Novel Vacuolar \(H^{+}\)-ATPase Complexes Resulting from Overproduction of Vma5p and Vma13p*

Received for publication, August 13, 2001, and in revised form, November 1, 2001
Published, JBC Papers in Press, November 20, 2001, DOI 10.1074/jbc.M107777200

Kelly Keenan Curtis and Patricia M. Kane‡
From the Department of Biochemistry and Molecular Biology, State University of New York, Upstate Medical University, Syracuse, New York 13210

The vacuolar \(H^{+}\)-ATPase (V-ATPase) is a multisubunit complex composed of two sectors: \(V_{1}\), a peripheral membrane sector responsible for ATP hydrolysis, and \(V_{0}\), an integral membrane sector that forms a proton pore. Vma5p and Vma13p are \(V_{1}\) sector subunits that have been implicated in the structural and functional coupling of the V-ATPase. Cells overexpressing Vma5p and Vma13p demonstrate a classic Vma+ growth phenotype. Closer biochemical examination of Vma13p-overproducing strains revealed a functionally uncoupled V-ATPase in vacuolar vesicles. The ATP hydrolysis rate was 72% of the wild-type rate; but there was no proton translocation, and two \(V_{1}\) subunits (Vma4p and Vma8p) were present at lower levels. Vma5p overproduction moderately affected both V-ATPase activity and proton translocation without affecting enzyme assembly. High level overexpression of Vma5p and Vma13p was lethal even in wild-type cells. In the absence of an intact \(V_{0}\) sector, overproduction of Vma5p and Vma13p had a more detrimental effect on growth than their deletion. Overproduced Vma5p associated with cytosolic \(V_{1}\) complexes; this association may cause the lethality.

The vacuolar \(H^{+}\)-ATPase (V-ATPase)\(^1\) is an electrogenic proton pump found throughout the endomembrane system (1, 2). The V-ATPase harnesses the energy derived from ATP hydrolysis to pump protons across membranes, creating an electrochemical gradient. V-ATPases share both sequence and structural similarities with F-type ATP synthases, and preliminary experiments indicate a fundamental similarity in their mechanisms (3). Few specific details about how V-ATPases couple ATP hydrolysis and proton transport are available, however, because the sequence similarities with F-ATPases lie primarily in the ATP-binding and proton pore subunits, not in the “stalk” subunits believed to be predominantly responsible for structural and functional coupling (4).

The Saccharomyces cerevisiae V-ATPase has been extensively studied and has proven to be an excellent model for all eukaryotic V-ATPases. To date, 13 subunits have been identified and assigned to either the peripheral cytoplasmic \(V_{1}\) sector or the membrane-associated \(V_{0}\) sector; however, a limited number of subunits have assigned function. The \(V_{1}\) sector subunit Vma1p contains the catalytic nucleotide-binding sites, whereas Vma2p possesses regulatory nucleotide-binding sites (2, 3). The \(V_{0}\) sector proteolipid subunits Vma3p, Vma11p, and Vma16p are thought to form the proton pore, probably in combination with the large integral membrane subunit Vph1p (5–7). The functions of the remaining subunits are poorly understood.

Vma1p and Vma2p are present in three copies per complex and are believed to reside within the bulky head domain seen in low resolution electron microscopic studies of the V-ATPase (8). The head domain appears to be attached to the membrane via two stalk regions. The composition of the stalk regions is unknown, but it is hypothesized that they may contain most of the subunits of undefined function and that these subunits could be involved in coupling the V-ATPase both structurally and functionally. The V-ATPase is a tightly coupled enzyme that only exhibits activity when the enzyme is fully assembled with all subunits at the membrane. When cells are deprived of glucose, \(V_{1}\) sectors disassemble from the membrane (9, 10). Disassembly results in the silencing of both sectors: \(V_{1}\) is inactive as an Mg-ATPase, and the \(V_{0}\) proton pore is closed (11–14). The free \(V_{1}\) and \(V_{0}\) sectors can be rapidly reassembled to form functional V-ATPase complexes upon glucose re-addition. This is thought to be a form of regulation of the V-ATPase that may assure that cytosolic ATP stores are conserved in times of starvation (15, 16).

