Arrangement of Subunits in the Proteolipid Ring of the V-ATPase*

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The vacuolar ATPases (V-ATPases) are multisubunit complexes containing two domains. The V1 domain (subunits A–H) is peripheral and carries out ATP hydrolysis. The V0 domain (subunits a, c, c’, c”, d, and e) is membrane-integral and carries out proton transport. In yeast, there are three proteolipid subunits as follows: subunit c (Vma3p), subunit c’ (Vma11p), and subunit c” (Vma16p). The proteolipid subunits form a six-membered ring containing single copies of subunits c’ and c” and four copies of subunit c. To determine the possible arrangements of proteolipid subunits in V0 that give rise to a functional V-ATPase complex, a series of gene fusions was constructed to constrain the arrangement of pairs of subunits in the ring. Fusions containing c” employed a truncated version of this protein lacking the first putative transmembrane helix (which we have shown previously to be functional), to ensure that the N and C termini of all subunits were located on the luminal side of the membrane. Fusion constructs were expressed in strains disrupted in c’, c”, or both but containing a wild copy of c to ensure the presence of the required number of copies of subunit c. The c-c”(ΔTM1), c’-c”(ΔTM1)-c’, and c’-c constructs all complemented the vma” phenotype and gave rise to complexes possessing greater than 25% of wild-type levels of activity. By contrast, neither the c-c’, the c’-c”(ΔTM1), nor the c”(ΔTM1)-c constructs complemented the vma” phenotype. These results suggest that functionally assembled V-ATPase complexes contain the proteolipid subunits arranged in a unique order in the ring.

The vacuolar (H+)–ATPases (V-ATPases)2 are a family of ATP-dependent proton pumps that acidify intracellular compartments like endosomes, lysosomes, and synaptic vesicles (1–6). Intracellular V-ATPases are important in processes such as membrane traffic, protein degradation, coupled transport, and the entry of many viruses and toxins, including influenza virus and anthrax toxin (1, 7). They also pump protons across the plasma membrane in certain cells, including renal intercalated cells, epididymal cells, osteoclasts, and tumor cells (4, 8–10). Plasma membrane V-ATPases in these cells are important in acid-base balance in the kidney, sperm maturation, bone resorption, and tumor metastasis, respectively.

The V-ATPases are multisubunit complexes containing two domains (1–6). The peripheral V1 domain is composed of eight subunits (A–H) and functions to hydrolyze ATP. The integral V0 domain is composed of six subunits and is responsible for proton transport. V0 domains from both yeast and mammalian sources contain a set of common subunits (a, c, c’, d, and e) (1, 11). In addition, the yeast V0 domain contains an additional proteolipid subunit (c”), whereas the V0 domain from at least some mammalian sources contains a glycoprotein subunit termed Ac45 (12).

The proteolipid subunits of the V-ATPase (subunits c, c’, and c”) are homologous to each other and to the c subunit of the F-ATPase (13, 14). The c subunit of the F-ATPase is an 8-kDa protein that contains two transmembrane helices (TM)s. The second TM contains a buried acidic amino acid that is critical for proton transport (15). The F-ATPase c subunits are arranged in a ring. In contrast with the F-ATPase, which contains only a single type of proteolipid subunit, the V-ATPases contain either two or three different proteolipid subunits. In yeast, all three proteolipid subunits have been shown to be essential for activity (14).

Subunits c and c’ have molecular masses around 17 kDa and contain four transmembrane helices. Subunit c” has a molecular mass of 23 kDa and contains a fifth transmembrane helix at the N terminus (14). Each proteolipid subunit contains a buried glutamic acid residue critical for proton transport. This critical residue resides in TM4 of subunits c and c’ but in TM3 of subunit c” (14). Subunit c” contains an additional buried glutamic acid residue in TM5, but this residue is not essential for activity. The essential glutamic acid residues in each subunit are thought to undergo reversible protonation and deprotonation during proton transport through the V0 domain.

