The yeast Saccharomyces cerevisiae vacuolar H\(^{+}\)-ATPase (V-ATPase) is a multisubunit complex responsible for acidifying intracellular organelles and is highly regulated. One of the regulatory subunits, subunit H, is encoded by the VMA13 gene in yeast and is composed of two domains, the N-terminal domain (amino acids (aa) 1–352) and the C-terminal domain (aa 353–478). The N-terminal domain is required for the activation of the complex, whereas the C-terminal domain is required for coupling ATP hydrolysis to proton translocation (Liu, M., Tarssio, M., Charsky, C. M., and Kane, P. M. (2005) J. Biol. Chem. 280, 36978–36985). Experiments with epitope-tagged copies of Vma13p revealed that there is only one copy of Vma13p/subunit H per V-ATPase complex. Analysis of the N-terminal domain shows that the first 179 amino acids are not required for the activation and full function of the V-ATPase complex and that the minimal region of Vma13p/subunit H capable of activating the V-ATPase is aa 180–353 of the N-terminal domain. Subunit H is expressed as two splice variants in mammals, and deletion of 18 amino acids in yeast Vma13p corresponding to the mammalian subunit H \(\beta\) isoform results in reduced V-ATPase activity and significantly lower coupling of ATPase hydrolysis to proton translocation. Intriguingly, the yeast Vma13p mimicking the mammalian subunit H \(\beta\) isoform is functionally equivalent to Vma13p lacking the entire C-terminal domain. These results suggest that the mammalian V-ATPase complexes with subunit H splice variant SFD-\(\alpha\) or SFD-\(\beta\) are likely to have different activities and may perform distinct cellular functions.

The vacuolar H\(^{+}\)-ATPase (V-ATPase) belongs to a family of V-type ATPases present in all eukaryotic organisms and functions as an ATP-dependent proton pump that transports protons across a lipid bilayer. The V-ATPase is required to acidify the lumen of cytoplasmic organelles, such as vacuoles, coated vesicles, endosomes, lysosomes, the Golgi apparatus, and chromaffin granules (1–3). Organellar acidification is essential for a variety of cellular processes such as receptor-mediated endocytosis, proteolysis of proteins, and proton-coupled transport of small molecules and ions (3). Although the V-ATPase is found mainly on intracellular organelles, it can also be found on the plasma membrane of intercalated cells in the distal nephron and osteoclasts, where it is required for urine acidification and bone resorption (4).

In the yeast S. cerevisiae, the V-ATPase is found primarily on the limiting membrane of the vacuole and to a lesser extent on Golgi and endosome membranes (3). The V-ATPase is essential for life in all eukaryotic organisms tested with the exception of some yeast strains, such as S. cerevisiae. In yeast, the loss of V-ATPase function results in the inability to acidify the vacuole, resulting in a pH-sensitive growth phenotype, making yeast an excellent model organism in which to investigate V-ATPase function (3).

The yeast V-ATPase is composed of 14 subunits, which are divided between two distinct sectors: a peripheral catalytic complex (V\(_1\)) and a membrane associated complex (V\(_0\)) (3). The V\(_1\) subcomplex is composed of eight subunits (A, B, C, D, E, F, G, and H), whereas the V\(_0\) subcomplex is composed of six subunits (a, c, c\(^\prime\), c\(^\prime\prime\), d, and e). The V-ATPase from other organisms (including mammals) is highly similar to the yeast enzyme complex in subunit composition of the V\(_1\) and V\(_0\) subcomplexes. Similar to the F\(_{0}\)F\(_{1}\)-ATP synthase (F-ATPase), the V-ATPase complex has an overall dumbbell shape with a central stalk made up of subunits D, F, and d connecting the catalytic domain (subunits A and B) to the ring of small transmembrane subunits c, c\(^\prime\), and c\(^\prime\prime\). There is also a peripheral stalk composed of the hydrophilic V\(_1\) subunits C, E, G, and H and the large transmembrane V\(_0\) subunit a. All subunits are required for a fully functional complex. The process of assembly and transport of the V-ATPase is a complex and multistep process requiring four endoplasmic reticulum-localized assembly factors (3, 5).

Subunit H is the only subunit of the V\(_1\) subcomplex that is not required for assembly and transport of the V-ATPase; however, subunit H is required for V-ATPase activation (6). Deleting the gene encoding subunit H (vma13Δ) in yeast results in an assembled V-ATPase complex that is trafficked to the vacuole but is inactive (6). In addition to its function to activate the fully assembled enzyme complex, subunit H is also needed to inhibit the hydrolase activity of the disassembled V\(_1\) subcomplex (7–9). The analysis of subunit H function in yeast has led to the model that subunit H plays an important role in the regulation of the activity of the enzyme complex.

Subunit H from yeast was the first V-ATPase subunit to have a high resolution crystal structure solved (10). The structure reveals that the protein is mainly \(\alpha\)-helical and contains two...
domains. The N-terminal domain (amino acids 1–352) consists of 17 consecutive α-helices that stack upon one another in a right-handed spiral to form a superhelix. The C-terminal domain (amino acids 353–478) has a similar overall structure to that of a right-handed superhelix. The C-terminal domain is required for activation of the V-ATPase, the subunit H per V-ATPase complex and that, whereas the N-terminal domain is sufficient to activate the ATP hydrolysis activity of the free V1-subcomplex through its interactions with the stalk subunit F (7).