Results from a variety of investigations have led to the hypothesis that the C and H subunits of the \(V_{1}\) sector (Vma5p and Vma13p, respectively) are instrumental in structural and functional coupling of the V-ATPase. When VMA5 is deleted from the yeast genome, both a fully assembled \(V_{0}\) subcomplex and a core \(V_{1}\) subcomplex are formed, whereas in most other \(V_{1}\) subunit deletion strains, the \(V_{1}\) core complex is fully or partially disassembled (17, 18). Glucose deprivation studies have shown that Vma5p is lost from both sectors during disassembly, suggesting that Vma5p may be directly involved in signaling \(V_{1}\) release from the membrane (9, 14). When VMA13 is deleted, the V-ATPase does assemble, but is inactive, indicating that Vma13p is an activator or a structural stabilizer (19). Vma13p is found in cytosolic \(V_{1}\) complexes formed in the absence of an intact \(V_{0}\) sector or released from the membrane by glucose deprivation (14). \(V_{1}\) sectors from a vma13Δ strain contain all of the other \(V_{1}\) subunits, but unlike cytosolic \(V_{1}\) complexes from wild-type cells, are active as Mg-ATPases (14). This suggests a more complex role for Vma13p as a regulator of the V-ATPase, in which it silences cytosolic \(V_{1}\), but activates assembled \(V_{1}\) \(V_{0}\) complexes. If Vma5p and Vma13p are involved in tightly coupling and regulating the V-ATPase, the levels of subunit within a cell could be important in V-ATPase function.
Overexpression of Yeast VMA13 and VMA5

**Experimental Procedures**

**Materials and Strains**—Molecular biology reagents were purchased from New England Biolabs Inc. LA-Tag was obtained from Panvera. Zymolyase 100T and Trans'N-Taq dye were from ICN. Dithiobis(succinimidyldimethylpropionate) was purchased from Pierce, and concanamycin A was purchased from Wako Bioproducts. N-Octyl β-D-glucopyranoside was purchased from Calbiochem. Precast 16% Tris-Tricine gels and a Mono-Q 1 column ion-exchange column were purchased from Bio-Rad. Monoclonal antibody 9E10 (anti-Myc epitope) was purchased from Santa Cruz Biotechnology. Alkaline phosphatase-conjugated goat anti-mouse and goat anti-rabbit antibodies were obtained from Promega. Oligonucleotides were purchased from MWG Biotech. All other chemicals were purchased from Sigma.

The yeast strains used in this study include wild-type strains SF838-5A and SF838-1Ds (17) and mutant strains SF838-1Dmsu5Δ (22), SF838-5Amsu5Δ (17), and YPH500msu5Δ (17). The W303 msu5Δ strain was constructed by one-step gene replacement using a msu5Δ::LEU2 disruption allele described previously (21). Media for growth of yeast strains were prepared as described by Sherman et al. (20) and in the laboratory of Fink et al. (21).

**Plasmid Constructions and Transformations**—To obtain the 2μ-VMA5 plasmid, VMA5 was subcloned from pRS316-VMA5 (22) into YEp352 (23) using XhoI and KpnI to release the VMA5 insert and to cleave the 2μ vector. To obtain the 2μ plasmids containing VMA13 and N-Myc-VMA13, VMA13 and N-Myc-VMA13 were subcloned from the corresponding pRS316 plasmids (24) into YEp352 using SacI and KpnI. To create flanking XhoI sites for insertion of VMA5 into the leu2-d vector, the VMA5 gene was amplified by LA-Tag polymerase using pRS316-VMA5 as a template and the following primers: 5′-GGCTC-GAGCCTTCAAGGAGATTG (primer 1) and 5′-GCGTACCTCA-CAGTTATTATCTTATTG (primer 2). VMA13 was amplified similarly using pRS316-VMA5 as a template and the following primers: 5′-GCTCTG-GAGCCTTCAAGGAGATTG (primer 1) and 5′-GCGTACCTCA-CAGTTATTATCTTATTG (primer 2). VMA13 was amplified similarly using pRS316-VMA3 as a template and the following primers: 5′-GGCTC-GAGCCTTCAAGGAGATTG (primer 1) and 5′-GCGTACCTCA-CAGTTATTATCTTATTG (primer 2). VMA13 was amplified similarly using pRS316-VMA5 as a template and the following primers: 5′-GGCTC-GAGCCTTCAAGGAGATTG (primer 1) and 5′-GCGTACCTCA-CAGTTATTATCTTATTG (primer 2). VMA13 was amplified similarly using pRS316-VMA5 as a template and the following primers: 5′-GGCTC-GAGCCTTCAAGGAGATTG (primer 1) and 5′-GCGTACCTCA-CAGTTATTATCTTATTG (primer 2). VMA13 was amplified similarly using pRS316-VMA5 as a template and the following primers: 5′-GGCTC-GAGCCTTCAAGGAGATTG (primer 1) and 5′-GCGTACCTCA-CAGTTATTATCTTATTG (primer 2). VMA13 was amplified similarly using pRS316-VMA5 as a template and the following primers: 5′-GGCTC-GAGCCTTCAAGGAGATTG (primer 1) and 5′-GCGTACCTCA-CAGTTATTATCTTATTG (primer 2). VMA13 was amplified similarly using pRS316-VMA5 as a template and the following primers: 5′-GGCTC-GAGCCTTCAAGGAGATTG (primer 1) and 5′-GCGTACCTCA-CAGTTATTATCTTATTG (primer 2). VMA13 was amplified similarly using pRS316-VMA5 as a template and the following primers:

