The E and G Subunits of the Yeast V-ATPase Interact Tightly and Are Both Present at More Than One Copy per V₁ Complex*

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The E and G subunits of the yeast V-ATPase are believed to be part of the peripheral or stator stalk(s) responsible for physically and functionally linking the peripheral V₁ sector, responsible for ATP hydrolysis, to the membrane V₀ sector, containing the proton pore. The E and G subunits interact tightly and specifically, both on a far Western blot of yeast vacuolar proteins and in the yeast two-hybrid assay. Amino acids 13–79 of the E subunit are critical for the E-G two-hybrid interaction. Different tagged versions of the G subunit were expressed in a diploid cell, and affinity purification of cytosolic V₁ sectors via a FLAG-tagged G subunit resulted in copurification of a Myc-tagged G subunit, implying more than one G subunit was present in each V₁ complex. Similarly, hemagglutinin-tagged E subunit was able to affinity-purify V₁ sectors containing an untagged version of the E subunit from heterozygous diploid cells, suggesting that more than one E subunit is present. Overexpression of the subunit G results in a destabilization of subunit E similar to that seen in the complete absence of subunit G (Tomashek, J. J., Graham, L. A., Hutchins, M. U., Stevens, T. H., and Klionsky, D. J. (1997) J. Biol. Chem. 272, 26787–26793). These results are consistent with recent models showing at least two peripheral stalks connecting the V₁ and V₀ sectors of the V-ATPase and would allow both stalks to be based on an EG dimer.

V-ATPases are ubiquitous proton pumps involved in acidification of organelles such as lysosomes, endosomes, and the Golgi apparatus in all eukaryotic cells (1). Eukaryotic V-ATPases are structurally very conserved; subunit compositions are highly similar between evolutionarily distant eukaryotes, and subunit sequences are as much as 80% identical between humans and yeast (1, 2). In addition, low resolution electron microscopy structures show a high level of similarity between humans and yeast (1, 2). In addition, low resolution electron microscopy structures show a high level of similarity between humans and yeast (1, 2). In addition, low resolution electron microscopy structures show a high level of similarity between humans and yeast (1, 2). In addition, low resolution electron microscopy structures show a high level of similarity between humans and yeast (1, 2). In addition, low resolution electron microscopy structures show a high level of similarity between humans and yeast (1, 2). In addition, low resolution electron microscopy structures show a high level of similarity between humans and yeast (1, 2).
image reconstruction of the *Neurospora crassa* V-ATPase have suggested that there might be two structurally distinct links, or potentially two stators, providing peripheral connections between *V*₁ and *V*₀. The authors of this work suggested that these two links contain subunits H and a and subunits E, G, and C, respectively (20). In this work, we focus on the interaction between subunits E and G of the yeast V-ATPase and the potential role of these subunits in stator structure and function. We provide evidence supporting a tight interaction between subunits E and G that requires the N-terminal third of subunit E, and we also show that there are more than one G and E subunit in each *V*₁ complex.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-HA² antibody, anti-Myc antibody, and anti-FLAG antibody, and anti-FLAG-agarose beads, and FLAG peptide were purchased from Sigma. Restriction enzymes were from New England Biolabs, and LA-Taq (Perkin-Elmer) was used for PCR.

**Plasmid and Strain Construction**—Construction of N-terminally Myc- and FLAG-tagged VMA10 plasmids was described previously (30, 31). In order to allow for selection of both plasmids simultaneously in a diploid cell, the Myc-tagged VMA10 gene was transferred to the low copy plasmid pRS316 (CEN6, URA3) and transformed into SF838-5Aa *vma10Δ::kanMX*. FLAG-tagged VMA10 in plasmid pRS315 (CEN6, LEU2) was transformed into SF838-5Aa *vma10Δ::kanMX*, and the two strains were mated to obtain the diploid strain containing both Myc-tagged and FLAG-tagged G subunits. Overexpression of VMA10 was achieved by cloning the N-terminally Myc-tagged VMA10 gene into plasmid YEp352 [2µ, LEU2] (32).