Analysis of V-ATPase subunit H in mammals has revealed two isoforms of this protein, which have been termed SFD (subunit H2) and SFD-β (subunit H3). The two isoforms, SFD-α and SFD-β, arise from alternate splice forms of the mRNA, such that SFD-β lacks 18 amino acids present in SFD-α, corresponding to amino acids 178–195 of the yeast subunit H (Vma13p) (14, 15). Although both splice variant mRNAs have been found in brain extracts, only the SFD-α isoform was associated with the V-ATPase complex extracted from brain chromaffin granules (14, 16).

In this report, we further characterize the function of subunit H/Vma13p in yeast. We show that there is only one copy of subunit H per V-ATPase complex and that, whereas the N-terminal domain is required for activation of the V-ATPase, the first 180 amino acids are not required for activation. We also show that yeast Vma13p constructed to mimic the mammalian SFD-β splice variant activates the ATP hydrolysis activity, but the efficiency of coupling ATP hydrolysis to proton translocation is greatly diminished. In addition, we show that the last α-helix of the N-terminal domain is required for V-ATPase activation and function. Last, we show that the minimal length of Vma13p required for ATPase activation extends from amino acid 180 to 353 of the N-terminal domain.

**EXPERIMENTAL PROCEDURES**

Strains and Culture Conditions—The yeast strains and plasmids used in this study are listed in Table 1. A vma13Δ::Kan’ yeast strain, AFY28, was generated in the W303-1B background (ATCC) by methods outlined in Wach et al. (17). The PEP4 disruption, pep4Δ, was generated as outlined by Nothever et al. (18) to create yeast strain AFY29. Plasmids were transformed into yeast strains using a high efficiency lithium acetate protocol (19). All yeast strains were cultured in S.D. minimal medium (0.67% yeast nitrogen base, 2% dextrose) supplemented with the appropriate amino acids or YEPD medium buffered to pH 5.0 using 50 mM succinate/phosphate. To test for a Vma- phenotype, saturated cultures were diluted to A600 = 0.25. Serial dilutions of the diluted culture were applied to YEPD plus 100 mM CaCl2 media and allowed to grow overnight before observing results.

Plasmid Construction and Epitope Tagging—All restriction enzymes used were purchased from New England BioLabs Inc., whereas Platinum Pfx DNA polymerase was purchased from Invitrogen.

A 3.2-kb DNA fragment containing VMA13 was excised from plasmid pRH490 with BamHI and XhoI and ligated into pRS316 (20) digested with the same enzymes to create pLG67. PCR-based site-directed mutagenesis was used to introduce a HindIII site (tAAGCTT) after the stop codon of VMA13 in plasmid pLG67, and an EcoRI site (gGAAATTCatg) was introduced immediately before the start codon using PCR-based site-directed mutagenesis to create pAF226. To introduce a single c-Myc epitope on the N terminus of Vma13p, PCR was used to amplify a 1.5-kb VMA13 DNA fragment with a 5′-primer (encoding a EcoRI site before the start codon, a c-Myc epitope directly after the start codon, and a 20-bp sequence of the VMA13 ORF) and a 3′-primer encoding a HindIII site after the stop codon. After treating the 1.5-kb DNA fragment with EcoRI

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### Table 1

S. cerevisiae strains and plasmids utilized in this study

| Strain   | Genotype                           | Source          |
|----------|------------------------------------|-----------------|
| W303-1B  | MATα ura3-1 leu2-3,112 trp1-1 ade2-1 his3-11,15 can1-100 | ATCC            |
| AFY28    | MATα ura3-1 leu2-3,112 trp1-1 ade2-1 his3-11,15 can1-100 vma13Δ::Kan’ | This study      |
| AFY29    | MATα ura3-1 leu2-3,112 trp1-1 ade2-1 his3-11,15 can1-100 pep4Δ vma13Δ::Kan’ | This study      |

### Plasmid Description

| Plasmid | Description                           | Source          |
|---------|--------------------------------------|-----------------|
| pRS316  | Centromere-based, low copy plasmid (pCEN-URA) | Ref. 20         |
| pRS315  | Centromere-based, low copy plasmid (pCEN-LEU) | Ref. 20         |
| pH490   | 3.2-kb BamHI-Xhol DNA fragment containing VMA13 subcloned into pBlueScript | Gift from Anraku laboratory |
| pLG67   | 3.2-kb BamHI-Xhol DNA fragment containing VMA13 subcloned into pRS316 | This study      |
| pAF200  | VMA13 containing HindIII site after stop codon subcloned into pRS316 | This study      |
| pAF201  | VMA13 containing EcoRI site before start codon and a HindIII site after stop codon subcloned into pRS316 | This study      |
| pAF202  | VMA13 containing EcoRI site before start codon, a NruI site before the stop codon, and a HindIII site after stop codon subcloned into pRS316 | This study      |
| pAF202  | VMA13::HA subcloned into pRS316 | This study      |
| pAF281  | cMycc::VMA13 subcloned into pRS316 | This study      |
| pAF282  | cMycc::VMA13 subcloned into pRS415 | This study      |
| pAF293  | vma13::3×ΔHA Δ180–195 subcloned into pRS316 | This study      |
| pAF300  | vma13::3×ΔHA Δ1–179 subcloned into pRS316 | This study      |
| pAF303  | vma13::3×ΔHA Δ331–353 subcloned into pRS316 | This study      |
| pAF309  | vma13::3×ΔHA Δ2–306 subcloned into pRS316 | This study      |
| pAF302  | vma13::3×HA (180–353) subcloned into pRS316 | This study      |
and HindIII, it was subcloned into pAF226 using the same restriction sites to make plasmid pAF281 (c-Myc-VMA13). The 3.2-kb DNA fragment containing c-Myc-VMA13 was excised from pAF281 with XhoI and SacI and subcloned into pRS415 (20) utilizing the same restriction sites to generate plasmid pAF282.