2 pnotes and vacuolar vesicles were suspended in cracking buffer (50 mM Tris-HCl (pH 6.8), 8 M urea, 5% SDS, and 5% β-mercaptoethanol) and subjected to SDS-PAGE, followed by immunoblotting. A titration was determined to determine a linear range of vacuolar vesicle load for each subunit antibody. Vph1p, Vma1p, Vma2p, and Vma5p, were detected using monoclonal antibodies 10D7, 8B1, 3D11, and 7A2, respectively (30). The Myc-tagged Vma13p subunit was detected using monoclonal antibody 9E10. Polyclonal antisera against the Vma4p and Vma8p V1 subunits were a generous gift from Tom Stevens. Binding of the antibodies was detected using alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies, followed by colorimetric development.

Immunoprecipitations from isolated vacuolar vesicles were executed as described previously (12). Immunoprecipitations were analyzed by SDS-PAGE, followed by silver staining (31). Biosynthetic labeling and non-denaturing immunoprecipitations from whole spheroplasts were performed as described (9) and analyzed on a Molecular Dynamics Storm PhosphorImager.

Growth of cells carrying different plasmids was monitored by two different methods. To obtain doubling times of liquid cultures, fresh transformants were diluted into fresh SD-Ura medium and shaken at 30 °C, and the density of the culture was measured with time by measuring the absorbance at 600 nm. Alternatively, freshly transformed strains were grown overnight in SD-Ura medium to log phase, and then cells were diluted in water to 1.0 A600/ml. Serial dilutions were made in water, and equal volumes of cells were transferred to plates for growth at 30 °C.

**Cytosolic V1 sectors were isolated from strains as described by Parra et al. (14) with the following modifications. The vma11Δ strain was transformed with the 2μ-VMA5 plasmid and grown overnight in SD-Ura medium. 2 liters of cells at a density of 6.5 × 106/ml were combined. 2-ml fractions were collected from the Mono-Q 1 column and precipitated overnight on ice using 10% trichloroacetic acid. Precipitated fractions were analyzed for the presence of several V1 subunits by immunoblotting as described above.

**Results**

**Overproduction of Vma5p and Vma13p**—To examine the effects of their overexpression, VMA5 and VMA13 were each subcloned into a multicopy (2μ) plasmid and transformed into the corresponding vma5Δ and vma13Δ strains, which lack the endogenous copies of the genes. Whole cell lysates were prepared from cells containing the genes expressed from multicopy plasmids and from the same mutants carrying the genes on a low copy (CEN) plasmid. Vma5p has been tagged at the N terminus with the Myc epitope (14). The resulting N-Myc-VMA5 fully complements the growth phenotype of the deletion strain and yields a VATPase with properties comparable to those of the wild-type enzyme (see below). This epitope-tagged version of VMA13 was used in all experiments. Western blot analysis of whole cell lysates prepared from the various strains showed that cells containing multicopy plasmid 2μ-VMA5 or 2μ-VMA13 displayed a 2-3-fold steady-state level of

**Fig. 1. Steady-state levels of overproduction of Vma5p and Vma13p.** A, vma5Δ cells were transformed with pRS316-VMA5 (CEN-VMA5) and YEp352-VMA5 (2μ-VMA5). B, vma13Δ strains were transformed with pRS193-VMA13 (CEN-VMA13Nmyc), or YEp352-VMA13Nmyc (2μ-VMA13Nmyc). Whole cell lysates were prepared as described under “Experimental Procedures” and analyzed by Western blotting. Membranes were probed with monoclonal antibodies directed against Vma5p and Vma2p (A) and monoclonal antibodies against Myc epitope-tagged Vma13p and Vma2p (B).
overexpression of the two subunits (Fig. 1).

To determine whether the overproduction of Vma5p or Vma13p had an effect on the V-ATPase, the cells were screened for a Vma− phenotype, characterized by sensitivity to elevated pH and Ca2+ levels (32, 33). The deletion strains carrying 2µ-VMA5 or 2µ-VMA13 exhibited a mutant growth phenotype, whereas those transformed with CEN-VMA5 or CEN-VMA13 did not (Fig. 2). This indicates that a 2-3-fold overproduction of Vma5p or Vma13p compromises V-ATPase activity.