Although evidence from quantitative amino acid analysis suggests that the proteolipid subunits of the V-ATPase form a six-membered ring (16), the arrangement of the proteolipids in the ring is not known. The purpose of this study was to determine which of the possible arrangements of the three proteolipid subunits give rise to a functional V-ATPase complex. This was addressed by construction of a series of gene fusions in which pairs of the proteolipid subunits were constrained to adopt a particular orientation. The results suggest that the V-ATPase proteolipid subunits in the functionally assembled complex adopt a unique orientation.

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2 The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine triphosphatase; F-ATPase, F1F0-ATP synthase; HA, influenza hemagglutinin; TM, transmembrane segment; ACMA, 9-amino-6-chloro-2-methoxyacridine; YEPD, yeast extract peptone dextrose; Me3SO, dimethyl sulfoxide.
EXPERIMENTAL PROCEDURES

Materials—Zymolyase 100T was obtained from Seikagaku America, Inc. Protease inhibitors (leupeptin, pepstatin, and aprotonin), the monoclonal antibody 3F10 (directed against the influenza hemagglutinin (HA) antigen) conjugated with horseradish peroxidase, mouse monoclonal antibody 8B1-F3 against the yeast V-ATPase A subunit, and the mouse monoclonal antibody 10D7 against the 100-kDa subunit a were from Invitrogen. Bacterial and yeast culture media were purchased from Difco. Restriction endonucleases, T4 DNA ligase, and other molecular biology reagents were from Invitrogen, Promega, and New England Biolabs. Concanamycin A, ATP, phenylmethylsulfonyl fluoride, and most other chemicals were purchased from Sigma.

Plasmid Construction and Epitope Tagging—We genetically fused the different proteolipid subunits to each other in six different combinations (Fig. 1a). The plasmid pYW3-16 encoding the fusion of c and c′(ΔTM1) was constructed by ligation of plasmid pTN3 containing VMA3 with a PCR product derived from pTN16 encoding a VMA16 lacking amino acids 1–48 but containing a single HA tag at the C terminus. The linker region connecting c and c′(ΔTM1) was 14 amino acids in length and included the C-terminal 6 amino acids of c and amino acids 49–54 of c′ (Fig. 1b). pYW16-3, pYW16-11, pYW11-16, and pYW11-3 were all constructed using the same strategy as pYW3-16 and contained linker regions of 14 amino acids (for pYW11-3) (Fig. 1b). pYW16-3, pYW11-16, and pYW11-3 contained single HA tags at the C terminus, whereas pYW16-11 contained three tandem HA tags at the C terminus. Initial attempts to construct a fusion protein containing c followed by c′ using the same strategy failed because of cleavage of the fusion protein in the linker region connecting the two subunits. To solve this problem, the linker region was replaced with the 14-amino acid linker from pYW3-16 (Fig. 1b), which then gave a stable fusion construct. pYW3-11 again contained a single HA tag at the C terminus. For construction of 11-3pRS316, an Xhol and Sphl fragment of pYW11-3 was recloned into pRS316. For construction of 3-16pRS316, an EcoRI and Sall fragment of pYW3-16 was recloned into pRS316. The oligonucleotides used for amplification of fusion genes are as follows: pYW3-11 forward, GTCGGATCCTTCTTGTTAAGGACATCCGCTTCTTTTTCGGGTTCCG, and pYW3-11 reverse, GAAACTAGTTAGCGTA-GTCGGGCACTGCTAGGG; pYW3-16 forward, GTCGGATCTTCTTTGTTAAGGACATCCGCTTCTTTTTCGGGTTCCG, and pYW3-11 reverse, GAAACTAGTTAGCGTA-GTCGGGCACTGCTAGGG; and the same reverse primer as pYW3-11; pYW11-3 forward, CCGGATGTACACTGAAATGTGTGCTCGT, and pYW11-3 reverse, CCGGAATCTTCTAGCCGCTAGTCCGGGCACGTGCTAGGG; pYW16-3 forward, GGGACGCGTGACTGAATTGTTGCTCTGTGCTC, and the same reverse primer as pYW3-11; pYW16-11 forward, AGCGGTGTCACTGACTGAAATGTGTGCTCGT, and pYW11-3 reverse, CCGGAATCTTCTAGCCGCTAGTCCGGGCACGTGCTAGGG; and pYW16-11 reverse, CCGGAATCTTCTAGCCGCTAGTCCGGGCACGTGCTAGGGTTAACCAGACAAACTCTTTGAGT.