The C terminus of the E subunit was tagged with 3HA (three copies of the hemagglutinin epitope) or green fluorescent protein (GFP) by integrating the tag along with the kanMX selectable marker at the 3′ end of the VMA4 gene as described by Longtine et al. (33). Briefly, cassettes containing the 3HA-kanMX and GFP-kanMX were PCR-amplified with oligonucleotides F2 (5′-CGGATCCCGGGGTTAATTAA-3′) and R1 (5′-GAAATTCGAGCTGGTATTTAAC-3′) as described (33), using plasmids pA6a-GFP-kanMX or pA6a-3HA-kanMX (generous gifts from Mark Longtine) as templates. Two ~200-bp fragments of VMA4 corresponding to a sequence just upstream from the stop codon (amplified with oligonucleotides VMA4-c1, 5′-GGGAATTCGCTGGGACAC-3′, and VMA4-c2, 5′-TGAATTACACGCTGGGACAC-3′) and a sequence downstream from the VMA4 stop codon (amplified with oligonucleotides VMA4-c3, 5′-GGGAATTCGCTGGGACAC-3′, and VMA4-c4, 5′-GGGAATTCGCTGGGACAC-3′) were created by PCR amplification from yeast genomic DNA. These fragments contain sequences overlapping the tag-containing cassettes and were combined with the tag fragments by fusion PCR of all three fragments using oligonucleotides VMA4-c1 and VMA4-200. The fusion products were introduced into yeast strain SF838-5Aa by lithium acetate transformation (34), and transformants were selected by growth on YEPD (yeast extract, peptone, 2% dextrose) medium containing 200 µg/ml G418. Formation of the correct fusion protein was confirmed by Western blotting using antibodies against both the E subunit and the HA or GFP tags. To test for retention of V-ATPase function in the presence of the tagged E subunits, the strains containing the integrated tags were grown on YEPD buffered to pH 7.5 containing 60 mM CaCl₂ (35). The strain containing the VMA4-3HA construct showed wild-type growth under these conditions, but the strain containing the VMA4-GFP construct did not grow, indicating that V-ATPase function was compromised. Vacuoles isolated from the VMA4-GFP-containing strain had little or no concanamycin-sensitive ATPase activity but did contain the VMA4-GFP protein; these vacuoles are used in Fig. 1. To construct a diploid strain containing both VMA4-3HA and the wild-type E subunit, the VMA4-3HA strain was mated to the congenic wild-type strain of the opposite mating type, SF838-5Aa. To construct a diploid strain containing both VMA4-3HA and FLAG-tagged VMA10, the SF838-5Aa *vma10Δ::kanMX* strain containing the pRS315-FLAG-VMA10 plasmid, described above, was mated to the SF838-5A *vma4Δ::3HA-KanMX* strain, and diploids were isolated under a dissecting microscope.

**Far Western Analysis**—To create constructs appropriate for expression from the promoter in the *in vitro* transcription/translation system, the start codons of the VMA4 and VMA10 genes were placed in proximity to the T7 promoter in plasmid pRS316. For VMA4, this was achieved by PCR amplification of VMA4 from genomic DNA with oligonucleotides 5′-CGGATACACATGTCCTCCGCG-3′ and 5′-GGTATA-CAAGCTGCTGGTGCG-3′, cloning the PCR fragment into pGEM-T-Easy, and then removing the VMA4 fragment with SacII and SplI for cloning into the same sites in pRS316. For VMA10, a fragment lacking the intron but retaining the N-terminal Myc tag was obtained by PCR amplification with oligonucleotides 5′-CAAGCCAGATGGAACACCAAGGC and 5′-CCATACCTTCTCCTTTACACCGG-3′ from pALTER-myc-VMA10 described by Charsky et al. (30). The fragment was introduced into pRS316 as described for VMA4. *In vitro* transcription and translation were carried out in the TNT Quick Coupled Transcription/Translation System (Promega). 1 µg of each plasmid was added to 40 µl of TNT Quick Master mixture containing 2 µl of [³⁵S]methionine, and the transcription/translation reaction was carried out for 1.5 h at 30 °C. The products were visualized by SDS-PAGE and autoradiography to confirm production of a protein of the correct size for the subunits. For far Western analysis (36), the entire 50-µl reaction mixture was combined with 6.5 ml of AC buffer (10% glycerol, 100 mM NaCl, 20 mM Tris, pH 7.6, 0.5 mM EDTA, 0.1% Tween 20) containing 2% nonfat dry milk and 10 mM dithiothreitol and incubated on ice before adding to the blot. Blots for far Western analysis were prepared by solubilizing vacuolar vesicles containing the indicated amount of protein in 1× Larmsml sample buffer (37), then separating by SDS-PAGE, and blotting to nitrocellulose. Blots were blocked in AC buffer containing 2% nonfat dry milk and 1 mM dithiothreitol and at the same time exposed sequentially to decreasing concentrations of

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²The abbreviations used are: HA, hemagglutinin; GFP, green fluorescent protein.
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guanidine-HCl for 30 min each, starting with 6 m guanidine-
HCl and decreasing to 3, 1, and 0.1 m in subsequent 30-min
incubations. The blot was then incubated without guanidine-
HCl overnight, then incubated with probe, and prepared as
described above for 3 h at 4 °C. Identical results, but a less
intense signal, were obtained when the guanidine denaturation
and renaturation steps were omitted.

Two-hybrid Analysis—To introduce VMA4 into two-hybrid
plasmid, VMA4 was amplified by PCR from wild-type yeast
genomic DNA with oligonucleotides VMA4-BamHI (5′-GGG-
GATTCTCAATGGCTATTTCTGTTTGG-3′) for introduction
of a BamHI site (underlined) just upstream from the ATG
(italicized) and VMA4-3′ (5′-GGTATAACGTGGTCG-
TCG). The PCR fragment was cloned into the pGEM-T Easy
vector and then cut with BamHI and Sall for ligation to pAS2
cut with the same enzymes. VMA10 was cloned into pACT2
by similar methods but was amplified with oligonucleotides
VMA10NBamHI (5′-GGGGAATCCATGCTATTTAG-3′) and VMA10-200
(5′-AGTAATCCAGTCCGATTGTTAG-3′).