Plasmid pAF279 was generated by first introducing a NruI site, via PCR-mediated site-directed mutagenesis, immediately before the stop codon of VMA13 in pAF226. Complementary oligonucleotides were synthesized so that when annealed together they formed an oligonucleotide duplex encoding a triple HA epitope flanked on both ends by NruI sites. The oligonucleotide duplex was incubated with NruI and subcloned into the NruI site generated in the modified pAF226 to yield a gene that encodes Vma13p with a 3× HA epitope tag on the C terminus (pAF279).

The truncated Vma13p mutants were generated by the following procedure. Forward primers were synthesized specifically for vma13Δ1–179, vma13Δ2–306, and vma13Δ2–353 (vma13-CT) by including an EcoRI site before the VMA13 ATG, the start codon, and 20–25 bases starting at the first codon after the specified deletion. PCR was performed using specific forward primers and 3′-primers that bind 30 bp after the stop codon with pAF279 as the template to generate fragments that encoded vma13Δ1–179, vma13Δ2–306, and vma13Δ2–352. The PCR fragment was incubated with EcoRI and HindIII, which was then subcloned into pAF279 that had also been incubated with EcoRI/HindIII to generate plasmids pAF290, pAF299, pAF301. To make the vma13Δ354–478 (vma13-N7) mutant, PCR fragments were generated using a 5′-primer that bound 20 bp from the start codon and a reverse deletion primer that contained 25 bases of the reverse complement corresponding to codons 346–353, an NruI site, stop codon, and HindIII site using pAF279 as the template. The PCR fragments were digested with EcoRI/HindIII and subcloned into pAF279 that had also been incubated with EcoRI/HindIII. The oligonucleotide duplex encoding the triple HA epitope was subcloned into the NruI site of the resulting plasmid to generate vma13Δ354–478/3×HA (pAF300).

To generate the plasmid encoding vma13Δ1–179,354–478 (vma13-(180–353)), the set of primers used to generate the vma13Δ1–179 construct was used with pAF300 as the template for the PCR. The resulting fragment was incubated with EcoRI/HindIII and subcloned into pAF279 with the same restriction sites to create pAF302.

To generate the internal deletion mutants, an inverse PCR strategy was used (21). Outward facing oligonucleotides were designed to delete base pairs encoding aa 180–195 from pAF279 to generate pAF293 or to delete base pairs encoding aa 331–352 from pAF279 to generate pAF303.

**Protein Preparation, SDS-PAGE, and Immunoblot Analysis**—Whole cell extracts and vacuolar membranes were prepared as described previously (22, 23). SDS-PAGE analysis was performed, and proteins were transferred to 0.2 μm nitrocellulose and probed with either an affinity-purified monoclonal anti-HA antibody (Covance Research Products Inc.) used at 1:3000 or the anti-Myc antibody 9E10 used at 1:5, as described previously (24). The monoclonal antibody 13D11, directed against Vma2p, was used at 1:1000 (Molecular Probes). Proteins were visualized using affinity-purified donkey anti-mouse secondary antibodies conjugated to horseradish peroxidase (1:20,000) (Jackson Laboratories) and chemiluminescence (Amersham Biosciences).

**Native Immunoprecipitation**—Immunoprecipitations were carried out as previously described (25). Briefly, 1 mg of C. _r_ E9 solubilized vacuolar membranes were precleared with the addition of 50 μl of a 50% slurry of Protein G-Sepharose 4 Fast Flow (Amersham Biosciences). Either 10 μl of affinity-purified rabbit anti-Myc antibodies (Santa Cruz Biotechnology) or 50 μl of rabbit anti-HA serum was added to the precleared supernatant and agitated at 4 °C for 1 h (26). 50 μl of a 50% slurry of protein G-Sepharose 4 fast flow was added and incubated at 4 °C for 1 h. The beads were collected with a brief centrifugation and washed three times with ice-cold phosphate-buffered saline plus 1% C12E9. After the washes were complete, the beads were resuspended in 200 μl of Thoriciner buffer (50 mm Tris, pH 6.8, 10% glycerol, 8 mm urea, 5% SDS, 5% β-mercaptoethanol) prior to SDS-PAGE analysis.

**Biochemical Methods**—For all biochemical assays, freshly prepared vacuolar membranes were prepared and used as previously described (23). The membrane protein content was analyzed by the modified Lowry protocol (27) and adjusted to final concentration of 1 mg of membrane protein/ml.

ATP hydrolysis was assayed by an enzyme-coupled assay, as previously described (23). Background ATPase activity, defined as activity determined in vacuolar membranes isolated from _vma13Δ_ yeast (AFY28), was subtracted from ATPase activities assayed from vacuolar membranes isolated from strains expressing wild type Vma13p or the mutant forms for each experiment. ATP-dependent proton translocation was measured in a Fluoromax fluorometer, as described previously (28), with the following modifications. Enriched vacuolar membranes (10 μg) were added to proton translocation buffer (20 mm HEPES, pH 7.0, 50 mm NaCl, 30 mm KCl, 5 mm MgSO4), which also contained 1 μm valinomycin and a 1 μm concentration of the fluorescent probe ACMA. Proton translocation was initiated with the addition of 2.5 mm ATP, and fluorescent quenching was monitored continuously until the proton gradient was collapsed, and fluorescence was recovered by the addition of 1 μm nigericin.