Strains and Culture Conditions—The yeast strains and plasmids used in this study are listed in Tables 1 and 2. HA-tagged forms of each proteolipid subunit (and the c′(ΔTM1) construct) were expressed in the strain in which the corresponding gene was disrupted. All fusion proteins were also HA-tagged. The c-c′ and c′-c fusions were expressed in a strain disrupted in subunit c′ but containing the endogenous copies of subunits c and c′. Similarly, the c-c′ and c′-c fusions were expressed in a strain disrupted in c′ but containing the endogenous copies of subunits c and c′. The c′-c″ and c-c′″ fusions were expressed in a strain disrupted in c′ and c″ but containing the endogenous copy of subunit c by expressing all fusions in strains containing the wild-type c gene, we could ensure that the requisite number of copies of subunit c to form a functional ring would be present. All yeast strains were cultured in synthetic dropout minimal media supplemented with the appropriate amino acids or YEPD media buffered to pH 5.5 using 50 mm succinate/phosphate. To test for a vma phenotype, saturated cultures were diluted to an absorbance at 600 nm of 0.20. Serial dilutions of the cultures were prepared in YEPD media buffered to pH 7.5, and growth was monitored on YEPD plates buffered to the same pH.

Transformation and Selection—Yeast cells were transformed using the lithium acetate method (17). The transformants were selected on ura minus plates (for 11-3pRS316 and 3-16pRS316) or histidine minus plates for other strains, and growth phenotypes of the mutants were assessed on YEPD plates buffered with 50 mm KH2PO4 or 50 mm succinic acid to either pH 7.5 or pH 5.5.

Protein Preparation, SDS-PAGE, and Immunoblot Analysis—Whole cell lysates were prepared using a modification of the previously described protocol (18); 5-ml yeast cultures were grown overnight at 30 °C to an absorbance at 600 nm of 1.0. Cells were collected and washed with 1 ml of cold extraction buffer (150 mm ammonium sulfate, 10% glycerol, 1 mm EDTA, 200 mm Tris (pH 8.0), 2 mm dithiothreitol, and protease inhibitors). Cells were resuspended in 200 μl of extraction buffer and vortexed with glass beads five times for 1-min pulses. Unbroken cells were removed by sedimentation for 3 min at 4000 rpm, and samples of the supernatant were collected. Vacuolar membrane vesicles were isolated as described previously (19). Whole cell lysates and vacuolar membranes were separated by SDS-PAGE on 4–15% gradient acrylamide gels, as described previously (20). Expression of the fusion proteins was analyzed by Western blotting using the horseradish peroxidase-conjugated monoclonal antibody 3F10 against HA, whereas subunit a and subunit A were detected using the monoclonal antibodies 10D7 and 8B1-F3, respectively, followed by a horseradish peroxidase-conjugated secondary antibody, as described previously (21, 22). Blots were developed using a chemiluminescent detection method obtained from Kirkegaard & Perry Laboratories.

