Mutational Analysis of the Stator Subunit E of the Yeast V-ATPase*

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Subunit E is a component of the peripheral stalk(s) that couples membrane and peripheral subunits of the V-ATPase complex. In order to elucidate the function of subunit E, site-directed mutations were performed at the amino terminus and carboxyl terminus. Except for S78A and D233A/T202A, which exhibited V1V0 assembly defects, the function of subunit E was resistant to mutations. Most mutations complemented the growth phenotype of vmaΔ mutants, including T6A and D233A, which only had 25% of the wild-type ATPase activity. Residues Ser-78 and Thr-202 were essential for V1V0 assembly and function. The mutation S78A destabilized subunit E and prevented assembly of V1 subunits at the membranes. Mutant T202A membranes exhibited 2-fold increased Vmax and about 2-fold less of VVoh assembly; the mutation increased the specific activity of VV0 by enhancing the kcat of the enzyme 4-fold. Reduced levels of V1V0 and V0 complexes at T202A membranes suggest that the balance between V1V0 and V0 was not perturbed; instead, cells adjusted the amount of assembled V-ATPase complexes in order to compensate for the enhanced activity. These results indicated communication between subunit E and the catalytic sites at the Aβ3 hexamer and suggest potential regulatory roles for the carboxyl end of subunit E. At the carboxyl end, alanine substitution of Asp-233 significantly reduced ATP hydrolysis, although the truncation 229–233Δ and the point mutation K230A did not affect assembly and activity. The implication of these results for the topology and functions of subunit E within the V-ATPase complex are discussed.

Vacuolar H^+ -ATPase (V-ATPase)1 proton pumps are present on vacuoles, lysosomes, endosomes, secretory vesicles, and Golgi of all eukaryotic cells where they maintain the acidic pH required for the multiple cellular processes achieved in these organelles (1–3). In kidneys, osteoclasts, neutrophils, and other specialized cells (3), V-ATPases are located also on the plasma membrane, where ATP-driven proton translocation to the extracellular space supports urine acidification, bone resorption, and cytosolic pH regulation among other processes.

Amino acid sequence conservation and heterologous genetic complementation of V-ATPase subunits from mammals and plants in yeast (5–11) have shown that V-ATPases are structurally and functionally highly conserved pumps. The yeast V-ATPase complex consists of 14 different subunits organized into two domains, V1 and V0 (1–3). ATP hydrolysis is catalyzed in V1, which is peripherally bound to the cytosolic side of the membrane and consists of subunits A–H. Integral to the membrane is V0, which forms the proton transporting domain and consists of subunits a, c, c’, c”, d, and e (2, 3, 12). Connecting V1 to V0 are one central stalk made of subunits D and F and one to three peripheral stalks consisting of subunits C, E, G, H, and the amino terminus domain of the V0 subunit a (13–17).

V-ATPases operate by a rotary mechanism of proton transport (18, 19) similar to that of the F-ATPases (20, 21), and both molecular motors share functional homolog subunits involved in rotation and catalysis (22). Similar to subunits β and α of the mitochondrial F-ATPase enzyme, subunits A and B form a hexamer where ATP binds and is hydrolyzed by the V0 domain. Subunit D is functionally equivalent to γ of F1 and constitutes the rotating central stalk tightly associated with the proteolipid rotor in V0. Comparable with subunits a and c from F0, the ring of proteolipid subunits (c’, c”, and c’) and the V0 subunit a form the path for proton transport across the membrane. Despite an overall structural similarity, there are important differences that distinguish V-ATPases from F-ATPases. One difference is the possibility of two or three peripheral stalks per V1V0, rather than one, as revealed by two-dimensional electron microscopy analysis (13, 14).

The number of peripheral stalks and the stoichiometry of subunit E per V-ATPase complex are important issues yet unresolved. Although quantitative amino acid analysis suggests the presence of only one subunit E (23) and two subunits G (24) per complex, there is a question of whether two or three subunits E are assembled (25–27). Cross-linking studies showed that subunit E extends from the top of V1 to V0 and interacts with the external surface of subunit B at the Aβ3 hexamer (28, 29). At the periphery of the Aβ3 hexamer, subunit E is in close proximity to the peripheral stalk subunits C, H, and G of V1 (24) and the amino end of the V0 subunit a, which is predicted to anchor V1 at the membrane (30–32). By making contact with all components of the peripheral stalk(s), subunit E (and possibly G) may represent the foundation for the peripheral stalks (17, 28, 29). Subunits E and G assemble into E-G dimers (33), and two E-G dimers, associated each with a B subunit, could represent the two peripheral masses detected by two-dimensional electron microscopy of the yeast V1 complex (34).

