VMA11 and VMA16 Encode Second and Third Proteolipid Subunits of the Saccharomyces cerevisiae Vacuolar Membrane H⁺-ATPase*

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The vacuolar membrane H⁺-ATPase (V-ATPase) of the yeast Saccharomyces cerevisiae is composed of peripheral catalytic (V₃) and integral membrane (V₀) domains. The 17-kDa proteolipid subunit (VMA3 gene product; Vma3p) is predicted to constitute at least part of the proton translocating pore of V₀. Recently, two VMA3 homologues, VMA11 and VMA16 (PPA1), have been identified in yeast, and VMA11 has been shown to be required for the V-ATPase activity. Cells disrupted for the VMA11 gene displayed the same phenotypes as those lacking either Vma3p or Vma11p; the mutant cells lost V-ATPase activity and failed to assemble V-ATPase subunits onto the vacuolar membrane. Epitope-tagged Vma11p and Vma16p were detected on the vacuolar membrane by immunofluorescence microscopy. Density gradient fractionation of the solubilized vacuolar proteins demonstrated that the tagged proteins copurified with the V-ATPase complex. We conclude that Vma11p and Vma16p are essential subunits of the V-ATPase. Vma3p contains a conserved glutamic acid residue (Glu¹⁰⁸) whose carboxyl side chain is predicted to be important for proton transport activity. Mutational analysis of Vma11p and Vma16p revealed that both proteins contain a glutamic acid residue (Vma11p Glu¹⁴⁵ and Vma16p Glu¹⁰⁸) functionally similar to Vma3p Glu¹⁰⁸. These residues could only be functionally substituted by an aspartic acid residue, because other mutations we examined inactivated the enzyme activity. Assembly and vacuolar targeting of the enzyme complex was not inhibited by these mutations. These results suggest that the three proteolipid subunits have similar but not redundant functions, each of which is most likely involved in proton transport activity of the enzyme complex. Yeast cells contain V₃ and V₀ subcomplexes in the vacuolar membrane and in the cytosol, respectively, that can be assembled into the active V₃V₀ complex in vivo. Surprisingly, loss-of-function mutations of either Vma11p Glu¹⁴⁵ or Vma16p Glu¹⁰⁸ resulted in a higher degree of assembly of the V₃ subunits onto the V₀ subcomplex in the vacuolar membrane.

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23 kDa with 56 and 35% amino acid identity to Vma3p, respectively (24, 25, 30, 31). Vma3p is a hydrophobic polypeptide chemically characterized as a proteolipid (soluble in chloroform/methanol) and is thought to constitute all or part of the proton translocating pore in \( V_{o} \) (5, 24, 25). Homologous subunits are found universally in V-type ATPase complexes characterized from various membrane sources, and all amino acid sequences determined for the subunits contain a conserved glutamic acid residue predicted to be critical for proton transmembrane activity (32). VMA11 was cloned by complementation of the growth defect of a calcium sensitive mutant, cls9 (30, 33). Unlike STV1, VMA11 is required for the activity and assembly of the vacuolar membrane V-ATPase complex (30, 33). VMA16 was originally identified as the growth defect of a calcium sensitive mutant, gene was cloned into pBluescript KS+ (30, 33). VMA11 was constructed as follows. A 1.8-kb TRP1 gene fragment in the upstream region of the VMA11 gene was replaced with a 0.85-kb HA :: TRP1 gene fragment in the downstream region of that gene (30, 33). The mutant was cured by transforming the plasmid by digestion with (blunt) fragment of pRHA150. pRHA150 was digested with \( Hpa \)II and \( Xho \)I and used to substitute for the chromosomal VMA11::HA gene in yeast. The sequence encoding the HA epitope was introduced into pRS306 (36), digested with HindIII, and used to substitute for the chromosomal VMA11::HA gene in yeast. The physiological function of this protein remained unclear.

In this work, the function of Vma11p and Vma16p was studied by examining the cellular localization of these proteins and by characterizing phenotypes of the cells carrying mutant forms of each protein. Our results indicate that Vma11p and Vma16p are novel \( V_{o} \) subunits of the V-ATPase complex essential for enzyme activity. We also report here that both proteins contain a glutamic acid residue important for function as found for Vma3p (34), and inactivating mutations of these residues influence the assembly status of the enzyme complex.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—Yeast strains used in this study are listed in Table I. RHA374, LYG11, and LYG10 are VMA3::HA, VMA11::HA, and VMA16::HA derivatives, respectively, of SF838–1D (35). YRH11a and RHP110 are isogenic to YPH499 (36) except \( \Delta \)vma16::TRP1 and \( \Delta \)vma16::HA::TRP1, respectively. RHA115 (VMA11::TRP1) was constructed by inserting a TRP1 gene fragment in the upstream region of the \( V_{1} \) in YPH499. RHA115 is Trp+ and Vma+. RHA161 (E145D), RHA117 (E145L), and RHA118 (E145Q) are vma11 mutant derivatives of RHA115. RHP1100-HRP1107 are isogenic to RHP110, except that each carries a wild type or mutant vma16 gene on a yeast low copy, centromere-based plasmid, pRS316 (36). RHP1108 is RHP110 harboring pLG34 (37).