How Is V-ATPase Activity Affected by Overproduced Vma5p and Vma13p?—The presence of a Vma phenotype generally suggests that the cell possesses <25% of the wild-type V-ATPase activity (34). We first examined the ability of the V-ATPase to acidify the vacuoles in these strains in vivo. Using the lysosomotropic dye quinacrine, we were able to qualitatively examine the acidification of vacuoles in the presence of excess Vma5p or Vma13p. Cells were stained with quinacrine and then visualized using fluorescence microscopy and Nomarski optics (26). Wild-type cells exhibited bright quinacrine staining that colocalized with the vacuole (Fig. 3A). Under the same conditions, vma5Δ cells and cells carrying 2µ-VMA13 displayed no quinacrine staining (Fig. 3, B and D), indicating that the vacuoles were not acidified. Cells overexpressing Vma5p did show some quinacrine staining localized to the vacuole, but the levels were generally less than in wild-type cells (Fig. 3C). These results suggest that, in the presence of excess Vma5p, it is still possible to establish a proton gradient across the membrane, but the gradient may not be as strong as in wild-type cells. Cells overexpressing Vma13p did not appear to establish a proton gradient sufficient for any vacuolar quinacrine accumulation. It was notable that we found many cases in which the bud was brightly stained compared with the mother cell in cells overexpressing Vma5p (Fig. 3C), but we currently have no explanation for this phenomenon.

To directly measure ATPase activity, vacuolar membranes were isolated from various strains, and concanamycin A-sensitive ATP hydrolysis was measured as described under “Experimental Procedures.” Overproduction of Vma5p or Vma13p moderately reduced the ATP hydrolysis activity of the V-ATPase, but not as much as expected from the growth phenotype. vma5Δ cells carrying VMA5 on a 2µ plasmid had 77% as much ATPase activity as the same cells carrying VMA5 on a CEN plasmid (Table I), even though their growth properties (Fig. 2) were dramatically different. Surprisingly, the V-ATPase retained up to 72% of its ATP hydrolysis activity in isolated vacuoles from the strain containing overproduced Vma13p, even though there was no quinacrine uptake in vivo (Fig. 3). A closer look at proton translocation by the V-ATPase was needed.
Overexpression of Yeast VMA13 and VMA5

Fig. 4. Effects of Vma5p and Vma13p overproduction on proton translocation into isolated vacuolar vesicles. 5 μg of isolated vacuolar vesicles from the indicated strains were placed in proton translocation buffer. Proton pumping was initiated by the addition of 2.5 mM ATP, and quinacrine fluorescence quenching over time was monitored.

enough to create a smaller proton gradient across the vacuolar membrane. It is somewhat surprising that this level of V-ATPase activity and proton pumping was insufficient to support growth of the cells at elevated pH and Ca2+ levels. The V-ATPase from cells containing overproduced Vma13p was capable of ATP hydrolysis, but was almost completely incapable of establishing a proton gradient. To our knowledge, this is the first time that such extensive functional uncoupling of a V-ATPase has been observed.

Overproduction of Vma5p and Vma13p Has a Structural Effect on the V-ATPase—The loss of the proton gradient in Vma13p-overproducing cells could arise either from a defect in the V-ATPase itself or from binding of Vma13p to another vacuolar protein that results in leakiness of the vacuolar membrane. Therefore, we determined whether the V-ATPase complex is structurally intact in cells overexpressing VMA5 and VMA13. The V-ATPase in wild-type vacuolar vesicles is stable to low salt washes, and several EDTA washes are incorporated into purification of the V-ATPase (36). In contrast, vacuolar vesicles isolated from cells containing 2μ-VMA5 or 2μ-VMA13 lost a substantial amount of ATPase activity after two washes in 1 mM EDTA. On average, specific ATP hydrolysis activity decreased by 47% in both 2μ-VMA5- and 2μ-VMA13-containing strains, whereas wild-type vesicles gained ~4% specific activity after washing. This indicates that the V-ATPase in cells containing overproduced Vma5p and Vma13p is structurally altered.

To determine whether the subunit composition of the V-ATPase is affected in the 2μ-VMA5- and 2μ-VMA13-containing strains, we took several approaches. First, the levels of various V1 and V0 subunits present at the vacuolar membrane were analyzed by Western blot analysis using antibodies against the Vph1p, Vma1p, Vma2p, Vma5p, and Vma4p V1 subunits of the V-ATPase. A set of titrations of vacuolar vesicles from vma13Δ cells transformed with the CEN-VMA13 or 2μ-VMA13 plasmid and from vma5Δ cells transformed with the CEN-VMA5 or 2μ-VMA5 plasmid as described under “Experimental Procedures.” The indicated amounts of vesicle protein were separated by SDS-12% PAGE and then analyzed by Western blotting. Blots were probed with monoclonal antibodies against Vph1p, Vma1p, Vma2p, Vma5p, and Myc-tagged Vma13p or with polyclonal antibodies against Vma4p and Vma8p. Vesicles from the 2μ-VMA13, 2μ-VMA5, and CEN-VMA5 cells are designated J, 2, and 3, respectively. B, immunoprecipitation of V-ATPase complexes from isolated vacuolar membranes. Vacuolar vesicles were isolated as described for A and then solubilized with 2% N-ethyl β-n-glucopyranoside. Solubilized vesicles were incubated on ice with monoclonal antibody 8B1 directed against Vma1p, which is capable of co-immunoprecipitating the entire V-ATPase, followed by protein A-Sepharose. Immunoprecipitated complexes were separated by SDS-PAGE on a 16.5% Tris-Tricine-acrylamide gel, followed by silver staining. A mock immunoprecipitation (containing antibody without solubilized vesicles) was conducted in parallel.