The acidification of yeast vacuoles was visualized using the lysosomotropic fluorescent dye quinacrine, as described previously (29). Briefly, log phase yeast cells (Λ400–1) were incubated in YEPD buffered to pH 7.6 with 100 mm HEPES in the presence of 200 μm quinacrine, and 50 μg/ml concanavalin A-TRITC (Invitrogen) was used to allow fluorescent visualization of the yeast cell surface. The cells were incubated at 30 °C for 10 min, after which they were cooled on ice for 5 min. The cells were washed twice with 100 mm HEPES, pH 7.6, containing 2% glucose. Finally, the cells were resuspended in wash buffer and immediately viewed. Images were acquired on a Zeiss Axioplan 2 fluorescence microscope using a ×100 objective and manipulated using AxioVision software (Zeiss).
Functional Dissection of the Vma13p N-terminal Domain

A)

| Protein expressed | Vma13p-HA | Vma13p-HA | myc-Vma13p |
|------------------|-----------|-----------|------------|
| Vma13p-HA        | S         | IP        | S          |
| myc-Vma13p       | S         | IP        | S          |
| Vma2p            | S         | IP        | S          |

B)

| Protein expressed | Vma13p-HA | Vma13p-HA | myc-Vma13p |
|------------------|-----------|-----------|------------|
| Vma13p-HA        | S         | IP        | S          |
| myc-Vma13p       | S         | IP        | S          |
| Vma2p            | S         | IP        | S          |

IP: HA

IP: myc

FIGURE 1. The assembled V-ATPase complex contains one copy of Vma13p. Enriched vacuolar membranes were isolated from yeast strain AFY28 (vma13Δ) containing either pAF282 (myc-VMA13), pAF279 (VMA13-HA), or both pAF282 and pAF279, as described under “Experimental Procedures.” The HA-tagged (A) or Myc-tagged (B) Vma13 proteins were immunoprecipitated using a polyclonal anti-HA or anti-Myc antibody from 1 mg of solubilized vacuoles. Protein from both the supernatant (S) and immunoprecipitated protein (IP) fractions were separated by SDS-PAGE, transferred to nitrocellulose, and probed with either anti-Myc, anti-HA, or anti-Vma2p antibodies.

RESULTS

The V-ATPase Complex Contains One Vma13p Subunit per Complex—To determine the number of Vma13p subunits per yeast V-ATPase complex, epitope-tagged copies of Vma13p were constructed and used in co-immunoprecipitation experiments. Vma13p was N-terminally tagged with the c-Myc epitope (Myc-Vma13p) or C-terminally tagged with the HA epitope (Vma13p-HA), and both epitope-tagged forms of Vma13p are fully functional. Plasmids encoding either a copy of Myc-Vma13p, a copy of Vma13p-HA, or both Myc-Vma13p and Vma13p-HA were transformed into vma13Δ yeast cells. Expression of epitope-tagged Vma13p was tested by immunoblot analysis of whole cell lysates using an antibody generated against either the c-Myc or HA epitope. Both epitope-tagged proteins were detected and migrated at the predicted size. Comparison of the steady-state levels of Myc-Vma13p and Vma13p-HA using an antibody that recognizes Vma13p showed that the cellular levels of Vma13p were the same independent of the epitope tag (data not shown).

Vacuoles were isolated from vma13Δ cells expressing epitope-tagged copies of Vma13p, and the membranes were solubilized with 1% C12E9 for co-immunoprecipitations. Native immunoprecipitations were performed on the solubilized vacuoles with antibodies recognizing the HA epitope (Fig. 1A) or the Myc epitope (Fig. 1B). The immunoprecipitated samples were subjected to SDS-PAGE and probed with antibodies against the HA epitope (Fig. 1A) or the Myc epitope (Fig. 1B) to determine if Vma13p was immunoprecipitated. Fig. 1A shows that Vma13p was immunoprecipitated with anti-HA antibodies from strains expressing only Vma13p-HA or both Vma13p-HA and Myc-Vma13p but not from strains only expressing Myc-Vma13p. The immunoprecipitated samples were also probed for the presence of the c-Myc epitope. Fig. 1A shows that Myc-Vma13p did not co-immunoprecipitate with HA-Vma13p from yeast expressing both Myc-Vma13p and HA-Vma13p. These results were independent of the epitope chosen for immunoprecipitation, as demonstrated by the results displayed in Fig. 1B.

To demonstrate that both the Myc-Vma13p and Vma13p-HA were associated with V-ATPase complexes, the immunoprecipitations were probed with antibodies specific to Vma2p, another V1 subunit. Fig. 1 shows that most of the Vma2p co-immunoprecipitated with the epitope-tagged Vma13p. As expected, when immunoprecipitated with anti-HA antibodies (Fig. 1A), all of the Vma2p was present in the supernatant lane from yeast expressing only the Myc-Vma13 polypeptide, and a significant portion (~50%) of Vma2p was present in the supernatant lane from immunoprecipitations of yeast expressing both Myc-Vma13p and HA-Vma13p. Again, these results were independent of the epitope chosen for immunoprecipitation, as demonstrated in Fig. 1B. These observations are consistent with the presence of two populations of the V-ATPase, one form containing a single copy of HA-Vma13p and the other containing one copy of Myc-Vma13p. We conclude from these data that there is only one copy of the Vma13p subunit per V-ATPase complex in yeast.