Deletion mutations were introduced into pAS2-VMA4 using the
QuikChange site-directed mutagenesis kit (Stratagene). All
mutations were confirmed by DNA sequencing (State Univer-
sity of New York Upstate Medical University DNA sequencing
core).

pAS2-VMA4, or the corresponding VMA4 mutant plasmid,
was cotransformed with pACT2-VMA10 into yeast strain
PJ69-4a (MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ
gal80Δ lys2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ (38)).
Cotransformants were selected on synthetic minimal media lacking
tryptophan (for pAS2) and leucine (for pACT2).
Cotransformed cells were tested for two-hybrid interaction by
transfer to supplemented minimal media lacking tryptophan,
leucine, histidine, and adenine (38). All the above VMA4- and
VMA10-containing plasmids were also cotransformed with the
empty pACT2 or pAS2-lamin plasmids, respectively. No growth
was observed for strains containing the empty plasmids on
medium lacking histidine and adenine.

Affinity Purification of Yeast V1—Affinity purification of V1
complexes via the FLAG-tagged G subunit was performed as
described in Zhang et al. (31) with the following modifications.
Supplemented minimal medium lacking leucine was used to
grow cells at all times, and the use of French pressure cell for
cell lysis was replaced by microfluidizer processor M-110L
(Microfluidics). After ultracentrifugation of the lysate, V1 was
cut with the same enzymes.

Other Biochemical Methods—Yeast whole cell lysates were pre-
pared for Western blotting from yeast cells grown to log phase
in selective medium. Protein derived from 1 A600 unit of cells was
solubilized in 100 μl of cracking buffer, and 12.5–25 μl of this
solution was subjected to electrophoresis and immunoblotting.
Immunoblots were probed as described by Charsky et al. (30).
Antibodies to the E subunit were the generous gift from Dan
Klionsky, University of Michigan. Vacuoles were prepared and
analyzed as described (39). Pulse-chase immunoprecipitations
under denaturing conditions were performed as described (40).

RESULTS

Interaction of the E and G Subunits—There is substantial
genetic and biochemical evidence that the V-ATPase E and G
subunits interact, with some of the most convincing evidence
coming from recent work with bacterially expressed and puri-
ified E and G subunits (26, 41). We took two different
approaches to further address the interaction between these
subunits. First, we expressed each subunit in an in vitro
transcription/translation system, labeled the expressed proteins
with [35S]methionine, and examined whether they would rec-
ognize any partner proteins on a blot of solubilized vacuolar
membranes separated by SDS-PAGE (a far Western blot). Fig. 1
shows far Western blots of yeast vacuolar proteins probed with
labeled E and G subunits. Both subunits recognized a single
band on the far Western blots, despite the fact that the solubi-
ilized wild-type vacuolar membranes contain all of the
V-ATPase subunits. The labeled E subunit recognized a band at
~16 kDa and the labeled G subunit recognized a band at ~27
kDa, the molecular masses of the G and E subunits, respec-
tively. To confirm the identities of the proteins recognized in the
two blots, we probed blots of vacuoles derived from the vma2Δ
strain, which lack all V1 subunits but contain other vacuolar
proteins (42), and vacuoles containing epitope-tagged versions
of the E and G subunits, in which there is a well defined shift in
molecular mass of the tagged subunits. As shown in Fig. 1B,
the labeled E subunit recognized a slightly larger protein in the
vacuoles containing a Myc-tagged G subunit, and the labeled G
subunit recognized an ~50-kDa protein in the vacuoles from a
strain with the E subunit tagged at the C terminus with GFP.
Both of the proteins recognized are at the molecular mass
expected for the tagged subunit. Neither labeled subunit inter-

To purify V1 via the C-terminal 3HA tag on the E subunit,
whole cell lysates were prepared from 3 liters of either the hap-
lloid strain containing VMA4-3HA as the sole copy of VMA4 or
the VMA4-3HA/VMA4 diploid strain. Lysates were prepared
as described above. The lysates were subjected to centrifuga-
tion at 52,100 rpm for 2 h in a 70 Ti rotor, and the resulting
supernatant was concentrated ~20–25-fold using an Ami-
con filter. This supernatant was incubated with 50 μl of
monoclonal anti-HA-agarose (Sigma) overnight at 4 °C with
shaking. After several washes with phosphate-buffered
saline, pH 7.4, bound material was eluted in cracking buffer
(8 M urea, 5% SDS, 50 mM Tris, pH 6.8, 1 mM EDTA) by
heating at 95 °C for 3 min. Sequential purification of V1 via
FLAG-tagged G and then the HA-tagged E subunit was per-
fomed by first isolating FLAG-G containing V1, complexes
from the soluble fraction of a VMA4-3HA × vma10Δ/
pRS315-VMA10 diploid on a FLAG-M2 affinity column as
described above, then eluting with FLAG peptide and immu-
noprecipitating Vma4-HA tagged complexes from the elu-
ant as described above.