Yeast cells were grown in YPD medium (1% yeast extract, 2% Bactopeptone (Difco), 2% glucose), YEP medium (1% yeast extract, 2% Bactopeptone, and 3% glycerol), or YNBD medium (0.67% yeast nitrogen base (Difco) and 2% glucose). YPD medium was buffered at pH 5.0 or 7.5 with 50 mM phosphate/succinate buffer as described previously (38). Yeast with high sensitivity of the cells was examined on YPD medium supplemented with 100 \( \mu \)g CaCl\(_2\) (39).

Disruption of the VMA11 Gene—Null vma11 mutants were constructed as follows. A 1.8-kb EcoRV-SpeI fragment containing the \( V_{1} \) gene was cloned into pBluescript KS+ (Strategene), creating pRHA150. pRHA150 was digested with Xhol and HindIII, blunted, and ligated to remove the \( Cl_{1} \) and \( Hin_{II} \) sites originating from the multicloning site of pBluescript KS+. The 0.6-kb \( Hin_{II}-Cl_{1} \) (blunt) fragment of pRHA151 was replaced with a 0.85-kb EcoRI-BglII (blunt) fragment of pJL280 (TRP1) (37) to create \( \Delta \)vma11::HA::TRP1 (pRHA163). The \( \Delta \)vma11::HA::TRP1 fragment was introduced into RHA163 by transformation and used to construct the disruption strain YRH11a by the method as described previously (38).

Isolation and Disruption of the VMA16 (PPA1) Gene—Two oligonucleotide primers, atcagactattgtagagagggaaattag (E145D), tatacagacactagaggagagaatatg (E145Q). Each primer introduces a unique restriction site (underlined) to screen for the introduced mutation. A 0.85-kb EcoRI-BglII (blunt) fragment from pJJ280 (TRP1) (37) was inserted at the SacI site of the resultant mutant plasmids as a marker for mutant selection. The SacI site is located 450 base pairs 5' of the initiating ATG, and insertion of the TRP1 gene into the wild type \( V_{1} \) gene at this position did not affect the cell growth or the V-ATPase activity (data not shown). Mutant vma16::HA::TRP1 fragments were introduced into the chromosomal \( V_{1} \) gene in YPH499 to yield RHA116–118, and vma16 mutants were selected by Trp+ phenotype, and introduction of the mutations was confirmed by Southern blot analysis of chromosomal DNA.

Site-directed Mutagenesis of the VMA11 Gene—A 3.4-kb XbaI-EcoRI::fragment containing the \( V_{1} \) gene was cloned into pRS316 (36) to create RHP1111. Site-directed mutagenesis of \( V_{1} \) was done by two sequential polymerase chain reaction reactions with \( pRP1111 \) and overlapping forward and reverse mutagenic primers as described (40). Nucleotide sequences of the mutant gene fragments were confirmed by DNA sequence analysis. The wild type and mutant \( V_{1} \) genes (listed in Table I) were introduced into RHP110 (\( \Delta \)vma16::TRP1) to yield RHP110 (pRHP111, wild type). RHP1101 (pRHP131, E108S), RHP1102 (pRHP132, E108L), RHP1103 (pRHP133, E108Q), RHP1104 (pRHP134, E108D), RHP1105 (pRHP135, E108V), RHP1106 (pRHP136, E108Q), and RHP1107 (pRHP137, E108K).

