radiolabeled for 10 min with [35S]methionine/cysteine and chased for the times indicated in minutes. Vph1p was immunoprecipitated from lysed cells, and samples were loaded into SDS-polyacrylamide gels and separated by electrophoresis, and the dried gel was exposed to a Phosphorimager cassette for detection. B, graphic depiction of Vph1p stability in WT, vma6Δ, and vma3Δ strains. The 0-min chase time point in all three strains was designated as 100%, and the percent of Vph1p remaining was plotted against time.

whether Vma9p is a peripheral membrane or integral membrane protein, membrane fractions from yeast were treated either with a gentle TE wash, the chaotropic agent Na2CO3, or the detergent Triton X-100. Na2CO3 treatment extracts peripheral proteins from pelleted membranes but not integral membrane proteins that can only be solubilized with detergents (43). Vph1p and Vma6p were utilized as integral and peripheral membrane protein controls, respectively. The integral membrane protein Vph1p was not solubilized by the TE or Na2CO3 treatments and remained with the pelleted membrane fraction (Fig. 4C). As expected, Vph1p fractionated with the supernatant only after solubilization with 0.5% Triton X-100. The peripheral membrane protein Vma6p was extracted from membranes with both Na2CO3 and Triton X-100 treatments. Similar to Vph1p, Vma9p was extracted into the supernatant fraction only by Triton X-100 treatment. These results demonstrate that Vma9p is an integral membrane subunit of the V-ATPase.

Vma9p has been identified in isolated vacuoles by Western blot analysis (24). The Vma9p-specific antibody was utilized to localize Vma9p by immunofluorescence in wild-type and mutant yeast strains. As expected, Vma9p clearly labeled the limiting membrane of the vacuole in wild-type cells (Fig. 5). Wild-type vacuolar Vma9p localization was perinuclear, encircling the DAPI-labeled nucleus in yeast cells lacking a V0 subunit (wt stv1Δ). This perinuclear labeling indicates that Vma9p localizes to the ER in yeast that fails to assemble a V0 subcomplex. Vma9p was also ER-localized in vma6Δ, vma11Δ, vma12Δ, vma16Δ, vma22Δ, and vph1Δ stv1Δ V0 mutant strains (data not shown). These results demonstrate that ER exit of Vma9p requires a fully assembled V0 subcomplex.

Vma9p Is Found in a V0-Vma21p Assembly Complex—The retention of Vma9p in the ER of yeast cells with defective V0 assembly led us to investigate whether Vma9p associates with the V-ATPase assembly factor Vma21p during normal V0 assembly. Previous work has shown that the ER-localized V0 subunits form a complex that interacts directly with the assembly factor Vma21p (Fig. 6A) (20). To determine whether Vma9p associates with the V0-Vma21p complex as illustrated (Fig. 6A), an HA epitope-tagged version of Vma21p was immunoprecipitated from wild-type yeast lysates. Vma9p was observed to co-immunoprecipitate with Vma21p-HA from extracts of wild-type cells (Fig. 6B). Vma9p did not precipitate from controls with yeast cells that lack an HA epitope-tagged version of Vma21p. The Vma9p-Vma21p interaction was preserved in vma1Δ yeast, which lack an assembled V1 subcomplex yet still assemble the V0 subcomplex (34). To better understand the relationship between Vma9p and the other V0 subunits, the Vma9p-Vma21p-HA interaction was examined in a series of mutants lacking individual V0 subunits. Vma21p-HA was immunoprecipitated from extracts of vma3Δ, vma6Δ, vma11Δ, vma12Δ, vma16Δ, and vph1Δ stv1Δ strains, and the precipitated protein fraction was probed for Vma9p. In all V0 subunit mutant strains examined, the Vma9p-Vma21p-HA interaction was greatly reduced or abolished as compared with wild-type strains. The Vma9p-Vma21p-HA interaction was also disrupted in yeast lacking the assembly factor Vma12p. In summary, Vma9p interacts with a Vma21p assembly complex in the ER, and this interaction was abrogated with the loss of any single V0 subunit.

To further examine the relationship between Vma9p and the other V0 subunits, the V0-Vma21p interaction in vma9Δ yeast was investigated. Vma21p-HA was immunoprecipitated from whole cell lysates of
Vma9p Analysis

FIGURE 5. Localization of Vma9p in wild-type, vma1Δ, vma3Δ, and vma21Δ cells. Immunofluorescence was performed on WT (SF838-1Da), vma9Δ (MACY10), vma1Δ (CY1), vma3Δ (LGY113), and vma21Δ (KYH3) cells as described under “Experimental Procedures.” Vma9p was visualized with a rabbit polyclonal antibody, followed by a goat anti-rabbit biotin antibody treatment, and a streptavidin-fluorescein isothiocyanate stain. The nucleus was visualized by DAPI. To observe vacuoles differentially, treatment. The nucleus was visualized by DAPI. To observe vacuoles differentially, labeling with DAPI. To observe vacuoles differentially, treatment. The nucleus was visualized by DAPI.