ATPase Activity and ATP-dependent Proton Transport—ATPase activity was measured using a coupled spectrophotometric assay (23). The reactions were carried out at 30 °C, and vacuolar membrane vesicles were incubated with Me3SO or 1 μM concanamycin A (in Me3SO) for 5 min prior to measurement of ATPase activity. ATP-dependent proton transport was measured by fluorescence quenching with the fluorescence probe 9-amino-6-chloro-2-methoxyacridine in transport buffer (50 mm NaCl, 30 mm KCl, 20 mm HEPES, 0.2 mm EGTA, 10% glycerol (pH 7.0)) as described (23) in the presence or absence of 1 μM concanamycin A.
Structure of V-ATPase Proteolipid Ring

RESULTS

Construction of Proteolipid Fusion Proteins—Previous results from studies of the bovine clathrin-coated vesicle and yeast V-ATPases suggest that the proteolipid ring of the yeast V-ATPase consists of four copies of subunit c and one copy of subunit c’ (16, 24). The arrangement of subunits in the proteolipid ring, however, is not known. To determine which arrangements of the subunits in the proteolipid ring are able to give rise to a functional V-ATPase complex, a series of six gene fusions was constructed between pairs of proteolipid subunit genes (Fig. 1a). These fusion constructs were then expressed in the appropriate deletion strains (Tables 1 and 2). By expressing proteolipid subunits as fusion constructs, the resultant oligomer is restricted in the possible arrangements that the proteolipid subunits can adopt. Assuming that each proteolipid subunit inserts in the ring with the same internal arrangement of transmembrane helices (see “Discussion”), there are five possible arrangements of the three proteolipid subunits in a six-membered ring (Fig. 2). In two of these, subunits c’ and c” are adjacent to each other, either clockwise or counterclockwise relative to each other (models A and B). In two other arrangements, subunits c’ and c” are separated by a single copy of subunit c (c’ and c” are again either clockwise or counterclockwise relative to each other, models C and D). In the final arrangement, subunit c’ and c” are on opposite sides of the six-membered ring separated by two copies of subunit c (model E). Each of these arrangements makes specific predictions about which proteolipid fusions should give rise to a functional V-ATPase (Table 3).

Topological studies of the yeast proteolipids suggest that subunit c” has five transmembrane helices with the N terminus on the cytoplasmic side of the membrane, whereas subunits c and c’ both have four transmembrane helices with both the N and C terminus on the luminal side of the membrane (25). It was therefore necessary to delete TM1 of subunit c” to ensure that construction of fusion proteins containing subunit c” at the C terminus did not change the way in which the transmembrane segments of subunit c” were inserted (Fig. 1a).

![Diagram of proteolipid subunit fusion proteins](image)

**Figure 1.** Proteolipid subunit fusion proteins. a, topological models of the six proteolipid fusion proteins created in this study. Transmembrane helices are numbered from the N terminus and are shown as follows: subunit c (gray gradient); subunit c’ (white); and subunit c” (solid gray). b, linker sequences used to fuse two proteolipids. The loop regions are shown in boldface, and the black lines below the sequence denote the amino acids derived from each proteolipid subunit.

| TABLE 1 | Yeast strains used in this study |
|---------|---------------------------------|
| Strain  | Genotype                        | Source/Ref. |
| TN101   | MATa, ura3-52, lys2-801, ade2-101, trp1::Δ63, his3::Δ200, leu2::Δ1, vma3::TRP1 | 26 |
| TN102   | MATa, ura3-52,lys2-801, ade2-101, trp1::Δ63, his3::Δ200, leu2::Δ1, vma11::TRP1 | 26 |
| TN103   | MATa, ura3-52,lys2-801, ade2-101, trp1::Δ63, his3::Δ200, leu2::Δ1, vma16::TRP1 | This study |
| YW100   | MATa, ura3-52, lys2-801, ade2-101, trp1::Δ63, his3::Δ200, leu2::Δ1, vma1::Kan’, vma16::TRP1 | 26 |
| LGY125  | SF388-1ds ade6 leu2 ura3 pep3-4 his4 vma3::kan’ vma11::Hyg’ | Dr. Tom Stevens |
| LGY139  | SF388-1ds ade6 leu2 ura3 pep3-4 his4 vma3::kan’ vma16::Nat’ | Dr. Tom Stevens |