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A. E subunit probe | G subunit probe
B. wt | Myc-Vma10p | Vma2Δ
wt | Vma4p-GFP | Vma2Δ

FIGURE 1. E and G subunits interact specifically in far Western blots of vacuolar proteins. A, vacuolar vesicles were isolated from wild-type (wt) cells, and vacuolar proteins were solubilized, separated by SDS-PAGE, and transferred to nitrocellulose. Increasing amounts of vacuolar proteins (8, 20, and 50 μg) were loaded for each blot. E and G subunits were synthesized individually and labeled with [35S]methionine by in vitro transcription and translation as described under “Experimental Procedures” and then used as probes to detect interacting proteins on the blot. Binding of the probes was detected by autoradiography, and the indicated sizes of the interacting bands were determined by comparison with molecular mass standards (not shown). B, in order to confirm identification of vacuolar proteins that bound the E and G subunit probes, vacuoles were isolated from cells containing Myc-tagged G subunit (myc-Vma10p) and GFP-tagged E subunit (Vma4p-GFP) as well as from a vma2Δ strain, which lacks the E subunit of the V-ATPase and assembles no V1 subunits at the membrane (42). 50 μg of vacuolar protein from each strain was loaded for the blots, and the blots were then probed with expressed E and G subunits as described above and under “Experimental Procedures.”

acted with anything in vacuoles from the vma2Δ strain. These results indicate that the E subunit is recognizing the G subunit on a far Western blot and the G subunit is recognizing the E subunit and support a tight and specific interaction between the two proteins.

We also probed the interaction between the E and G subunits in the two-hybrid assay, using a system in which successful interaction between the GAL4 activation and binding domains could be assessed by expression of two different nutritional marker genes. In this system, interaction between two proteins is manifest as growth of the transformed cells on medium lacking histidine and/or adenine, and the growth rate of the cells provides a semi-quantitative measure of the strength of the interaction being measured. The results are shown in Fig. 2. Cells containing VMA10, the gene encoding the G subunit, attached to the GAL4 activation domain (pACT-VMA10) along with VMA4, the gene encoding the E subunit, attached to the GAL4 binding domain (pAS2-VMA4), are able to grow well on medium lacking histidine and adenine (Fig. 2), indicating high level expression of the HIS3 and ADE2 genes placed under control of GAL4-driven promoters. In contrast, neither the pACT-VMA10 nor the pAS2-VMA4 plasmid permitted significant growth when combined with an empty version of the companion plasmid. These results also support the presence of a tight interaction between the E and G subunits.

One of the strengths of the two-hybrid method is that it allows rapid assessment of regions required for binding between proteins without requiring that mutant proteins be capable of assembly into a larger complex. The E subunit is highly conserved with an overall sequence identity of 30–35% and sequence similarity of 53–58% when compared with homologs from Drosophila melanogaster (GenBank™ accession number NP_730957.1), human (GenBank™ accession number AAP35792.1), Caenorhabditis elegans (GenBank™ accession number AAK67210.1), Arabidopsis thaliana (GenBank™ accession number NP_187468.1), and Schizosaccharomyces pombe (GenBank™ accession number CAB11186.1). Sequence identities vary somewhat in different regions of the E subunit, and this might suggest regions of interaction. The C-terminal 50 amino acids of Vma4p (amino acids 183–233) are 42–50% identical with the sequences cited above, whereas amino acids 100–183 are only 25–30% identical, and amino acids 13–100 are 35–39% identical. We made constructs of Vma4p lacking these different regions and tested their interaction with Vma10p as described above. Deletion of the C-terminal amino acids, which appear to be the most conserved region, did not affect interaction between Vma4p and Vma10p. Two-hybrid constructs containing only the first 100 amino acids of Vma4p interacted with Vma10p as strongly as the full-length construct, but deletion of either amino acids 13–98 or 13–78 completely prevented the interaction. These data suggest that the N-terminal third of the E subunit is critical for the E-G subunit interaction. Although they still permitted interaction with the G subunit, the two C-terminal deletions of the E subunit did appear to slow the growth of yeast cells, even under the +his, +ade conditions that do not require two-hybrid interactions. A dominant negative effect of overexpression of the N-terminal 83 amino acids of the yeast E subunit has been reported previously (50), and we believe that the truncated E subunit two-hybrid constructs are mimicking this effect.