Fig. 5. V-ATPase subunit composition in vacuolar vesicles. A, quantitative immunoblotting of V-ATPase subunits in isolated vacuolar membranes. Vacuolar vesicles were isolated from vma13Δ cells transformed with the CEN-VMA13 or 2μ-VMA13 plasmid and from vma5Δ cells transformed with the CEN-VMA5 or 2μ-VMA5 plasmid as described under “Experimental Procedures.” The indicated amounts of vesicle protein were separated by SDS-12% PAGE and then analyzed by Western blotting. Blots were probed with monoclonal antibodies against Vph1p, Vma1p, Vma2p, Vma5p, and Myc-tagged Vma13p or with polyclonal antibodies against Vma4p and Vma8p. Vesicles from the 2μ-VMA13, 2μ-VMA5, and CEN-VMA5 cells are designated J, 2, and 3, respectively. B, immunoprecipitation of V-ATPase complexes from isolated vacuolar membranes. Vacuolar vesicles were isolated as described for A and then solubilized with 2% N-ethyl β-n-glucopyranoside. Solubilized vesicles were incubated on ice with monoclonal antibody 8B1 directed against Vma1p, which is capable of co-immunoprecipitating the entire V-ATPase, followed by protein A-Sepharose. Immunoprecipitated complexes were separated by SDS-PAGE on a 16.5% Tris-Tricine-acrylamide gel, followed by silver staining. A mock immunoprecipitation (containing antibody without solubilized vesicles) was conducted in parallel.
vesicles from the strains overproducing Vma5p and Vma13p did not contain elevated levels of these subunits.

To determine the assembly state of the V-ATPase from the different strains by an independent method, we performed immunoprecipitations from a separate isolation of vacuolar vesicles. Using monoclonal antibodies capable of co-immunoprecipitating the entire V-ATPase complex, we confirmed that overexpression of Vma13p resulted in lower levels of the V1 sector subunit Vma4p and also observed lower levels of Vma8p (Fig. 5B). Co-immunoprecipitation confirmed that strains overproducing Vma5p had an apparently wild-type subunit composition.

Finally, we purified the V-ATPase from isolated vacuolar vesicles by glycerol gradient fractionation (36). We found that V-ATPase complexes purified from wild-type, 2μ-VMA5-containing, and 2μ-VMA13-containing strains all peaked at a similar glycerol density (data not shown). This result was anticipated because the V-ATPase from Vma5p-overproducing strains resembled the wild-type enzyme composition. The V-ATPase from Vma13p-overproducing strains had reduced levels of some smaller stalk subunits, but this may not generate a sufficient change in size or structure in the enzyme to have a major effect on its fractionation pattern.

Taken together, these data indicate that the V-ATPase from cells overproducing VMA13 is both functionally and structurally defective. It is notable, however, that the structural defects cannot be accounted for by the coordinate loss of the V1 subunits from the membrane that is seen both in most V1 subunit deletion mutants and in certain V1 point mutants. Instead, certain stalk subunits appear to be specifically lost, and this could help to account for the functional uncoupling of ATP hydrolysis and proton transport. In contrast, the structural defects of the V-ATPase from Vma5p-overproducing cells appear to be much more subtle; the overall stability of the enzyme is reduced, but the subunit composition does not appear to be altered.

**Growth Defects of Strains Overexpressing Vma5p and Vma13p**—If overexpression of Vma5p and Vma13p simply destabilizes the V-ATPase, then the effects of overexpression of these subunits should never be worse than the effects of their deletion. In performing the experiments above, however, we noticed that the growth defects of overproducing strains could be quite pronounced. Quantitative analysis of doubling times for strains containing the 2μ-VMA5 and 2μ-VMA13 plasmids in glucose-containing medium indicated that the growth defects were comparable to those of vma deletion cells (see below), but the level of overexpression indicated in Fig. 1 (2–3-fold) was also quite modest. We therefore addressed whether high level expression of VMA5 and VMA13 is toxic to cells by an alternate method. Unfortunately, the most commonly used methods for inducible expression of yeast genes require either growth of cells on galactose, which can cause disassembly of the V-ATPase complex, or the addition of heavy metals, which can be toxic to cells with impaired V-ATPase activity. Therefore, we decided to overexpress VMA5 and VMA13 by cloning the genes into a system in which very high plasmid copy numbers can be maintained in strains that fail to assemble one of the V1 sectors.