FIGURE 6. Vma9p is associated with the V0-Vma21p assembly complex. A, model looking down on the Vma21p V-ATPase V0 assembly complex in the endoplasmic reticulum. Vph1, -3, -6, -9, -11, -16, and -21 represent Vph1p, Vma3p, Vma6p, Vma9p, Vma11p, Vma16p, and Vma21p respectively. B, extracts from WT (SF838-1Da), vma1Δ (CY1), vma3Δ (LGY113), vma6Δ (CBY1), vma11Δ (LGY114), vma16Δ (LGY115), vph1Δ stv1Δ (KEV9), and vma12Δ (DIY102) yeast harboring plasmid pKH28 (VMA21::HA) were subjected to native immunoprecipitation of Vma21p-HA using anti-HA antibodies. As a negative control WT (no tag) SF838-1Da cells harboring plasmid pKH14 (VMA21) were similarly immunoprecipitated. Input (I) and immunoprecipitation (IP) samples were prepared and Western blots generated as described under “Experimental Procedures.” Blots were probed with Vma9p antibody; lanes correspond to 5% of input.

wild-type and vma9Δ cells, and these fractions were analyzed for the presence of Vma3p, Vma6p, Vma11p, and Vph1p. As expected, all V0 subunits assayed were found to co-immunoprecipitate with Vma21p-HA from wild-type cells (Fig. 7). Except for Vph1p, loss of Vma9p did not affect the steady state levels of the other V0 subunits. In yeast lacking Vma9p both the Vph1p-Vma21p-HA and Vma6p-Vma21p-HA interactions were significantly reduced or abolished (Fig. 7, A and B). However, the V0 proteolipid subunits Vma3p and Vma11p interacted strongly with Vma21p-HA even in the absence of Vma9p (Fig. 7, B and C). These results indicate that the proteolipid proton-translocating ring (Vma3p, Vma11p, and we expect Vma16p) forms a complex with Vma21p in the ER independent of Vma9p, but surprisingly Vph1p and Vma6p association with this complex requires Vma9p.

DISCUSSION

Our results and the work of other groups demonstrate that Vma9p is an integral membrane protein of the V-ATPase V0 subcomplex (this work and see Refs. 23 and 24). The genetic analysis of the VMA9 gene in yeast and C. elegans provides important in vivo evidence that Vma9p (subunit e) is required for V-ATPase function (8). The embryonic cellular hyperfusion observed in C. elegans fus-1 mutants is also observed when other V-ATPase subunits are eliminated by RNA interference, indicating that the hyperfusion phenotype observed in C. elegans reflects the loss of V-ATPase function and not a specific function of subunit e. The presence of a subunit e homolog associated with the purified bovine chromaffin granule V-ATPase complex provides solid evidence that subunit e is an important component of the mammalian V-ATPase complex as well (22–24). Plants, fungi, and animals are all predicted to express a small hydrophobic protein homologous to yeast subunit e (Vma9p), indicating that subunit e is likely to be an essential component of the V-ATPase complex in all eukaryotes (8, 24, 44).

Loss of Vma9p results in the elimination of in vivo acidification of yeast vacuoles (23), and the elimination of all V-ATPase activity associated with vacuolar membranes (24). Biochemical analysis reveals that Vma9p is an integral membrane protein that localizes to vacuolar membranes, and Vma9p co-purifies with the V-ATPase from isolated vacuoles (this work and see Ref. 24). The decreased half-life of Vph1p in vma9Δ cells is consistent with our previous findings that Vph1p is rapidly degraded in yeast cells lacking a V0 subcomplex (19, 30, 41). Taken together, the available data indicate that by all criteria Vma9p behaves biochemically and functionally as a subunit of the V0 subcomplex (this work and see Ref. 25).

Vma9p localizes to the vacuole in wild-type cells and in cells lacking the V1 subcomplex (vma1Δ cells), indicating that Vma9p transport to and stability in the vacuolar membrane does not require an assembled V1 subcomplex (34). In contrast, Vma9p is localized to the ER in yeast strains with defective V0 biogenesis. Loss of any single V0 subunit (Vma3p, Vma6p, Vma11p, and Vma16p) or V-ATPase assembly factor (Vma12p, Vma21p, or Vma22p) results in Vma9p ER localization. The inability of Vma9p to exit the ER in yeast cells lacking any V0 subunit
translocation ring, which appears to directly interact with Vma21p through Vma11p (20).

What role does Vma9p (subunit e) play in the V₀ subcomplex of the V-ATPase? One clue comes from the fact that the proteolipid V₀ subunits (Vma3p, Vma11p, and Vma16p) all contain an essential glutamate residue in one of their transmembrane helices (14). These acidic residues are thought to serve as carriers for protons translocating through the membrane (14). There are no acidic residues in the predicted transmembrane regions of Vma9p. Thus, it is unlikely that Vma9p participates in the proton translocation. Another clue comes from the observation that the proteolipids form a complex with Vma21p in the ER independent of Vma9p, yet the association of Vma9p and Vph1p (20) with this Vma21p-V₀ complex is absolutely dependent on the presence of all three proteolipids. Taken together with the independence of Vma9p and Vph1p for their association with the V₀-Vma21p assembly complex, these observations have led us to propose that Vma9p (subunit e) together with Vph1p (subunit a) constitute the stator that interfaces with the rotating proteolipid ring (Fig. 7D).

Because of the Vma9p/Vph1p/Vma6p interdependence, we have positioned Vma9p with contacts to Vph1p and Vma6p, although the presence of such direct interactions has not yet been tested experimentally. Establishing the close contact interactions between Vma9p/subunit e and the other V₀ subunits remains an important goal for the future.

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