How Many E and G Subunits Are Present in Each V1 Complex?—Although the data described above strongly support interaction between the E and G subunits, they did not provide any evidence of E-E or G-G interactions or insights into the stoichiometry of these subunits in the intact yeast V-ATPase complex.
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FIGURE 2. Two-hybrid analysis of E-G subunit interactions. Wild-type VMA4 (subunit E) and the indicated VMA4 deletion mutants were cloned into the pAS2 two-hybrid vector as described under “Experimental Procedures.” Wild-type VMA10 (subunit G) was cloned into the pACT2 two-hybrid vector. Each of the wild-type and mutant pAS2-VMA4 plasmid constructs were cotransformed independently with the pACT2-VMA10 plasmid, and cotransformants were selected on supplemented minimal media lacking tryptophan and leucine (+ade and +his). To test for interaction between the wild-type or mutant E subunits and subunit G, cells were transferred to selective medium that also lacked histidine and adenine. Growth on these −his and −ade plates requires that the indicated E subunit construct is able to interact with the G subunit; failure to grow indicates that no two-hybrid interaction occurs. None of the E subunit constructs were able to grow on −his and −ade when combined with an empty pACT2 plasmid (data not shown).

To address the question of stoichiometry more thoroughly, we introduced two different tagged G subunits into a diploid yeast cell, and we asked whether the two different tags were ever present in the same complex in vivo. We have previously described versions of the yeast G subunit tagged at the N terminus with Myc or FLAG epitope tags (30, 31). Both proteins complemented the growth defects of a vma10Δ strain, which lacks any other copy of the G subunit, and supported wild-type levels of V-ATPase activity in yeast vacuoles when expressed from a low copy plasmid. We constructed a diploid strain in which both copies of the VMA10 gene were disrupted and introduced the FLAG- and Myc-tagged G subunits on two low copy plasmids. Under these conditions, the overall level of G subunit expression should approximate that in the normal diploid cell. Both tagged proteins are expressed in the diploid cell as shown in Fig. 3A. V1 sectors were released into the cytosol by depriving the cells of glucose and then affinity-purified via the FLAG-tagged G subunit as described previously (31). The affinity-purified material was then probed for the presence of the Myc-tagged G subunit. Fig. 3A shows that the V1 complexes purified by affinity chromatography via the FLAG-tagged G do contain Myc-tagged G subunit, in addition to the FLAG-tagged G subunit. This indicates that there must be more than one G subunit per V1 complex. We further purified V1 by gel filtration chromatography (Fig. 3B). As expected, the Myc-tagged G was purified with the FLAG-tagged G and intact V1 in fractions 7–10 (31), 3 supporting the argument that both G subunits are present in assembled V1 complexes.

To address the stoichiometry of the E subunit, we introduced a triple hemagglutinin (3HA) tag onto the C terminus of this subunit by genomic tagging. A haploid yeast strain containing only the 3HA-tagged E subunit was able to grow on YEPD medium buffered to pH 7.5 containing 60 mm CaCl2, indicating that the tagged subunit supports V-ATPase function in vivo (data not shown). A single genomic copy of the 3HA-tagged E subunit was introduced into a heterozygous diploid yeast strain containing only one wild-type copy of the E subunit gene, in order to create a strain containing both 3HA-tagged and untagged E subunits at approximately normal levels. Because the tagged and untagged forms of the E subunit have different relative molecular masses, we sought to test whether the untagged E subunit could be isolated with the tagged E subunit, which would suggest that there are at least two E subunits per V1 complex. The 3HA tag was used for affinity purification of V1 complexes on an anti-HA antibody column. The Coomassie-stained gel in Fig. 4A indicates that the complexes isolated via the 3HA-tagged E subunit binding to the anti-HA antibody column had a subunit composition similar to V1 complexes isolated via the FLAG-tagged G subunit (31). (Western blotting of the HA-purified material with antibodies to the A and B subunits further supported the identification of the complex as V1; data not shown.) The anti-HA purified V1 contained trace amounts of subunit C not seen in the FLAG-purified material, because this FLAG purification was done from a deletion mutant lacking subunit C. (It was previously shown that FLAG-tagged V1 purified from wild-type cells also contains trace amounts of subunit C (31).) The HA-purified material also contains heavy and light chains of the anti-HA antibody on the column, because elution of the 3HA epitope requires denaturing conditions, whereas the FLAG-tagged V1 can be eluted by competition with the FLAG peptide.

In order to more conclusively identify the tagged and untagged E subunits, V1 was purified via FLAG affinity column from a strain containing the FLAG-tagged G subunit or via an HA affinity column from a haploid strain containing only HA-tagged E subunit and a diploid strain containing both HA- and untagged E subunit. Immunoblots from all three samples were probed with antibody to the native E subunit or anti-HA antibody. Significantly, anti-HA-purified V1 from the diploid strain contains both the untagged E subunit, running at the same size as the E subunit in V1 purified by affinity chromatography against FLAG-tagged G subunit, and a higher molecular weight band above the untagged E subunit that is not seen in the anti-FLAG purified V1 but is the only band present in the haploid HA-tagged E subunit strain. This band is recognized by the HA antibody in the Western blot shown in Fig. 4B, whereas the lowest molecular mass E subunit band is not. This result suggests that untagged E subunit is purified along with the HA-tagged E subunit from the diploid strain and that there are at least two E subunits per V1 complex.