![Fig. 6. High level overexpression of Vma5p and Vma13p is lethal to yeast cells.](image)

VMA13, were transformed with an empty leu2-d vector or the same vector containing VMA5 or VMA13. When cells were grown on minimal medium lacking uracil, on which there is little overexpression of VMA5 and VMA13, all of the transformants were able to grow (Fig. 6A). When cells are plated onto minimal medium lacking leucine, the plasmid must be amplified at high levels to support growth, and the VMA5 and VMA13 genes would also be amplified. Cells bearing the leu2-d plasmid carrying VMA5 and VMA13 were not viable on medium lacking leucine, even at low pH (pH 5.7) (Fig. 6B). This suggests that highly overexpressed Vma5p and Vma13p complicate the growth of yeast cells beyond the Vma growth defects characteristic of vma mutations and that overexpressed VMA5 and VMA13 may exhibit a “gain of function” that is deleterious to the cell.

Gain-of-function mutations in the V-ATPase have not been described previously, but there are at least two obvious ways that alterations in V-ATPase structure could generate novel and potentially damaging activities. The peripheral V1 sector of the enzyme and the membrane-bound V0 sector exhibit considerable structural independence. Free V1 or V0 sectors are synthesized and stable in strains that fail to assemble one of the sectors (18), and free V1 and V0 sectors exist in a dynamic equilibrium with fully assembled V-ATPase complexes in wild-type cells (16, 38). One important characteristic of the free V1 and V0 sectors is that their activities appear to be silenced; free V1 sectors are not active as MgATPases, and free V0 sectors do not appear to be open proton pores. If the MgATPase activity of free V1 sectors was activated or the V0 proton pore were opened in the presence of overproduced Vma5p and Vma13p, then a new and potentially deleterious function of the V-ATPase subcomplex might be revealed. To test this possibility, we examined whether overexpression of Vma5p and Vma13p would generate an additional growth defect in vma mutants that lack either an intact V1 or V0 sector, but assemble the other sector. These mutants have already lost all V-ATPase activity and thus exhibit a full Vma- phenotype. Therefore, any additional growth defect would suggest an additional defect arising from Vma5p or Vma13p overproduction.

Yeast vma2Δ mutants lack the B subunit of the V1 sector, but assemble intact V0 sectors at the vacuole (12). vma11Δ mutants...
lack the c’ subunit, one of the three essential proteolipids of the V₀ sector, but assemble intact cytoplasmic V₁ sectors (7, 17). Table II describes the doubling times of a variety of mutant and wild-type strains containing either the empty vector or the 2μ vectors containing VMA5 and VMA13 described above. The wild-type strain exhibited little difference in its doubling time in minimal medium (pH 5.7) whether it contained the empty plasmid or the VMA5 and VMA13 multicopy plasmids. Similarly, the vma2Δ strain grew more slowly than the wild-type strain under all conditions, as reported previously for VMA2 plasmid or the vector containing VMA5 or VMA13. Log-phase cultures of all strains were diluted into fresh SD-Ura medium; growth of the strains was monitored by periodically measuring the absorbance of the culture at 600 nm; and the doubling times during log-phase growth of the cultures were determined from growth curves.

| Strain | Doubling time (h) |
|--------|-------------------|
| Wild-type (W303) + YEp352-empty | 1.6 |
| Wild-type + YEp352-VMA5 | 1.5 |
| Wild-type + YEp352-VMA13 | 1.4 |
| vma2Δ + YEp352-empty | 2.8 |
| vma2Δ + YEp352-VMA5 | 2.5 |
| vma2Δ + YEp352-VMA13 | 2.9 |
| vma11Δ + YEp352-empty | 2.3 |
| vma11Δ + YEp352-VMA5 | 3.3 |
| vma11Δ + YEp352-VMA13 | 3.4 |

Table II: Doubling times of wild-type cells and vma mutants overexpressing VMA5 and VMA13

Vma5p shows variable association with the immunoprecipitated complexes and is indicated by *. The positions of molecular mass standards are shown on the left (arrows) and correspond to the following molecular masses (from top to bottom): 200, 96, 69, 43, 29, and 18.4 kDa. Vma5p appears to destabilize fully assembled V₁ complexes (data not shown). We assayed MgATPase activity in all of the fractions containing the assembled V₁ complexes, including fractions 20–22, which were shown to contain fully assembled V₁ complexes in the previous studies, but did not observe any MgATP hydrolysis (data not shown).