The V-ATPase Complex with Vma13p Mimicking the Mammalian Subunit H Splice Form β Is Uncoupled—Subunit H exists as two isoforms in mammals, SFD-α and SFD-β, resulting from alternative splicing (15, 16), whereas in yeast, there is only one form of subunit H (Vma13p) (6). The alternate splice form SFD-β results in an internal deletion of 18 amino acids (aa 176–194; Fig. 2) compared with the full-length SFD-α form. An alignment of the protein sequences from the two mammalian subunit H isoforms with the yeast Vma13p sequence shows that the internal deletion corresponds to amino acids 178–195 of Vma13p (Fig. 2B). Zhou et al. (15, 16) reported that the two mammalian isoforms of subunit H activated the mammalian V-ATPase similarly for both Mg2⁺ ATPase activity and proton pumping. However, due to the limitation of the system, it was not possible to test the in vivo functions of the two subunit H splice isoforms.

To determine if an equivalent Vma13p β splice form is functional, an internal deletion mutant was created by inverse PCR to delete the coding sequence for amino acid residues 180–195 (vma13Δ180–195) (Fig. 2B). The plasmid coding for Vma13Δ180–195p was transformed into vma13Δ yeast and checked for complementation. Fig. 3A shows that yeast expressing Vma13Δ180–195p grew on media containing 100 mM CaCl₂, which indicates that the V-ATPase is assembled and
However, \textit{vma13}/H9004 180–195 yeast did not grow as well as \textit{VMA13} wild type yeast cells. Because yeast cells possessing as little as 25% of wild type V-ATPase activity do not exhibit a Vma phenotype (31, 32), a more sensitive method was used to assess V-ATPase function.

A vital vacuolar staining dye, quinacrine, was used to test the acidification state of vacuoles in cells expressing either Vma13p or Vma13/H9004 180–195p. Quinacrine is a weakly basic fluorescent dye that accumulates in low pH compartments within the cell, such as the yeast vacuole (22). Fluorescence microscopy shows that yeast cells with a functional V-ATPase take up quinacrine and have brightly fluorescent vacuoles, whereas cells with a compromised or dysfunctional V-ATPase have weakly fluorescent to nonfluorescent vacuoles. Cells expressing wild type Vma13p contained fluorescent vacuoles, displayed as green disks within the yeast cell (outlined in red using concanavalin A-TRITC), whereas cells expressing Vma13Δ180–195p had a lower level of fluorescence as compared with wild type (Fig. 3B). Taken together with the results of reduced growth on media containing 100 mM CaCl$_2$, the reduced levels of quinacrine staining in \textit{vma13}Δ180–195 yeast indicates that the V-ATPase complex in these cells is not fully functional.

To investigate the basis for the reduced acidification, biochemical assays were conducted on isolated vacuoles from yeast expressing Vma13Δ180–195p. Western analysis with an anti-HA antibody indicated that the levels of the mutant protein present in isolated vacuoles were similar to the levels of vacuole-associated Vma13p in wild type cells (Fig. 3C). An immunoblot with an antibody specific to the V$_1$ subunit Vma1p indicates that the samples were equally loaded and the ratio of wild type Vma13p and Vma13Δ180–195p remained approximately the same (Fig. 3C). This result indicates that Vma13Δ180–195p binds to the vacuole-associated V-ATPase just as well as wild type Vma13p, suggesting that the lower level of vacuolar acidification must be attributed to a less functional
Functional Dissection of the Vma13p N-terminal Domain

TABLE 2
ATPase activity from vacuolar membranes

ATPase activity was assayed on 10 μg of vacuolar membrane protein as described under “Experimental Procedures.” The percentage of wild type (WT) activity is defined as the ratio of ATPase activity seen in isolated vacuolar membranes with that of the ATPase activity determined from vacuolar membranes isolated from yeast expressing wild type Vma13p. Background ATPase activity in vacuolar membranes isolated from vma13Δ yeast (AFY28) was subtracted from ATPase activities assayed from vacuolar membranes isolated from strains expressing wild type Vma13p or the mutant forms for each experiment. The percentage of inhibition by concanamycin A was measured in the presence of either 100 mM concanamycin or 50 μM N-ethylmaleimide. Vacuolar membranes from strains expressing Vma13Δ331–353p or Vma13Δ2–306p had no detectable ATPase activity.

| Genotype         | ATPase activity* | WT activity | Average inhibition by concanamycin A* | Average inhibition by N-ethylmaleimide* |
|------------------|------------------|-------------|--------------------------------------|---------------------------------------|
|                  | μmol/mg/mg       | %           | %                                    | %                                     |
| VMA13            | 0.86 ± 0.12      | 100         | 76                                   | 82                                    |
| vma13Δ180–195    | 0.45 ± 0.09      | 52          | 53                                   | 73                                    |
| vma13Δ1–179      | 0.79 ± 0.08      | 92          | 72                                   | 80                                    |
| vma13-NT         | 0.39 ± 0.06      | 45          | 29                                   | 62                                    |
| vma13(180–353)   | 0.28 ± 0.10      | 32          | 23                                   | 68                                    |

* n = 6.

V-ATPase complex. ATPase activity assays revealed that vacuoles isolated from yeast expressing Vma13Δ180–195p had 52% of wild type ATPase activity (Table 2). This high level of ATPase activity for the Vma13Δ180–195p V-ATPase seems inconsistent with the growth and acidification phenotypes of vma13Δ180–195 yeast cells, since previous reports indicate that yeast cells exhibiting a Vma− phenotype typically have <25% of wild type V-ATPase activity (31, 32).