Because complexes containing the 3HA-tagged E subunit cannot be readily eluted from the anti-HA antibody column, we were not able to further purify the V1 complexes by gel filtration. To eliminate the possibility that the untagged E subunits were associating with 3HA-tagged E subunits in a dimer or another small complex, we constructed a diploid strain containing both the FLAG-tagged G subunit and HA-tagged E subunit, along with untagged copies of each subunit. V1 complexes were first affinity-purified on a FLAG column, as described above, and then complexes eluted by FLAG peptide were immunoprecipitated with anti-HA antibody. As shown in Fig.

3 M. Ohira and P. M. Kane, unpublished data.
This strongly argues that association of tagged and untagged E subunits cannot be attributed to a cytoplasmic E subunit dimer but instead that intact V₁ complexes contain at least two E and at least two G subunits.

Coordination of E-G Subunit Stoichiometry in Vivo—Tomashok et al. (43) provided initial evidence of a genetic interaction between the E and G subunits by demonstrating that, in contrast to the other yeast V₁ subunits, the E subunit is destabilized in the absence of the G subunit. In addition, Fethiere et al. (41) found that yeast G subunit expressed in E. coli is stabilized by coexpression of the E subunit. These results and the indications of a tight E-G subunit interaction in Figs. 1 and 2 suggest that the relative stoichiometry of these two subunits might be tightly controlled in vivo, but this has not been tested in a strain expressing endogenous E and G subunits. To test this, we overexpressed the G subunit in yeast cells and examined the effects on V-ATPase activity. Yeast cells lacking V-ATPase activity show a distinctive Vma phenotype characterized by optimal growth at pH 5 and little or no growth at elevated pH and/or calcium concentrations. As shown in Fig. 5A, yeast cells lacking the G subunit (vma10Δ) exhibit a Vma phenotype, but this phenotype is complemented by expression of the tagged G subunit on a low copy plasmid. Growth of cells expressing subunit G from a high copy plasmid was like that of the vma10Δ strain, however, suggesting that the V-ATPase is not functional. To probe the reason for the loss of V-ATPase function, whole cell lysates were prepared from cells producing varied levels of subunit G and probed for the presence of the A, B, E, and G subunits. Fig. 5B shows that the G subunit is indeed overexpressed from the multicopy plasmid but, significantly, that the steady state level of the E subunit is greatly reduced in the strain overexpressing subunit G. The A and B subunits are present at constant levels in all of the strains.

The lower level of subunit E in the strain overexpressing subunit G could arise from transcriptional or post-translational effects. Because the E subunit protein was destabilized in a vma10Δ strain, we did a pulse-chase experiment and monitored the stability of newly synthesized E subunit protein in the G subunit overexpressing versus the wild-type strain. Newly synthesized E subunit protein was biosynthetically labeled during a 5-min pulse with Tran35S-label and then immunoprecipitated at various times of chase. The decay of the newly synthesized E subunit in each strain is shown in Fig. 5C. The E subunit protein is quite stable in the wild-type strain but is degraded much more rapidly in the overexpressing strain, similar to the effects reported in a vma10Δ strain. The strain containing the low copy plasmid showed an initial increase in turnover, possibly because low copy plasmids are often present at slightly more than one copy per cell (44). Because the steady state level of the E subunit in cells expressing subunit G from a low copy plasmid appears to be very similar to the level in wild-type cells (Fig. 5B), either the initial instability of the newly synthesized E subunit is somehow balanced by an increase in long term stability or increased synthesis or we are not detecting the ~15% difference in E subunit levels between the wild-type and low copy plasmid-containing cells suggested by this pulse-chase study. Nevertheless, these results do indicate that not only is the E subunit dependent on the presence of subunit G for stability, it is also...
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**Figure 4.** V1 sectors contain more than one E subunit. A, V1 sectors were isolated either via anti-FLAG affinity chromatography from a haploid strain containing a FLAG-tagged G subunit or via anti-HA affinity chromatography from strains containing a C-terminal 3HA-tagged E subunit (E-HA3). The purified V1 samples were obtained from the following: 1) a heterozygous diploid containing a FLAG-tagged G subunit or via anti-HA affinity chromatography from strains containing a C-terminal 3HA-tagged E subunit (E-HA3). The purified V1 complexes from diploids containing both FLAG-tagged G and HA-tagged E subunits (FLAG-purified), whereas the remainder of the total expressed and labeled subunit binds to the blot, depletion of the subunit probe is unlikely to account for the specificity of this signal, but it is still possible that molecular features required for other interactions could not be preserved through the blotting, denaturation, and renaturation process. The two-hybrid data (Fig. 2) not only provide further support for an E-G subunit interaction but also indicate that this interaction cannot occur without amino acids 13–78 of subunit E. In an examination of pairwise interactions of subunits expressed in vitro, Jones et al. (26) found that removal of the first 38 amino acids of subunit E prevented interaction with subunit G, whereas removal of amino acids 1–19 did not. Fethiere et al. (41) noted that the N terminus of subunit E is predicted to have a high propensity for coiled-coil formation and suggested it might form a coiled-coil with subunit G. We found previously that a number of mutations in the N-terminal 55 amino acids of subunit G affect V-ATPase function; some of these, specifically mutations Y46A or K55A, mimicked the effects of a G subunit deletion and destabilized the E subunit (despite the presence of a stable G subunit) (30). Taken together, all of these data suggest that the E and G subunits interact via their N-terminal regions, both of which are predicted to be highly helical and possibly capable of coiled-coil formation.