**DISCUSSION**

Vma5p and Vma13p have been implicated in the structural and functional coupling of the V-ATPase. Based on this, we had hypothesized that overexpression of one or both of these two subunits might inactivate the V-ATPase by dissociating the fully assembled complex into free V₁ and V₀ sectors. Although we did observe a Vma− phenotype characteristic of inactivation of the V-ATPase, the structural basis of this phenotype is less straightforward. This study does provide new insights into the functional roles of these two subunits, however. Overproduction of Vma13p results in an assembled V₁V₀ complex that is capable of ATP hydrolysis, but not proton transport. This is the first reported example of a fully uncoupled V-ATPase complex. Overexpression of Vma5p appears to destabilize fully assembled V-ATPase complexes by an unknown mechanism, but more significantly, drives association of Vma5p with cytosolic...
V1 complexes. We hypothesize that this association may be responsible, at least in part, for the fact that overexpressed Vma5p has even more damaging effects on cells than does a total loss of V-ATPase activity. It is significant that these structural and functional phenotypes of overproduction have not been seen for deletion or point mutations in any yeast V-ATPase subunit and thus are completely novel. We have not determined whether the effects of Vma5p or Vma13p overexpression arise during biosynthesis of the V-ATPase, through some type of exchange or sequestering of subunits in the cytosol or by another mechanism; but we describe the implications of these phenotypes below.

Overexpression of Vma13p Functionally Uncouples ATP Hydrolysis and Proton Translocation—Vma13p is a member of the peripheral V1 sector, but one of its distinctive characteristics is that it does not appear to be essential for association of V1 subunits with V0 sectors in isolated vacuoles (19). Vacuoles from vma13Δ cells lack all V-ATPase activity, but do assemble other V1 subunits at the membrane; deletion of other V1 subunits appears to prevent association of the remaining peripheral subunits with the membrane (19). Cytosolic V1 sectors isolated from vma13Δ cells also contain all of the other V1 subunits, but do exhibit some MgATPase activity, suggesting they have lost some of the “silencing” of MgATPase activity that usually accompanies release of V1 from the membrane (14). Based on this behavior of vma13Δ mutants, Vma13p is believed to act as an activator of V1V0 complexes and as an inhibitor of free V1 complexes. It has been hypothesized that it achieves this dual role by stabilizing a "coupled" conformation of V1V0 that is competent for ATP-driven proton pumping and then undergoing a conformational change when V1 is released from the membrane that prevents catalysis by free V1 (14, 39).

Interpretation of the data from Vma13p overproduction in this context would suggest that, in the presence of extra copies of Vma13p, an intermediate conformation is present in which Vma13p has moved out of its V1 inhibitory conformation to allow MgATP hydrolysis to occur, but V1 and V0 are not attached in a manner compatible with ATP-driven proton pumping. From the data presented here and the information available about Vma13p binding and function, we cannot distinguish whether the V-ATPase is functionally uncoupled because the V0 is an open proton pore or because conformational changes generated by ATP hydrolysis cannot be relayed to the proton pore, perhaps because of a structural defect in one of the stalks. Partial loss of Vma4p and Vma8p, two putative stalk subunits, would be consistent with either functional defect; further experiments may allow us to distinguish between these two possibilities. It is notable that a milder uncoupling defect was also observed for several vma8Δ mutants (40). How does overproduction of Vma13p generate the uncoupled V1V0 complexes? If Vma13p has binding sites on both the V1 and V0 sectors, as suggested by its role in activating coupled ATP hydrolysis and proton transport and by cross-linking (39) and two-hybrid (41) experiments, excess Vma13p might allow binding of individual Vma13p molecules to V1 and V0. Isolated vacuoles do not contain excess Vma13p (Fig. 5A), but this does not eliminate the possibility that low affinity binding of a second Vma13p is responsible for generating the final complex we observed. The data showing normal levels of Vma4p and Vma8p in cytosolic V1 complexes (Fig. 7) argue against an alternative model in which excess Vma13p sequesters these subunits from the cytosol, preventing their assembly with V1. It is notable that mammalian V-ATPases contain two isoforms of subunit H (Vma13p) (42, 43), and there is evidence indicating that both isoforms may be present at nonequivalent sites in a single V-ATPase complex (39). There is no evidence of a second Vma13p isoform in yeast, but it is possible that overexpression allows inappropriate binding of a second Vma13p to a site similar to that occupied by the second isoform in mammalian cells and thus alters the properties of the yeast enzyme.
Several other features of Vma13p must also be considered in interpreting these data, however. Vma13p is unique among the V-ATPase subunits in its ability to interact with other, non-V-ATPase complexes. Surprisingly, human VMA13 was shown to bind the human immunodeficiency virus-1 Nef protein and more recently was demonstrated to play a critical role in facilitating Nef internalization (44). In yeast, Vma13p was recently demonstrated to interact with the Golgi-localized ecotransferase Ynd1p and to act as an inhibitor of its activity (45). Although Vma13p appeared to act in the context of fully assembled V-ATPase complexes (whether active or inactive) in its regulation of Ynd1p, overexpressed Vma13p generated even lower levels of Ynd1p-dependent ADPase activity than VMA13 expressed at wild-type levels, probably due both to down-regulation of Ynd1p levels and further inhibition of the enzyme by excess Vma13p. It is possible that the full spectrum of Vma13p-binding partners and cellular functions is not yet known, and phenotypes of VMA13 overexpression could reflect its activities in these other complexes as well. This could help to account for the gain of function that we observed when VMA13 was overexpressed at modest levels in vma11Δ cells or at high levels in wild-type cells. We do not believe that alternative Vma13p functions account for the uncoupled V-ATPase and proton pumping activities in isolated vacuoles, however, because the V-ATPase complexes show specific structural defects. It is also significant that modest levels of VMA13 overexpression in an otherwise wild-type background (Fig. 1) yield a characteristic V− phenotype, suggesting that a specific V-ATPase defect predominates under these conditions.