| TABLE 2 | Plasmids used in this study |
|---------|------------------------------|
| Plasmid | Gene                        | Protein expressed | Strain expressed in | Source/Ref. |
| pRS413  | None                        | None              | TN103               | 26 |
| pTN3    | VMA3::I::HA                  | c                 | TN101               | 26 |
| pTN11   | VMA11::I::HA                 | c’                | TN102               | 26 |
| pTN16   | VMA16::I::HA                 | c’                | TN103               | 26 |
| pTN16-TM1 | VMA16(Δ2–41)-I::HA          | c’-(ΔTM1)         | TN103               | This study |
| PYW3-11 | VMA3::VMA11::I::HA           | c’-c              | TN102               | This study |
| PYW11-3 | VMA3::VMA3::I::HA            | c’-c              | TN102               | This study |
| PYW3-16 | VMA16::VMA3::I::HA           | c’-(ΔTM1)         | TN103               | This study |
| PYW16-3 | VMA16(Δ1–41)-I::VMA3::I::HA  | c’-(ΔTM1)-c’     | YW100               | This study |
| PYW11-16| VMA11::VMA16(Δ1–41)-I::HA   | c’-(ΔTM1)-c’     | YW100               | This study |
| 11-3pRS316 | VMA11::VMA16::I::HA      | c’-c              | LGY125              | This study |
| 3-16pRS316 | VMA3::VMA16(Δ1–48)-I::HA | c’-(ΔTM1)        | LGY139              | This study |
and others have shown previously that strains expressing subunit c’ from which TM1 had been deleted still gave rise to a functional V-ATPase complex (25, 26). Thus, c’(ΔTM1) was used in place of c’ in construction of fusion proteins in this study.

Care was taken to define the transmembrane segments as well as the minimal length of the loop connecting the two proteins. We defined the transmembrane helix/connecting loop boundaries of the yeast proteolipids by comparing them to the NtpK subunit of the V-type (Na+)-ATPase from Enterococcus hirae (27). If the connecting loop between the fused proteins is too short, it may not allow the two halves of the protein to adopt a native conformation or insert properly into the membrane. However, if the connecting loop is too long, it could allow for another c subunit to insert between the two halves of the fusion construct. Using a model of the c12 ring from the Escherichia coli F-ATPase and the c10 ring from E. hirae V-ATPase (15, 27), we estimate that a loop length of 10–14 amino acids is sufficient to connect adjacent subunits without allowing an additional copy of subunit c to insert between them. Therefore, all proteolipid fusions were constructed with a linker length of 10–14 amino acids.

![Diagram of V-ATPase proteolipid ring](image)

**FIGURE 2.** Possible arrangements of the three proteolipid subunits (c, c’, and c”) in a six-membered proteolipid ring of the yeast V-ATPase. Subunit c is shown in white, subunit c’ is shown in dark gray, and subunit c” is shown in light gray. The proteolipid ring is viewed from the luminal side of the membrane, with the arrangement of helices within each subunit modeled after that observed in the c ring of the Na+ V-ATPase from E. hirae (27). A five TM model of subunit c’ is assumed in this diagram.

**TABLE 3**

Ability of fusion constructs to form proteolipid rings shown in Fig. 2

| Model A | Model B | Model C | Model D | Model E | Result |
|---------|---------|---------|---------|---------|--------|
| c-c’    | +       | +       | +       | +       | +      |
| c-c’(ΔTM1) | -       | +       | +       | +       | +      |
| c’-c    | -       | +       | +       | +       | +      |
| c’(ΔTM1)-c’ | +       | -       | +       | +       | +      |
| c’(ΔTM1)-c | -       | +       | +       | +       | +      |

* + indicates the proteolipid ring shown in the corresponding model in Fig. 2 can be formed from the fusion construct indicated, whereas - indicates the ring cannot be formed.