A number of current models for V-ATPase structure incorporate a dimer of the G subunit, but the evidence for a G subunit dimer is primarily from in vitro analysis of pairwise subunit interactions (26, 45). The yeast G subunit expressed and purified from *E. coli* formed an elongated dimer, and dimer formation is dependent on the correct ratio of the E and G subunits. These results also suggest that an E-G subunit interaction is likely to occur shortly after these subunits are synthesized.

**DISCUSSION**

Most current models of V-ATPases assign both the E and G subunits to the peripheral stalk, but these models vary considerably in how they envision these subunits associating with each other and with subunits C, H, a, and A. The results reported here highlight the tight association between subunits E and G. Such an interaction was suggested by Fethiere et al. (41) based on isolation of a tight E-G complex from *E. coli* expressing the yeast E and G subunit genes from a bicistronic plasmid. The far Western blot shown in Fig. 1 suggests that the G subunit interacts strongly with subunit E, even when presented with other subunits of the V-ATPase present on the blot. Because only a small proportion of the total expressed and labeled subunit binds to the blot, depletion of the subunit probe is unlikely to account for the specificity of this signal, but it is still possible that molecular features required for other interactions could not be preserved through the blotting, denaturation, and renaturation process. The two-hybrid data (Fig. 2) not only provide further support for an E-G subunit interaction but also indicate that this interaction cannot occur without amino acids 13–78 of subunit E. In an examination of pairwise interactions of subunits expressed in vitro, Jones et al. (26) found that removal of the first 38 amino acids of subunit E prevented interaction with subunit G, whereas removal of amino acids 1–19 did not. Fethiere et al. (41) noted that the N terminus of subunit E is predicted to have a high propensity for coiled-coil formation and suggested it might form a coiled-coil with subunit G. We found previously that a number of mutations in the N-terminal 55 amino acids of subunit G affect V-ATPase function; some of these, specifically mutations Y46A or K55A, mimicked the effects of a G subunit deletion and destabilized the E subunit (despite the presence of a stable G subunit) (30). Taken together, all of these data suggest that the E and G subunits interact via their N-terminal regions, both of which are predicted to be highly helical and possibly capable of coiled-coil formation.

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Stoichiometry of the Yeast V-ATPase E and G Subunits

S. cerevisiae V-ATPase, which is composed of 11 subunits (denoted A-G), is a ubiquitous enzyme involved in key physiological processes such as acidification of intracellular vesicles and maintenance of the proton gradient across the mitochondrial inner membrane. Overexpression of the G subunit, which is essential for the catalytic cycle of the enzyme, destabilizes subunit E. This can be due to (1) competition between G subunit dimerization and E-G complex formation, (2) excess subunit G because binding to subunit G is critical for E subunit stability, (3) excess subunit G because binding to subunit G destabilizes subunit E, or (4) competition between G subunit expression and E subunit expression. The stoichiometry of E and G subunits reported for the bovine clathrin-coated vesicle V-ATPase, which is derived from quantitative amino acid analysis, is also observed in S. cerevisiae. However, others have argued that both of these subunits are present in multiple copies in the V0 sector of the enzyme (47). Isolation of M. tuberculosis clathrin-coated vesicle V-ATPase, which was derived from quantitative amino acid analysis, also provides support for a G subunit dimer by suggesting that there might be two G subunits per V1 (46). We cannot directly test this model.

Although recovery of the FLAG epitope from the column was eliminated by deletion of amino acids 1–38 (45), Yeast G subunit expressed in vitro was also able to bind to a glutathione S-transferase-G subunit fusion protein bound to a column (26). Significantly, there was no E subunit present in either of these experiments, and the region required for G subunit dimerization appears to be very similar to that required for formation of an E-G subunit complex. Fethiere et al. (41) found that the G subunit expressed in E. coli was significantly more stable when expressed with the E subunit, conditions where an E-G subunit complex is formed. One possibility is that in the absence of the E subunit, two G subunits can form a coiled-coil similar to that formed by the N-terminal section of subunit E and the G subunit. There might even be competition between the E subunit and other G subunits for binding to the N terminus of subunit G. Although we have no direct evidence for this, the data shown in Fig. 1 would appear to suggest that given a choice the G subunit binds to subunit E more readily than to subunit G on the far Western blot. Perhaps more significantly, a competition between G subunit dimerization and E-G complex formation might help to explain the effects of G subunit overexpression in vivo (Fig. 5). It is possible that in the presence of excess subunit G, G subunit dimerization (or multimerization) begins to compete with E subunit binding to subunit G. This result in destabilization of the E subunit soon after synthesis, because binding to subunit G is critical for E subunit stability in vivo (43). Further experiments are needed to directly test this model.