Overexpression of Vma5p Generates a Novel Cytosolic V1 Complex—Although both Vma13p and Vma5p have been viewed as structurally and functionally bridging V1 and V0 complexes, there are both similarities and differences in the phenotypes of vma13Δ and vma5Δ strains. Both deletions exhibit the full range of V− growth defects, and both allow assembly of both V1 and V0 sectors than any other V1 subunit deletion strain (18, 19, 22). Isolated vacuoles from vma5Δ strains contain very low levels of V1 sector subunits, however (22). Vma5p is also lost from both sectors when V1 and V0 sectors dissociate during glucose depletion and is reassembled with both sectors upon glucose re-addition (9). These data are consistent with a critical role for Vma5p in attachment of V1 and V0 and possible involvement in signaling their reversible disassembly in vivo. It has also been proposed that release of Vma5p from V1 could play a role, in cooperation with Vma13p inhibition, in the silencing of MgATP hydrolysis in cytosolic V1 sectors (14).

The data shown here demonstrate that excess Vma5p can affect both fully assembled V-ATPase complexes and free V1 complexes. V-ATPase complexes in isolated vacuoles are destabilized by the presence of excess Vma5p, but the specific structural defect is subtle because the complexes have an apparently normal subunit composition. This type of subtle defect in V-ATPase structure is also characteristic of a number of VMA5 point mutations 2 and may indicate that Vma5p is responsible not only for the crude attachment of V1 sectors to V0 but also for more subtle features underlying “correct” attachment of the two complexes. Significantly, there is no evidence of uncoupling of V1 and V0 activities in isolated vacuoles; ATP hydrolysis and proton pumping are decreased by roughly the same amount in vacuoles from VMA5-overexpressing strains (Table 1 and Fig. 4). The effects of VMA5 overexpression on cytosolic V1 sectors are somewhat clearer. Vma5p has never been isolated with cytosolic V1 sectors from glucose-deprived cells or yeast cells containing only cytosolic V1 sectors because of deletion of a V0 subunit (14). However, it does coprecipitate with V1 from vma11Δ cells overexpressing VMA5. In this case, excess VMA5 may allow binding to a low affinity site on V1 that cannot be bound in the presence of normal Vma5p levels. Although we did not observe Mg-ATPase activity in our assays of the ion-exchange fractions, these fractions contained lower levels of subunit protein than we assayed in the past (14). It remains an intriguing possibility that Vma5p bound to cytosolic V1 complexes activates some level of futile ATP hydrolysis and that this accounts for the deleterious gain of function observed when Vma5p is overproduced at high levels or in the presence of high levels of cytosolic V1 (in a vma11Δ strain). Further experiments will be necessary to test this possibility. Vma5p has not yet been shown to participate in other complexes as Vma13p does, so it is more difficult to attribute overexpression phenotypes to non-V-ATPase-associated activities.

Physiological Relevance of V1 Subunit Overexpression Studies—Although we have focused on the biochemical significance of the complexes formed in the presence of VMA5 and VMA13 overexpression, these results do have physiological relevance, particularly for VMA5. The human VMA5 gene was the only V-ATPase subunit gene identified among those genes whose mRNA levels change in response to cytomegalovirus infection; VMA5 mRNA levels were 8-fold higher 24 h after viral infection (46). VMA5 mRNA levels were 8.9-fold higher after yeast cells were treated for 10 min with 0.4 M NaCl; but in this case, several other V-ATPase subunit genes were coordinately up-regulated (35). Protein levels were not determined in either of these studies, so it remains to be determined whether and to what level Vma5p is overproduced; but the studies reported here suggest that the functional consequences of even 2–3-fold overproduction of Vma5p could be very significant.

Acknowledgments—We thank Tom Stevens for providing antibodies and plasmids used in this study, Richard Cross for the use of a spectrophotometer, and Mark Schmitt and Dave Amberg for helpful discussions.

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Kelly Keenan Curtis and Patricia M. Kane

J. Biol. Chem. 2002, 277:2716-2724.
doi: 10.1074/jbc.M107777200 originally published online November 20, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107777200

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