In Vivo Function and Stability of V-ATPase Containing Proteolipid Subunit Fusions—To address the functional role of different fusions, we first tested the ability of these constructs to complement the phenotype of yeast strains from which the various proteolipid genes have been deleted. It has been shown previously that yeast lacking any of the V-ATPase proteolipid genes display a conditional lethal phenotype (vma−) characterized by an inability to grow at pH 7.5 but retaining the ability to grow at pH 5.5 (28). Data from our laboratory and others have shown previously that plasmid-borne VMA3, VMA11, VMA16, or VMA16ΔTM1 were able to complement their own disruption (25, 26). The c-c’(ΔTM1), c’(ΔTM1)-c’, and c’-c fusion proteins were all able to confer wild-type growth on YEPD media buffered at pH 7.5, whereas the c-c’, c’-c’(ΔTM1), and c’(ΔTM1)-c constructs could not (Fig. 3). We also observed that the c’-c’(ΔTM1) fusion construct did not support growth at pH 7.5 of the vma3Δ,vma16Δ double deletion strain, whereas the c’-c fusion construct did not support growth at pH 7.5 of the vma3Δ,vma11Δ double deletion strain (data not shown). This latter result is consistent with the need for multiple copies of subunit c, which cannot be provided by the fusion constructs alone in the double deletion strains.

Next the expression and stability of the fusion proteins as well as their ability to assemble into a V-ATPase complex was tested. Western blot analysis was performed on whole cell lysates using the monoclonal antibody 10D7 for subunit a, 8B1-F3 for subunit A, and 3F10 for the HA tag. Because there is currently no available antibody raised against any of the proteolipid subunits, an HA epitope tag was added at the C-terminal end of all constructs. Three HA tags were added to the C terminus of c’(ΔTM1)-c’ because of the weak signal observed with only one tag. All the proteolipid fusions had apparent molecular masses of 32–36 kDa, whereas the monomers (c, c’, or c’(ΔTM1)) had a molecular mass of 17 kDa (Fig. 4). The lower level of antibody staining of the c’(ΔTM1)-c construct in whole cell lysate may reflect altered reactivity of the HA epitope in this construct or the low expression or degradation of the fusion protein in this case.
mutant strain. These results demonstrate that five of the six fusion proteins are expressed and are stable. Previous studies have shown that the loss of subunit c or subunit d results in reduced levels of subunit a (29), likely because of reduced subunit a stability when not assembled into V0. In contrast, because V1 assembles independently of V0 (29, 30), V1 subunits are unaffected by the deletion of V0 subunits. To determine whether the proteolipid fusion constructs were able to stabilize subunit a, Western blotting was performed on whole cell lysates. As expected, lysates from all strains, including that carrying the empty vector, showed normal levels of subunit A (Fig. 4). Lysates isolated from strains expressing c-c'-(ΔTM1), c'-c''-(ΔTM1)-c', or c'-c showed almost the same level of subunit a relative to strains carrying the c, c', or c''-(ΔTM1) controls. Strains carrying the c-c', c'-c''-(ΔTM1), or c''-(ΔTM1)-c showed reduced levels of subunit a, suggesting that these proteolipid fusions do not assemble properly.

To confirm this assembly defect and to test for proper targeting of the V-ATPase subunits to the vacuolar membrane, partially purified vacuoles were subjected to SDS-PAGE, and Western blot analysis was performed. As can be seen in Fig. 5, Western blots of isolated vacuoles carrying c-c''-(ΔTM1), c'-(ΔTM1)-c', or c'-c showed slightly reduced levels of subunits a and A relative to vacuoles isolated from the strains expressing the monomer of the three proteolipid subunits, whereas the c-c', c'-c''-(ΔTM1), and c''-(ΔTM1)-c constructs showed dramatically reduced levels of subunits a and A, showing that the latter three constructs were unable to assemble into either a stable V0 or V1V0 complex. These results suggest that the fusions c-c', c'-c''-(ΔTM1), and c''-(ΔTM1)-c cannot form the proper arrangement of proteolipid subunits in the V0 domain, whereas the c-c''-(ΔTM1), c'-(ΔTM1)-c', and c'-c are able to do so.