To determine if the decreased acidification of the vma13Δ180–195 vacuoles was due to a lower rate of proton pumping (22), we assayed proton translocation using an assay that measures acidification-dependent fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA). Fig. 4 shows V-ATPase activities and ACMA quenching for the vacuoles isolated from the various yeast strains. As expected, wild type vacuoles exhibited strong ACMA fluorescence quenching upon ATP addition, which was reversed upon the addition of the proton ionophore, nigericin. However, ACMA quenching for vma13Δ180–195 vacuoles was greatly reduced as compared with wild type vacuoles. By calculating the initial rate of proton pumping and the ATPase activity, we can estimate a relative coupling ratio for the V-ATPase associated with the isolated vacuole membranes (12). The coupling ratio for the V-ATPase containing Vma13Δ180–195p was only ~40% of the coupling ratio for the wild type V-ATPase. These results indicate that deletion of residues 180–195 of yeast subunit H results in a V-ATPase in which the ATP catalytic activity is poorly coupled to proton pumping. Since yeast Vma13Δ180–195p was constructed to mimic the mammalian subunit H β splice variant, these results suggest that the mammalian V-ATPase complex with the two different subunit H splice variants (SFD-α and SFD-β) may have distinctly different activities and cellular functions.

Interestingly, deletion of subunit H residues 180–195 produces the same effect as deleting the entire C terminus (vma13-NT). Liu et al. (12) reported that deletion of the subunit H C-terminal domain (Vma13-NT) produced a V-ATPase complex in which the ATPase activity is largely uncoupled from proton pumping. When the two mutants, vma13Δ180–195 and vma13-NT, were compared side by side, the yeast behaved almost identically in growth (Fig. 3A) and quinacrine uptake (Fig. 3B) as well as the ATPase activities and proton pumping from isolated vacuolar vesicles (Fig. 4). The Vma13-NT protein is missing the entire C-terminal domain, whereas Vma13Δ180–195p is only missing the amino acids making up an internal helix in the N-terminal domain (Fig. 2). The drastic consequences of deleting residues 180–195 in subunit H could be the result of misalignment of the C-terminal domain relative to the N-terminal domain, resulting in the inability of subunit H to confer normal coupling of the ATPase activity to proton pumping.

The First 179 Amino Acids of Vma13p Are Not Required for Function—To further investigate the function of the Vma13p N-terminal domain, we generated a form of Vma13p lacking amino acids 1–179 (Vma13Δ1–179p; Fig. 2). The plasmid encoding Vma13Δ1–179p was transformed into vma13Δ yeast and checked for complementation. Fig. 5A shows that vma13Δ1–179 yeast grew on media containing 100 mM CaCl₂ and the growth was indistinguishable from wild type yeast, indicating that expression of the mutant protein resulted in a fully functional V-ATPase complex. vma13Δ1–179 yeast exhibited normal vacuole acidification, as revealed by normal levels of quinacrine uptake (Fig. 5B). In addition, Western analysis revealed that the expression level of Vma13Δ1–179p was very similar to that of the wild type Vma13p, and the approximate ratio to the V₁ Vma1p remained the same (Fig. 5C). Finally, biochemical analysis of vma13Δ1–179 vacuoles showed that the V-ATPase activity and proton pumping was similar to vacuoles isolated from yeast expressing the full-length Vma13 protein (Fig. 4 and Table 2). From these data, we conclude that the first 179 amino acids of yeast subunit H are not required for a fully functional and coupled V-ATPase.

The Last Helix of the N-terminal Domain of Vma13p Is Required for Function—To further investigate the role of Vma13p in V-ATPase coupling efficiency, we deleted the helix that connects the N-terminal and C-terminal domains (Fig. 2, helix shown in red). The sequence encoding the last helix in the N-terminal domain (amino acids 331–353) was removed by inverse PCR, creating vma13Δ331–353. This helix is predicted to orient the C-terminal domain relative to the N-terminal domain. The plasmid coding for Vma13Δ331–353p was transformed into vma13Δ yeast and checked for complementation. vma13Δ331–353 yeast failed to grow on media containing CaCl₂, and these cells did not accumulate quinacrine in their vacuoles (Fig. 6, A and B), indicating that the V-ATPase con-
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V-ATPase activity and proton pumping in vacuole membranes isolated from yeast with various forms of Vma13p. Enriched vacuolar membranes were isolated from yeast strain AFY28 (vma13Δ) containing either pAF279 (VMA13), pAF290 (vma13Δ1–179), pAF293 (vma13Δ180–195), pAF300 (vma13-NT), or pRS316 (empty vector), as described under “Experimental Procedures.” A, 10 μg of vacuolar membranes were assayed for total ATPase activity as described under “Experimental Procedures.” The percentage of wild type activity (% WT activity) is defined as the ratio of ATPase activity seen in isolated vacuolar membranes with that of the ATPase activity determined from vacuolar membranes isolated from yeast expressing wild type Vma13p. B, strains used in A were stained with quinacrine and concanavalin A-TRITC as described under “Experimental Procedures.” The panels on the left show quinacrine depicted in green and concanavalin A-TRITC depicted in red. The panels on the right in B show cell morphology as viewed by Nomarski optics. C, immunoblot analysis of vacuoles isolated from strains described in A. 10 μg of protein were separated by SDS-PAGE, transferred to nitrocellulose, and probed with either anti-HA antibodies specific to the HA epitope present on the wild type and mutant Vma13p or antibodies specific to Vma1p.