Protein Preparation, SDS-PAGE, and Western Blot Analysis—Preparation of vacuolar membrane fractions and purification of the V-ATPase were previously described (3, 5). When vacuolar membranes were prepared from strains (RHP1100-RHP1108) expressing Vma16p from pRS316 (URA3)-based plasmids, cells were grown in YNB (uracil) supplemented with 0.5% casamino acids (Difco). Each vacuolar membrane fraction was assayed for the vacuolar membrane marker, dipetidyl aminopeptidase B (DPAP-B) (43), and a purification index, which was expressed as the ratio of the specific DPAP-B activity in the vacuolar membrane to the activity in a spheroplast preparation, was determined. Vacuolar membrane fractions with a purification index of >30 (5) were used throughout this study. Protein extracts of whole cell and vacuolar membrane fractions were prepared as described by Hill and Stevens (44). Proteolipid subunits were purified by chloroform/methanol extraction of whole cell extracts or vacuolar membranes (45). Crude membrane and cytosolic fractions were prepared as follows. Yeast cells...
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TABLE I
Strains and plasmids used in this study

| Strains | Plasmids | Description | References |
|---------|----------|-------------|------------|
| SF838–1D | pRS316 | centromere-based, low copy plasmid (pCEN-URA3) | 36 |
| YPH499  | pRHA150 | 1.8-kb EcoRV-Spe I VMA11 gene fragment cloned into pBluescript KS+ | This study |
| YPH501  | pRHP111 | 3.4-kb XhoI-Eco R1 VMA16 gene fragment cloned into pRS316 | This study |
| YHR11a  | pRHP110 | same as pRHP111 except vma16 E108D | This study |
| RHP110  | pRHP112 | same as pRHP111 except vma16 E108V | This study |
| RHP1103 | pRHP113 | same as pRHP111 except vma16 E188D | This study |
| RHP1104 | pRHP114 | same as pRHP111 except vma16 E188V | This study |
| RHP1105 | pRHP115 | same as pRHP111 except vma16 E188Q | This study |
| RHP1106 | pRHP116 | same as pRHP111 except vma16 E108Q | This study |
| RHP1107 | pRHP117 | same as pRHP111 except vma16 E108Q | This study |
| RHP1108 | pRHP118 | same as pRHP111 except vma16 E188Q | This study |

RESULTS
Construction of a ppa1 (vma16) Null Mutant—Vma3p and its homologue, Vma11p, have been shown to be required for activity and assembly of the V-ATPase complex (30). To determine whether another Vma3p homologue, Ppa1p (Vma16p) (31), is also required for expression of the V-ATPase activity, we constructed and characterized a ppa1 null mutant. One of the two copies of the PPA1 gene in a wild type diploid strain, YPH501 (36), was disrupted by a DNA fragment replacing ~80% of the PPA1 reading frame with the TRP1 gene (Fig. 1A). The resulting PPA1Δppa1::TRP1 diploid cells were sporulated, tetrads were dissected, and spores were checked for viability and segregation of the TRP1 marker. Spores were grown on YPD medium buffered at pH 5.0. Spore viability for the heterozygous diploid was almost identical to that of the wild type parent cells, and no anomalous segregation pattern was observed (data not shown). These results indicate that the Δppa1 mutant is viable.

PPA1 (VMA16) Is Required for the V-ATPase Activity—Δppa1 cells displayed growth phenotypes characteristic of mutants disrupted for VMA3, VMA11, or other genes required for the V-ATPase activity (VMA genes) (4, 23) (Table II). These include inability to grow in YPD medium buffered at pH 7.5 (17, 48), YPD medium containing 100 mM CaCl₂ (33, 48), or medium containing glycerol as a sole carbon source (YPG) (33). The Δppa1 cells also failed to accumulate quinacrine in their vacuoles, which indicates that the mutant vacuole is not acidified (Table II). In addition, vacuolar membrane fractions isolated from the Δppa1 cells lacked bafilomycin A₁-sensitive ATPase activity (Table II). These results suggest that the PPA1 gene is required for V-ATPase activity. Thus, the PPA1 gene is an additional member of the VMA genes. The name PPA (for
proteolipid of proton ATPase) seems to be confusing because the name is used by another gene with unrelated function (PPA2; inorganic pyrophosphatase). We propose here to rename the gene VMA16.

**VMA16 (PPA1) Is Required for the Assembly of the V-ATPase Complex—**Δvma3 and Δvma11 mutant cells fail to assemble V-ATPase subunits onto the vacuolar membrane (25, 30, 49). In addition, steady state levels of two integral subunits (100- and 17-kDa V$_0$ subunits) were decreased significantly in the Δvma16 mutant (RHP110) (Fig. 2). The observed phenotypes of Δvma16 mutant cells fail to assemble the V-ATPase subunits that are peripherally associated with the membrane (all the V$_1$ and the 36-kDa V$_0$ subunit). Pulse-chase experiments of the 100-kDa subunit in Δvma16 cells showed that the subunit was synthesized normally compared with wild type but degraded more quickly by nonvacuolar protease(s) in the mutant cells (data not shown). An increased rate of degradation of the 100-kDa subunit was also observed in Δvma3 and Δvma11 cells (data not shown).