ATPase and Proton Transport Activity of V-ATPase Complexes Containing Proteolipid Fusion Proteins—To determine the effect of the fusion constructs on activity of the V-ATPase, both concanamycin-sensitive ATPase activity and ATP-dependent proton transport (as assessed by quenching of ACMA fluorescence) were measured in isolated vacuoles as described under “Experimental Procedures.” The data shown in Fig. 6 represents the ATPase and proton transport activities sensitive to 1 μM concanamycin. The activities are expressed relative to those measured for vacuolar membranes isolated from a yeast strain expressing wild-type subunit c'. For ATPase activity, this corresponds to 0.51 μmol of ATP/min/mg of protein. Representative traces for ATP-dependent ACMA quenching as a measure of proton transport are shown in Fig. 7. Previous results have suggested that retention of 20% of wild-type V-ATPase activity is sufficient to confer on cells a wild-type growth phenotype (31, 32). As expected, vacuolar membranes from strains expressing the c-c''-(ΔTM1), c'-(ΔTM1)-c', or c'-c fusion proteins had more than 25% of the wild-type ATPase and proton pumping activities, whereas vacuolar membranes from cells expressing the c-c', c'-c''-(ΔTM1), or c''-(ΔTM1)-c fusions showed almost no activity. The activities observed for the functional constructs are due to V-ATPase because both ATPase and proton transport activities are inhibitable by the specific V-ATPase inhibitor concanamycin. These data indicate that, although the c-c''-(ΔTM1), c'-(ΔTM1)-c', or c'-c fusion con-
structures do not give fully active complexes, the resultant complexes do possess significant V-ATPase activity.

**DISCUSSION**

The results presented in this study suggest that the three proteolipid subunits of the yeast V-ATPase adopt a particular arrangement in the proteolipid ring. Thus, of the five possible arrangements of proteolipid subunits described in Fig. 2, only that described by model B is consistent with the data obtained (Table 3). In this model, subunit c’ is adjacent to and counterclockwise from subunit c” as viewed from the luminal side of the membrane, with the four copies of subunit c making up the remainder of the proteolipid ring. Moreover, although the results do not rule out other possible arrangements of transmembrane segments within each proteolipid subunit, they do place some constraints on the relative arrangements in adjacent subunits (see below).

The proteolipid ring of the V-ATPases plays a crucial role in proton transport. The V-ATPases, like the F-ATPases, operate by a rotary mechanism (33, 34). ATP hydrolysis in the $V_1$ domain drives rotation of a rotary complex that includes the proteolipid ring of $V_0$ (34). Rotation of the proteolipid ring relative to subunit a within the $V_0$ domain drives unidirectional proton transport from the cytoplasmic to the luminal side of the membrane, as originally proposed for the F-ATPases (35, 36). Subunit a is held fixed relative to the catalytic head of $V_1$ by peripheral stalks or stators (37, 38) and is thought to provide access channels (hemi-channels) for protons to reach and leave the critical carboxyl groups on the proteolipid ring (1). Evidence for such hemi-channels has been obtained for the F-ATPase a subunit (39). Subunit a also contains a critical arginine residue in TM7, which interacts with the carboxyl groups on the proteolipid ring (40–42), thereby displacing protons from these sites into the luminal hemi-channel following rotation. By functioning as the proton acceptors and donors during rotary catalysis, the proteolipids serve an essential function in proton transport.