Amino acids 1–179 of Vma13p are not required for function. A, a serial dilution of strain AFY29 (vma13Δ) carrying either plasmid pAF279 (VMA13), pRS316 (empty vector), or pAF290 (vma13Δ1–179) grown on YEPD, pH 5.0, or YEPD plus 100 mM CaCl2 to test for a Vma13p phenotype. B, strains used in A were stained with quinacrine and concanavalin A-TRITC as described under “Experimental Procedures.” The panels on the left show quinacrine depicted in green and concanavalin A-TRITC depicted in red. The panels on the right in B show cell morphology as viewed by Nomarski optics. C, immunoblot analysis of vacuoles isolated from strains described in A. 10 μg of protein were separated by SDS-PAGE, transferred to nitrocellulose, and probed with either anti-HA antibodies specific to the HA epitope present on the wild type and mutant Vma13p or antibodies specific to Vma1p.

Amino acids 331–353 of Vma13p are required for function. A, a serial dilution of strain AFY29 (vma13Δ) carrying either plasmid pAF279 (VMA13), pRS316 (empty vector), or pAF303 (vma13Δ331–353) grown on YEPD, pH 5.0, or YEPD plus 100 mM CaCl2 to test for a Vma13p phenotype. B, strains used in A were stained with quinacrine and concanavalin A-TRITC as described under “Experimental Procedures.” The panels on the left show quinacrine depicted in green and concanavalin A-TRITC depicted in red. The panels on the right in B show cell morphology as viewed by Nomarski optics. C, immunoblot analysis of vacuoles isolated from strains described in A. 10 μg of protein were separated by SDS-PAGE, transferred to nitrocellulose, and probed with either anti-HA antibodies specific to the HA epitope present on the wild type and mutant Vma13p or antibodies specific to Vma1p.

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353p (Table 2). We conclude that Vma13Δ331–353p binds to the V-ATPase complex but fails to activate its enzyme activity.

The ARM6 N-terminal Domain Facilitates Binding of the C-terminal Domain to the V-ATPase—To further test the function of the Vma13p interdomain helix (amino acids 331–353), we constructed a mutant that contained the last armadillo motif of the N-terminal domain (ARM6) (10) attached to the C-terminal domain (Vma13Δ2–306p; see Fig. 2). Yeast expressing Vma13Δ2–306p failed to grow on media containing 100 mM CaCl2 (Fig. 7A), and the vacuoles from these yeast failed to accumulate quinacrine dye (data not shown), indicating that the vacuoles were not acidified. Despite the lack of V-ATPase function, Western analysis revealed that Vma13Δ2–306p was expressed at the same relative level as wild type Vma13p (Fig. 7B).

Liu et al. (12) reported that Vma13p lacking the N-terminal domain (Vma13-CT) bound and activated the V-ATPase complex very poorly. To directly compare V-ATPase binding/activation of the Vma13-CT protein with our Vma13Δ2–306p protein, we isolated vacuoles from yeast expressing either wild type Vma13p, Vma13Δ2–306p, or Vma13-CT, and these proteins were analyzed by Western blotting. Fig. 7B shows that Vma13Δ2–306p associated with vacuolar membranes to the same extent as wild type Vma13p, whereas Vma13-CT associated with vacuole membranes at very reduced levels. Interestingly, these results indicate that whereas the Vma13p C-terminal domain with the ARM6 region (Vma13Δ2–306p) associates with the V-ATPase complex, it is unable to activate the V-ATPase.

Vma13 Amino Acids 180–353 Constitute the Minimal Region Required for V-ATPase Activation—The results reported above suggest that the region of Vma13p required for activation of the V-ATPase complex might be amino acids 180–353. To test this hypothesis, we constructed a mutant form of Vma13p that contains this minimum region (Vma13-(180–353)).
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(180–353)p. Yeast expressing Vma13-(180–353)p showed growth similar to yeast expressing Vma13-NT (Fig. 8A), and quinacrine uptake indicated that vma13-(180–353) vacuoles were acidified (Fig. 8B). However, the level of quinacrine accumulation appeared to be slightly reduced relative to that of yeast expressing the entire Vma13 N-terminal domain (Vma13-NT). Western analysis revealed that Vma13-(180–353)p was expressed at the same level as Vma13-NT (Fig. 8C). From these data, we conclude that amino acid residues 180–353 constitute the minimal sequence of Vma13p needed for activation of the V-ATPase.

DISCUSSION

Subunit H is a unique protein in the V-ATPase complex in that it is the only subunit required for activation but not trafficking or assembly of the V-ATPase complex (1). Previous studies have revealed a critical role for subunit H in regulating the V-ATPase complex activity and in coupling ATP hydrolysis to proton pumping (3, 7, 9, 12). In this paper, we have investigated further the role of subunit H/Vma13p in the regulation of the yeast V-ATPase complex and in doing so have identified a novel mechanism by which the mammalian V-ATPase complex may be regulated.

The first goal of the study was to define the stoichiometry of subunit H in the yeast V-ATPase complex, motivated in part by the discovery of two splice isoforms of subunit H in mammals (15). Our results demonstrate that there is only a single copy of Vma13p in the assembled V-ATPase complex. Our findings agree with recent results from mass spectrometry, which suggested that there is likely to be only a single copy of subunit H per yeast V-ATPase complex (33). Given the strong similarity between V-ATPase complexes from yeast and humans (3), we predict that there is a single copy of subunit H per V-ATPase complex in organisms as diverse as yeast and humans.