**Construction of Strains Expressing Functional Epitope-tagged Vma11p and Vma16p—**The observed phenotypes of Δvma3, Δvma11, and Δvma16 mutant cells were indistinguishable from one another. The simplest interpretation of these results would be that Vma11p and Vma16p are second and third proteolipid subunits of the V-ATPase. However, a possible function for these proteins in the assembly and/or vacuolar targeting of the enzyme complex could also account for the phenotypes of the mutant cells (44–46). To further investigate the function of Vma11p and Vma16p, we examined the cellular localization of these proteins. Vma11p and Vma16p were tagged at the C terminus with a nine-amino acid epitope of the influenza virus hemagglutinin (HA) (41) (Fig. 1A) to allow detection of the proteins by anti-HA antibodies. Recombinant DNA fragments coding for the tagged proteins (Vma11p-HA and Vma16p-HA) were constructed, and the chromosomal copies of the respective genes were replaced with the tagged alleles (VMA11::HA and VMA16::HA). Resultant strains expressing Vma11p-HA (LGY11) or Vma16p-HA (LGY10) grew as well as wild type cells (SF838–1D, and SF838–1D) on YPD medium buffered to pH 7.5, supplemented with 100 mM CaCl$_2$, and YPG. Vascular acidification was normal in these strains as indicated by quinacrine uptake into the organelles (data not shown), and the V-ATPase specific activity in isolated vacuolar membrane fractions was similar to that in the wild type membranes (1.83, 1.14, and 1.23 μmol ATP hydrolyzed/min/mg protein for SF838–1D, LGY11, and LGY10, respectively). We conclude that the tagged Vma11p and Vma16p were functional.

**TABLE II**

| Strains          | Growth$^a$ | Vascular acidification$^b$ | V-ATPase activity$^c$ |
|------------------|-----------|---------------------------|-----------------------|
| Wild type        | ++        | ++                        | +                     |
| Δvma16           | ++        | ++                        | +                     |
| E108D            | ++        | ++                        | +                     |
| E108Q            | +         | +                         | 2                     |
| E108L            | +         | +                         | 0                     |
| E188D            | ++        | ++                        | ND$^d$                |
| E188V            | ++        | ++                        | ND                    |
| E188Q            | ++        | ++                        | ND                    |

* a pH 5, YPD buffered to pH 5; pH 7.5, YPD buffered to pH 7.5; CaCl$_2$ YPD supplemented with 100 mM CaCl$_2$, YPG containing glycerol as a sole carbon source. ++, grows as well as wild type cells; +, grows well but slower than wild-type cells; −, no growth.

* b Vascular acidification was monitored by quinacrine staining in vivo.

* c Baflomycin A$_1$-sensitive ATPase activity in vacuolar membrane fractions (nmol ATP hydrolyzed/min/mg protein). The ATPase activity was assayed at 30°C as described under “Experimental Procedures.” The vascular membrane fractions were isolated from cells grown in YNBD medium to minimize plasmid loss during cell growth. This presumably is the reason for the low V-ATPase activity measured in this series of experiments as compared with that in other experiments (cultured in rich medium (YPD), Table III). The purity of the fractions was not affected by growing the cells in YNBD medium.

* d ND, not determined.

In addition, the levels of the integral subunits (100- and 17-kDa V$_0$ subunits) were decreased significantly in the Δvma16 cells. Loss of Vma16p did not affect the cellular levels of the V-ATPase subunits that are peripherally associated with the membrane (all the V$_1$ and the 36-kDa V$_0$ subunit). Pulse-chase experiments of the 100-kDa subunit in Δvma16 cells showed that the subunit was synthesized normally compared with wild type but degraded more quickly by nonvacuolar protease(s) in the mutant cells (data not shown). An increased rate of degradation of the 100-kDa subunit was also observed in Δvma3 and Δvma11 cells (data not shown).

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3 Although we had previously reported a decrease in the steady state level of the 36-kDa subunit in yeast cells lacking Vma3p (20), more recent kinetic analyses indicate that the 36-kDa subunit is equally stable in wild type, Δvma3, Δvma11, and Δvma16 cells (L. A. Graham and T. H. Stevens, unpublished results).
HA-tagged Vma11p and Vma16p Are Localized on the Vacuolar Membrane—Localization of Vma11p-HA and Vma16p-HA was analyzed by indirect immunofluorescence microscopy. Anti-HA antibodies bound to the outline of vacuoles in LGY11 (VMA11::HA) and LGY10 (VMA16::HA) cells were fixed, spheroplasted, and stained with anti-HA antibodies. Cells were viewed by Nomarski optics to observe cell morphology (Nomarski) and epifluorescence microscopy using a filter specific for fluorescein to observe anti-HA stain (Fluorescence).