The stoichiometry of E and G subunits reported for the bovine clathrin-coated vesicle V-ATPase, which was derived from quantitative amino acid analysis, also provides some support for a G subunit dimer by suggesting that there might be two G subunits per V1 (46). However, others have argued that both of these subunits are present in multiple copies based on staining intensities in Coomassie-stained gels (2). This result in destabilization of the E subunit soon after synthesis, because binding to subunit G is critical for E subunit stability in vivo (43). Further experiments are needed to directly test this model.

The stoichiometry of E and G subunits reported for the bovine clathrin-coated vesicle V-ATPase, which was derived from quantitative amino acid analysis, also provides some support for a G subunit dimer by suggesting that there might be two G subunits per V1 (46). However, others have argued that both of these subunits are present in multiple copies based on staining intensities in Coomassie-stained gels (2). We used an entirely different approach to assess subunit stoichiometry in the yeast V1, Figs. 3 and 4, similar to the approach used to demonstrate which protein subunits are present in multiple copies in the V0 sector (47). Isolation of Myc-tagged G subunit with V1 isolated by affinity chromatography via a FLAG-tagged G subunit provides strong support for the presence of multiple G subunits in each V1 complex. Although recovery of the FLAG epitope from the column appears to be higher than recovery of the Myc epitope, this is to be expected, because if there were two G subunits per V1 complex, and the different tagged subunits were randomly assorted, only one-half of the total Myc-tagged G subunit would be recovered in complexes containing FLAG-tagged G subunit. The experiment in Fig. 3 cannot distinguish between the presence of two G subunits per V1 and more than two G subunits. The results shown in Fig. 4 also argue for two or more copies of the E subunit per V1 complex. As with the tagged G subunit experiment, we cannot determine whether there are two, or more than two, E subunits per V1 complex, but the experiment does support the presence of more than one E in each complex.

These experiments are consistent with data suggesting that the E and G subunits stain more strongly than other V1 subunits but do not agree with the E subunit stoichiometry determined for the clathrin-coated vesicle V-ATPase (2, 46). We cannot

FIGURE 5. Overexpression of subunit G in vivo destabilizes subunit E. A. SF838-5Av (wt, wild-type) or SF838-5Av vma10Δ cells transformed with no plasmid (vma10Δ), myc-VMA10 on a low copy plasmid (CEN-VMA10), or myc-VMA10 on a high copy plasmid (2μ-VMA10) were streaked on YEPL medium buffered to pH 5 (pH 5) or YEPL medium buffered to pH 7.5 containing 60 mM CaCl2 (pH 7.5 + Ca2+). Better growth at pH 5 than at elevated pH and calcium concentrations is characteristic of loss of V-ATPase activity. B. Whole cell lysates were prepared from the strains indicated as in A. Two different 2μ-VMA10 transformants are shown because there may be some variation in plasmid number between transformants. Protein from each strain was transferred to nitrocellulose as described in Materials and Methods. Wild-type (A) and vma10Δ cells transformed with myc-VMA10 on a low copy (●) or multicopy (○) plasmid were converted to spheroplasts and labeled with Tran35S-label for 5 min followed by varied periods of chase, as described (40). All samples were solubilized and immunoprecipitated with polyclonal antiserum against the E subunit, followed by protein A-Sepharose. Radioactivity in the 27-kDa band (the E subunit) was quantitated on a PhosphorImager and normalized to the amount in the band present at 0 min chase for each sample. Error bars represent the range of two independent immunoprecipitations.
account for the discrepancy at this time. Subunit composition of the yeast and bovine enzymes appears to be very similar. However, there is evidence for two different H subunits in the bovine enzyme, whereas the yeast enzyme appears to have only one, so a genuine species-specific difference in stoichiometry is possible (19, 46). The experiments reported here were also performed on free V₁, whereas those on the bovine enzyme used assembled V₁V₀, but both the EM structures and biosynthetic labeling of the subunits in immunoprecipitated V₁ and V₁V₀ complexes do not suggest that any V₁ subunits other than subunit C are lost during disassembly (31, 48, 49).

EM images of both yeast V₁ and Neurospora V₁V₀ complexes have suggested that there are two peripheral masses adjacent to the hexameric A/B head group of V₁ (20, 31). Given the evidence described above that the EG complex could be a fundamental building block of the stator and that both of these subunits are present in more than one copy, it is tempting to speculate that both of the peripheral masses are stators containing EG complexes, perhaps bound to different sets of stator subunits such as C and H, as suggested by Venzke et al. (20) Consistent with such an arrangement, Fethiere et al. (23) have recently provided evidence for a 1:1 complex of the E, G, and C subunits, and the E-C interaction was shown to require the N terminus of the E subunit (26). Lu et al. (50) demonstrated previously that the H subunit interacts with the N terminus of the E subunit. In the future, visualization of tagged E and G subunits bound to antibody in V₁ and V₁V₀ complexes by electron microscopy could help to resolve both the stoichiometry of these subunits and their arrangement in the complex.

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