The mammalian homologue of subunit H has been shown to be expressed as two splice variants, SFD-α and SFD-β (15). The differential splicing results in an internal deletion of 18 amino acids, where the SFD-α is the full-length version of the molecule and SFD-β contains the internal deletion. The role of these two isoforms in regulating the activity of the mammalian V-ATPase remains to be determined. Yeasts contain just the full-length form of subunit H corresponding to SFD-α, whereas the SFD-β form would correspond to an internal deletion of amino acid residues 179–195 in yeast Vma13p (6, 10). We have shown that the yeast subunit H with the internal deletion that mimics SFD-β, Vma13Δ180–195p, is capable of activating the yeast V-ATPase. Yeast expressing Vma13Δ180–195p exhibited slight growth defects, and acidification of the vacuoles in these cells was somewhat compromised. Furthermore, when the activity of the V-ATPase was examined by biochemical methods, the complexes containing Vma13Δ180–195p had slightly lower ATPase activity but significantly diminished proton pumping activity compared with the wild type V-ATPase.

The effects of deleting amino acids 180–195 from yeast subunit H can be understood in the context of two different models. First, the crystal structure of yeast subunit H shows that the N-terminal peptide folds back and interacts with a shallow groove formed by the N-terminal domain (10). The 180–195 deletion removes some of the residues that interact with the N-terminal peptide, and it is possible that interaction of the N-terminal peptide is important for subunit H regulation of the V-ATPase complex. Alternatively, removing the α-helix encoded by amino acids 180–195 could result in misalignment of the C-terminal domain, which has been shown to be important for coupling ATP hydrolysis to proton pumping (12). In support of the latter model, we found that the V-ATPase complex with Vma13Δ180–195p was uncoupled to the same extent as Vma13p lacking the entire C-terminal domain (Vma13-NT) (12) (Table 2 and Fig. 4). Since Vma13p is a superhelix composed of α-helices stacked upon each other in angles varying from 18 to 33° (10), removing a complete α-helix from the molecule would be predicted to result in twisting the C-terminal domain relative to the N-terminal domain. Therefore, removing the α-helix encoded by amino acids 180–195 could result in misaligning the C-terminal domain such that the Vma13Δ180–195p is no longer able to couple the ATPase activity with proton translocation. These results suggest that the mammalian V-ATPase complexes with subunit H splice variants SFD-α or SFD-β are likely to have different activities and may perform distinct cellular functions. This hypothesis awaits an analysis of the effects of the selective expression of the two subunit H splice variants in cell culture.

To test whether the entire subunit H N-terminal domain is required for activation of the V-ATPase complex, we deleted the first half of this domain (Vma13Δ1–179p). Vma13Δ1–179p was found to be fully functional; vma13Δ1–179 yeast exhibited no growth defects, their vacuoles were acidified normally, and the Vma13Δ1–179p-containing V-ATPase had normal ATPase activity that was fully coupled to proton translocation. Therefore, whereas the subunit H N-terminal domain is required for V-ATPase activation (12), our results indicate that the first half of this domain is not required for this activation.

To further examine the relationship between the subunit H N-terminal and C-terminal domains in regulating the V-ATPase, we deleted the helix that connects these domains (aa 331–353). Removing this helix should not disrupt the groove formed by the N-terminal domain but would be predicted to alter the positioning of the C-terminal domain relative to the N-terminal domain. Although this mutant subunit H (Vma13Δ331–353p) was stable and associated with the vacuolar V-ATPase complex, it failed to activate the enzyme. These results support the notion that the positioning of the C-terminal domain relative to the N-terminal domain in subunit H is very important for activation and coupling of the V-ATPase.

Liu et al. (12) reported that the subunit H C-terminal domain is required for coupling proton translocation to ATP hydrolysis. They found that Vma13-CT activated the ATPase activity to a very low extent (<2%). We report here that the addition of the last superhelix of the subunit H N-terminal domain (aa 306–353; the ARM6 repeat) dramatically increased the binding of the C-terminal domain to the vacuolar V-ATPase. Whereas the addition of the ARM6 repeat dramatically improved binding, the Vma13Δ2–306p protein did not activate V-ATPase activity. These results emphasize the importance of other regions of the subunit H N-terminal domain in activating the V-ATPase complex.
To investigate the minimal region of Vma13p required for activation of the V-ATPase complex, we deleted the entire C-terminal domain and the first 179 amino acids of the N-terminal domain. The subunit H fragment containing just amino acids 180–353 (Vma13-(180–353)p) activated the V-ATPase activity as well as the entire N-terminal domain (Vma13-NT) and, like Vma13-NT, the Vma13-(180–353)p-associated V-ATPase was significantly uncoupled. However, both Vma13-NT and Vma13-(180–353)p were sufficiently coupled to allow at least partial acidification of the vacuole in vivo, as revealed by quinacrine accumulation and growth on media containing CaCl₂. From these data, we conclude that residues 180–353 are the minimal portion of Vma13p required for binding to and activation of the V-ATPase complex.

In summary, our results together with the work of Liu et al. (12) lead to a detailed model for the activation and coupling of the V-ATPase by subunit H. The subunit H C-terminal domain is required for efficient coupling of ATP hydrolysis and proton translocation. It is also clear that the subunit H N-terminal domain is required for productive binding of subunit H to the V-ATPase and that it is the second half of this domain that is largely responsible for binding. In addition, our studies suggest that the alignment of the subunit H N-terminal and C-terminal domains is critically important for the coupling of the V-ATPase. Finally, these results have important implications for the possible regulation of the V-ATPase by the SFD-α and SFD-β splice variants of subunit H in mammalian cells.

Acknowledgments—We thank Emily Coonrod and Laurie Graham for careful reading of the manuscript and all of the members of the Stevens laboratory for helpful discussions.

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