Fig. 3. Immunolocalization of Vma11p-HA and Vma16p-HA. SF838–1D (wild type; lane 1, 20 μg of proteins), RHA374 (VMA3::HA; lane 2, 2.5 μg), LGY11 (VMA11::HA, lane 3, 20 μg), or LGY10 (VMA16::HA; lanes 4, 20 μg) cells were examined by Western blot analysis with anti-HA antibodies. The HA-tagged proteins are indicated by arrowheads.

The signal intensity of Vma16p-HA (Fig. 4, lane 1) was similar to that of Vma11p-HA (lane 3) and was 5–10 times lower than that of Vma3p-HA (lane 2; eight times less amount of proteins was loaded in lane 2). Although we cannot estimate the exact molar ratio of the three proteins by this method, this result suggests that Vma16p and Vma11p exist at lower levels than Vma3p in the vacuolar membrane. The tagged proteins gave only weak signals in both immunofluorescence microscopy and Western blot analysis when expressed in cells containing normal vacuolar protease activities, though the cells still displayed Vma-phenotypes (data not shown). The C-terminal ends of these proteins may be facing inside the lumen of the vacuole, and the tag might be removed by the vacuolar proteases. However, other interpretations of the data cannot be ruled out at this time.

HA-tagged Vma11p and Vma16p Are Components of the V-ATPase Complex—We next examined whether Vma11p and Vma16p are included in the V-ATPase complex. Vacular membrane proteins from LGY11 and LGY10 cells were solubilized with 2W3–14 and fractionated through a 20–50% glycerol density gradient. Fractions from the gradients were assayed for V-ATPase activity and subjected to Western blot analysis with anti-V-ATPase subunit antibodies. Fig. 5 shows the distribution of two V-ATPase subunits (100-kDa V₀ and 69-kDa V₁ subunits) and the tagged proteins in the gradient. As reported previously (18, 20), V-ATPase subunits fractionate into two peaks, one that cofractionates with the ATPase activity and contains both the V₀ and V₁ subunits (V₀V₁ (VMA11, fractions 8–10; LGY10, fractions 7–9) and the other that migrates to lower density near the dipeptidyl aminopeptidase B protein (DPAP-B; ~120-kDa vacuolar membrane protein (43)) and contains only the V₀ subunits (LGY11, fraction 12; LGY10, fraction 11). The second peak in the lower density fractions is predicted to represent the V₀ subcomplex not assembled with V₁ subunits (20, 27, 50). The distribution patterns of Vma11p-HA and Vma16p-HA matched exactly with those of the 100- (Fig. 5) and 36-kDa (Ref. 20 and data not shown) V₀ subunits, indicating that the two proteins are physically associated with both the active V₀V₁ complex and the V₀ subcomplex in the vacuolar membrane.

Vma11p Glu₁⁴⁵ and Vma16p Glu₁⁰⁸ Are Important for the V-ATPase Activity—The glutamic acid residue at position 137 (Glu₁⁴⁵) in Vma3p lies in the center of the predicted fourth transmembrane domain of the subunit (Fig. 1, A and B) (24, 25, 34). Mutational analysis of this residue has suggested that the carboxyl side chain at this position is critical for proton translocating activity (34). We examined whether Vma11p and Vma16p also contain an acidic residue functionally similar to Vma3p Glu₁⁴⁵. Vma11p Glu₁⁴⁵, Vma16p Glu₁⁰⁸, and Vma16p Glu₁⁸⁸ (Fig. 1, A and B) were modified by site-directed mutagenesis. No other acidic residues are found in the predicted
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**FIG. 5.** Detection of Vma11p-HA and Vma16p-HA in glycerol gradient fractions containing the detergent-solubilized V-ATPase complex. 500 µg of vacuolar membranes prepared from either LGY11 (VMA11::HA) or LGY10 (VMA16::HA) were solubilized in 1% (w/v) ZW3-14 and fractionated by centrifugation for 20 h at 175,000 × g through a 12-ml 20–50% glycerol gradient. 750-µl fractions were collected, and 100 µl was assayed immediately for ATPase and DPAP-B activity. Proteins were precipitated from each fraction by the addition of 5% trichloroacetic acid (final concentration), separated by SDS-PAGE, and probed with antibodies specific for the 100- and 69-kDa V-ATPase subunits and the HA epitope.