Because of its importance in proton transport, it is of interest to determine the structure of the proteolipid ring of the V-ATPases. A 3.9-Å resolution structure has been reported for the proteolipid ring of the F-ATPase from yeast mitochondria (43). The structure reveals a 10-membered ring with the outer part of the ring composed of TM2 of subunit c and the inner portion composed of TM1 (43). Individual c subunits have the TM1/TM2 loop facing the cytoplasmic side of the membrane, where it can interact with other subunits of the rotor. The critical acidic residues are located near the middle of TM2, although the structure is not of sufficiently high resolution to visualize the orientation of these groups. Modeling studies of the F-ATPase c ring based on the NMR structure of subunit c as well as cross-linking studies suggest that the buried carboxyl group is oriented inward toward a pocket formed between adjacent c subunits (15).

A 2.1-Å resolution structure has also been reported for the proteolipid ring of the (Na$^+$)-V-ATPase from the archaebac-
teria *E. hirae* (27). Like the eukaryotic V-ATPase subunits c and c’, the archaeobacterial protein is composed of four TMs with the critical carboxyl group located in TM4. Because the *E. hirae* ring contains 10 copies of this protein, the ring is composed of 40 transmembrane helices, twice as many as the mitochondrial c ring. Nevertheless, the buried carboxyl groups (which are bound to Na⁺) are located near the middle of TM4 and are oriented toward a common pocket formed by TM2, TM3, and TM4.

Unlike the proteolipid rings from both the F-ATPase and the (Na⁺)-V-ATPase, which contain only a single species of c subunit, the eukaryotic V-ATPases contain either two (for mammals) or three (for fungi) distinct proteolipid subunits. Moreover, mutagenesis studies in yeast indicate that all three proteolipid subunits are essential to form a functional V-ATPase complex and do not replace one another upon gene disruption (14). Subunit c”, which is common to all eukaryotic V-ATPases, is unique both in having an additional transmembrane helix at the N terminus and in containing an additional (nonessential) buried carboxyl group in TM5. By placing the essential carboxyl group in TM3 rather than TM5, an irregularity is introduced in the spacing of the carboxyl groups around the ring (Fig. 2). This irregularity may affect the ability of the free V₀ domain to passively conduct protons. In fact, one important difference between V₀ and F₀ is that free V₀ does not carry out passive proton conduction (44). This property is important because an essential mechanism of regulating V-ATPase activity *in vivo* involves reversible dissociation of the complex into its component V₁ and V₀ domains (45). The data in this study support the relative orientation of subunits c, c”, and c’ shown in model B. In this model, TM4 of subunit c is in proximity to TM2 of subunit c”, and TM5 of subunit c’ is in proximity to TM1 of subunit c’. As a result, helices containing essential glutamic acid residues are spaced evenly around the proteolipid ring except for the border of subunits c and c”, where they are in adjacent helices, and subunits c” and c’, where there is a gap lacking any critical acidic residue.

The proteolipid ring of the V₀ domain from eukaryotes also appears to differ from that in *E. hirae* in size. Early measurements of subunit stoichiometry using quantitative amino acid analysis suggested 5–6 copies of c plus c’ (not distinguishable by SDS-PAGE) and a single copy of subunit c” (16). Later studies employing epitope-tagged subunits demonstrated that yeast V-ATPase complexes contain single copies of subunits c and c” but multiple copies of subunit c (24). This study shows that the c”-c’ fusion is expressed, stable, and functional, further confirming that there are only single copies of the c’ and c” subunits present in the V₀ proteolipid ring. Thus the eukaryotic proteolipid ring appears to be both more complex and significantly smaller than that from archaeobacteria.

Attempts were made by our laboratory and the Stevens laboratory (University of Oregon) to construct homodimers of subunit c to establish whether the functional complex contains an even or odd number of c subunits. Tandem repeat DNA sequences of subunit c were constructed in plasmids and amplified in *E. coli* deficient in homologous recom-
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