transmembrane domains of the two proteins. *vma11* mutant strains (*vma11* E145D, E145L, E145Q) were constructed by replacing the chromosomal copy of the gene with the mutant alleles. *vma16* mutants were constructed by introducing mutant genes (*vma16* E108D, E108L, E108Q, E108V) and *vma16* E188D, E188Q, E188V) into RHP110 (Δ*vma16*) on a low copy, centromere-based plasmid, pRS316 (36). Tables II and III summarize the growth phenotypes and V-ATPase activities of the mutant cells compared with wild type cells. Of the three residues examined, mutants of the Vma11p Glu145 and Vma16p Glu108 displayed phenotypes similar to cells expressing Vma3p Glu137. These residues could be functionally substituted by an aspartic acid residue, but other mutations altering these residues examined, mutants of the Vma11p Glu145 and Vma16p Glu108 displayed phenotypes similar to cells expressing Vma3p Glu137. These residues could be functionally substituted by an aspartic acid residue, but other mutations altering these residues might alter the secondary or tertiary structure of these polypeptides, thus inhibiting the assembly and/or targeting of the V-ATPase complex. We therefore examined whether the V-ATPase complex was assembled onto the vacuolar membrane in the *vma11* Glu145 and *vma16* Glu108 mutant cells. Neither of the mutations inhibited the assembly of the V-ATPase subunits onto the vacuolar membrane (Fig. 6, A and B), although the amounts of membrane-bound V1 subunits appear to be increased in the inactive *vma11* and *vma16* mutant cells (Fig. 6, A, lanes 3 and 4, and B, lanes 3–5) and will be discussed in a later section. Solubilization and density gra-

**FIG. 6.** Assembly of V-ATPase subunits in wild type, *vma11* Glu145 and *vma16* Glu108 cells. A, detection of V-ATPase subunits in wild type and *vma11* mutant vacuolar membranes. Proteins in vacuolar membrane fractions (∼10 µg) isolated from RHA115 (wild type, lane 1), RHA116 (*vma11* E145D, lane 2), RHA117 (*vma11* E145L, lane 3), RHA118 (*vma11* E145Q, lane 4), and YRH11a (Δ*vma11*, lane 5) were subjected to Western blot analysis with anti-V-ATPase subunit antibodies. The levels of the 17-kDa subunit were analyzed by examining the amount of polyethylene in chloroform/methanol extracts of the vacuolar membrane fractions. The anti-100-kDa subunit polyclonal antibody recognized two polypeptides of 100 and 75 kDa. The 75-kDa species (asterisk) is a proteolytic product of the 100-kDa subunit (3). B, detection of V-ATPase subunits in wild type and *vma16* mutant vacuolar membranes. V-ATPase subunits were detected as described above in vacuolar membrane fractions (∼10 µg/lane) isolated from RHP1100 (wild type, lane 1), RHP1101 (vma16 E108D, lane 2), RHP1102 (E108L, lane 3), RHP1103 (Δ*vma16*, lane 4), and YRH110 (Δ*vma11*, lane 5). C, distribution of the 60-kDa V1 subunit in wild type (*RHA 115*) and *vma11* Glu137 mutant (*RHA 116–118*) cells. Cells were spheroplasted, lysed, and fractionated into membrane (P) and cytosol (S) fractions. Proteins in each fraction (corresponding to ∼1 A600 of cells) were resolved by SDS-PAGE, and the distribution of the 60-kDa V1 subunit was analyzed by Western blot analysis.
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GluE145 and GluE108 mutations resulted in a higher degree of assembly of the V1 subunits to the V0 subcomplex in the vacuolar membrane (Fig. 6, A, lanes 3 and 4, and B, lanes 3–5). The levels of V0 subunits in vacuolar membranes were kept at relatively unchanged levels (Fig. 6, A and B). These mutations did not affect the steady state levels of the V-ATPase subunits (data not shown) but appeared to change the distribution of the 60-kDa V1 subunit (Fig. 6C) from cytosol to the membrane fractions. Fig. 7 shows that the second peak of the V0 subunits (RHA115, fractions 8–10) was not prominent in the mutant membrane (RHA117, fraction 8). Similar changes in the ratio of V0V1 and V0 subunits during solubilization and fractionation on the gradient fractions remained unchanged from that in the membrane (data not shown), indicating that there was no specific loss of V0 subunits during solubilization and fractionation on the gradient. These results suggest that the assembly and/or retention of V1 subunits to the V0 subcomplex on the vacuolar membrane was promoted in the vma11 E145L and vma16 E108L mutant cells.

To further investigate the assembly status we also compared the effects of nitrate on the wild type and mutant V-ATPase complexes. Nitrate (50–100 mM) causes the stripping of V1 from V0 only in the presence of Mg2+ and ATP (3). The dissociation of V1 by these reagents is thought to be triggered by a conformational change induced by the binding of ATP to a nucleotide binding site in V1 (20). V1 subunits in the vma11 E145L mutant complex were released from the membrane by the treatment with nitrate and Mg-ATP (Fig. 8). Incubation in a buffer without Mg-ATP or nitrate was not effective at stripping V1 from the membranes (data not shown). Therefore, the mutant enzyme complex is likely to retain a structure that can

FIG. 7. Detection of V0V1 and V0 complexes in glycerol gradient fractions containing solubilized vacuolar membrane proteins. 850 μg of vacuolar membranes prepared from either RHA115 (A, wild type) or RHA117 (B, vma11 E145L) were solubilized in 1% W3–14 (w/v) and fractionated by centrifugation for 14 h at 177,000 × g through a 10-ml 20–50% (2.5% steps) glycerol gradient. 750-μl fractions were collected, and aliquots (100 μl each) were assayed for activity of the V-ATPase and DPAP-B. Proteins were precipitated from each fraction by the addition of 5% trichloroacetic acid (final concentration), separated by SDS-PAGE and detected by staining with Coomassie Blue (Coom.). The numbers in italics under the lanes represent V-ATPase and DPAP-B activities for the corresponding fraction. Lanes without numbers on the bottom contained activity less than 1 nmol/min/ml fraction. Distribution of the V-ATPase subunits through the fractions was examined by Western blot analysis with antibodies specific for the 100-, 69-, and 36-kDa V-ATPase subunits. Gradient fractions are identified with even numbers starting at the bottom of the gradient.

indicate that the vma11 GluE145 and vma16 GluE108 mutations do not inhibit the assembly and vacuolar targeting of the V-ATPase subunits. The mutant complex appeared to migrate to denser fractions than the wild type complex (Fig. 7, A and B). The reason for this difference in the behavior of these complexes is currently unknown. Although sometimes one or two additional polypeptides were observed by Coomassie staining of the peak fractions, of the mutant V-ATPase complex, these proteins were not consistently present in the peak fractions. It is most likely that these polypeptides are polypeptides are the breakdown products of the V-ATPase subunits. However, we cannot exclude the possibility that these proteins are specifically associated with the mutant complex and thus changing its mobility in the density gradient.

Surprisingly, the inactivating Vma11p GluE145 and Vma16p

FIG. 8. Subunit stripping by ATP and nitrate. Wild type (RHA115) or vma11 E145L mutant (RHA117) vacuolar membranes were suspended in a solution containing 50 mM Tris-Mes, pH 6.9, 5 mM MgCl2, 25 mM KCl, 100 mM KNO3, and 5 mM ATP. After incubation at 4 °C for 30 min, the suspension was centrifuged at 100,000 × g for 1 h to yield pellet (P) and supernatant (S) fractions. Proteins in each fraction were subjected to SDS-PAGE analysis, and V-ATPase subunits were detected by Coomassie Blue staining (A) or by Western blot analysis with anti-69-kDa subunit monoclonal antibody (B).
bind ATP and execute the conformational change triggered by the nucleotide binding.

**DISCUSSION**

In this paper, we showed that Vma11p and Vma16p are novel proteolipid subunits of the yeast V-ATPase. The major evidence that supports our conclusion is as follows: 1) Cells disrupted for either *VMA11* or *VMA16* are defective in the activity and assembly of the V-ATPase. 2) The functional HA-tagged Vma11p and Vma16p reside on the vacuolar membrane and cofractionate with the V-ATPase complex when the tagged subunits contain an inactive but fully assembled V-ATPase complex in the vacuolar membrane, suggesting that the functions of the two proteins are required during the catalytic reaction. Therefore, the yeast V-ATPase contains three different proteolipid subunits in its complex. It is unlikely that these subunits are isoforms of different V-ATPases, because mutations affecting any of these proteins completely inhibit the V-ATPase activity in the vacuolar membrane (25, 30, 34, 48).

No polypeptide corresponding to Vma11p (17 kDa) or Vma16p (23 kDa) has yet been identified by previous purification studies (3, 5). This might be due to poor staining of these subunits by Coomassie Blue as has been observed for the proteolipid subunits of the V- and F_{0}F_{1}-type ATPases (25, 51). The levels of the HA-tagged subunits suggest that Vma11p and Vma16p are present at lower levels than Vma3p in the vacuolar membrane, although the exact molar ratio of the three proteolipid subunits in the enzyme complex remains to be determined. This might also be the reason that only Vma3p has been identified by previous biochemical studies of the V-ATPase.

In this work, we demonstrated that the three yeast V-ATPase proteolipid subunits exhibit many common properties. The primary structures of these proteins are similar to one another, and all three subunits are essential for activity and assembly of the enzyme complex (24, 25, 30, 48). Furthermore, each contains a glutamic acid residue that lies in a predicted transmembrane domain and is required for V-ATPase activity (Glu^{137} in Vma3p, Glu^{145} in Vma11p, and Glu^{108} in Vma16p) (Fig. 1) (34). These results indicate that the three subunits share a common function, which is most likely to be involved in the proton transport activity of the enzyme complex.

The phenotypes of the mutants altering the conserved glutamic acid residues in Vma3p, Vma11p, and Vma16p are similar but not identical. Generally, mutations at Vma3p Glu^{137} appeared to be more severe than those of the other two. For example, Vma3p E137D appears to be partially active (34), whereas Vma11p E145D and Vma16p E108D are fully functional. Only vma3 E137Q, but not vma11 E145Q and vma16 E108Q, inhibits cell growth in YNB medium buffered to pH 7.5 when each of the mutant genes is introduced into wild type haploid cells on a low copy, centromere-based plasmid (pHS316). This dominant negative phenotype produced by the vma3 E137Q mutation, together with the relative abundance of Vma3p-HA in the vacuolar membrane, may suggest that Vma3p is the major proteolipid subunit in the enzyme complex. The coated vesicle proton pump is reported to contain six copies of the 17-kDa proteolipid subunits (52), and binding of N,N'-dicyclohexylcarbodiimide to a single copy of this subunit is sufficient to abolish the enzyme activity (53). Providing that both wild type and mutant subunits are expressed in the same cells and incorporation of a single mutant 17-kDa subunit inhibits the enzyme activity, the fraction of the active ATPase complex is expected to increase as the copy number of the subunit decreases. In this scenario, the defects resulting from mutations in Vma3p would be more detrimental. Of course, the mutant phenotypes might reflect the difference in the functions of the conserved glutamic acid residues in the three proteins. It will be important in the future to establish the function, subunit interaction, and stoichiometry of these proteins individually.

Our present work raises many questions about the proteolipid subunit content of V-type ATPases in other organelles and organisms. The ~20-kDa V_{0} subunits present in the V-type ATPases of bovine chromaffin granules and coated vesicles have been reported to exhibit similar biochemical properties to those of the 17-kDa proteolipid subunits (52, 54) and thus are candidates for a “second” proteolipid subunit in their enzyme complexes. It should be noted that cDNA fragments capable of encoding all or part of Vma16p homologues have been isolated from plant, mouse, human, and nematode. Although yeast cells appear to have two V-ATPase complexes, one containing Vph1p and the other containing Stv1p (14, 29), it is highly likely that all three proteolipid subunits function in both V-ATPase complexes, because ∆vma3, ∆vma11, and ∆vma16 mutants each display phenotypes identical to ∆stv1Δvph1 double mutants (25, 29, 30, 48).

The inactivating mutations of Vma11p Glu^{145} and Vma16p Glu^{108} do not inhibit the assembly and targeting of the V-ATPase subunits but instead promote the binding of the V_{1} subunits to the V_{0} subcomplex on the vacuolar membrane, resulting in an increase in the population of inactive but fully assembled V_{0}V_{1} complex. These results suggest that the equilibrium of assembly/disassembly of the V_{1} subunits to the V_{0} subcomplex is altered in the mutant cells. Reversible assembly of the V_{1} subunits to the V_{0} domain in vivo has been found to be responsive for physiological changes. Kane (27) has shown that dissociation and reassembly of V_{0} and V_{1} can occur rapidly in vivo in response to changes in nutrient conditions. Sumner et al. (55) reported that the inactivation of the *Munduca sexta* plasma membrane V-ATPase during molting parallels the dissociation of V_{1} subunits from V_{0} in the membrane. In this context, it will be interesting to investigate whether the V-ATPase complex senses changes in a proton motive force loaded across the vacuolar membrane or an ATP potential in the cytosol under different nutritional conditions.

In summary, Vma11p and Vma16p are novel proteolipid subunits of the yeast V-ATPase, which show properties similar to Vma3p. Despite the structural and functional similarities of these polypeptides, our results reveal that a single V-ATPase complex must contain at least one copy of each of the three proteolipid subunits to the V_{0}V_{1} complex.

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