The structure and number of peripheral stalks could play a role regulating V-ATPase function by reversible disassembly, a mechanism exclusive of the V-type pump (35–37). In the ab-

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1 The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine triphosphatase; V1V0, V-ATPase proton pump; MOPS, 4-morpholineethanesulfonic acid; MES, 4-morpholinepropanesulfonic acid; AMP-PNP, 5’-adenyl-β,γ-imidodiphosphate.

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Gene, was maintained in fully supplemented minimal medium lacking /H9004 subunits in a glucose-dependent manner (17, 39–42). Enzyme aldolase have been shown to interact with V-ATPase and endosomal membranes (RAVE) complex and the glycolytic subunit E within the V1Vo complex. Our results support a been characterized. This study, aimed at mapping functionally essential function of V-ATPases during yeast cell cycle progress-

The mutation D145G resulted in defective assembly of the function identified one functional mutation, the only V-ATPase (17, 24, 38) and dissociation of subunit C (35). New interactions acts with the A3B3 hexamer. At the carboxyl end half of subunit TCTTTGATTGATCGACC-3

/H11032-GACAAGATTGAAATTAACAACGC-

/H11032- AAACAAAATG-3

\[ Y160A, 5 \]

\[ T6A, 5 \] ACTGC-3

\-CGATGTCCTCCGCTATTGCTGCTTTGACACCA-

/H11032

sence of glucose, V1 dissociates from V0, and disassembly stops ATP hydrolysis and proton transport and helps preventing energy depletion. V1 inactivation is accomplished, at least in part, by major structural changes involving subunits D and H (17, 24, 38) and dissociation of subunit C (35). New interactions between subunit H and the central stalk subunit D are formed in V1 complexes free of V0 (24), where subunit H may bridge subunits E and G (17). Furthermore, reversible disassembly is a post-translational event (35), and interactions between V-ATPase subunits and other cellular proteins impart an additional level of regulation. The regulator of ATPase of vacuolar and endosomal membranes (RAVE) complex and the glycolytic enzyme aldolase have been shown to interact with V-ATPase subunits in a glucose-dependent manner (17, 39–42).

Genetic and biochemical studies have revealed the importance of subunit E for V1V0 assembly (43, 44) and its potential regulatory role by interacting with RAVE and other proteins (17, 41, 42). Genetic screens searching for loss of subunit E function identified one functional mutation, the only V-ATPase temperature-sensitive mutant that has been described (44).

The mutation D145G resulted in defective assembly of the V-ATPase complex and was very informative by showing the essential function of V-ATPases during yeast cell cycle progression. However, this is the only mutant for subunit E that has been characterized. This study, aimed at mapping functionally important regions along the open reading frame of subunit E, revealed new insights into the structural organization of the subunit E within the V1V0 complex. Our results support a model where the amino end of subunit E interacts with subunits H and G and Ser-78 is essential for E subunit stability and assembly. Thr-202 probably orients toward V1 and interacts with the Aβ2 hexamer. At the carboxyl end half of subunit E, Thr-202 has the capacity to regulate ATP hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Material and Strains**

Zymolase 100T was purchased from ICN. Concanamycin A was purchased from Wako Biochemicals. Octyl-β-D-glucopyranoside was from Calbiochem. Alkaline phosphatase-conjugated secondary antibodies were purchased from Promega. The QuikChange mutagenesis kit was from Stratagene. Amphotyls, prestained broad range molecular weight protein markers, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Bio-Rad. All other reagents were purchased from Sigma.

The vmaΔ strain, SF838-1Da vma4 (Mata ade6 leu2-3,112 ura3-52 pep4-3 his3-519 gal2 vma4::URA3) (43), was obtained from Patricia M. Kane. The wild-type strain referred to throughout the study is the wild-type strain (44).

**Site-directed Mutagenesis**

Mutagenesis was performed using the QuikChange site-directed mutagenesis kit from Stratagene according to the manufacturer’s protocol. The vma4 gene cloned in the BamHI site of the CEN vector pRS315 was used as template. The primers used for mutagenesis (shown below) and their complementary oligonucleotides (not shown) were as follows with the substitution sites underlined and the position of deletion indicated by an asterisk: S2A, 5′-CCGCTATATTGATCGACCAGCC-3′; T9A, 5′-CCGCTATATTGATCGACCAGCC-3′; S78A, 5′-GCTTTGCAAGACAGTTAAGGCGAGCAGCC-3′; T202A, 5′-GACAGAAGTTAAGGCGAGCAGCC-3′; K230A, 5′-GTCCTCCTCAAGACAGACGCGCTTCTCTTGAGGCAGC-3′; D233A, 5′-GACAGAAGTTAAGGCGAGCAGCC-3′. The mutant T202A served as template to generate the double mutant T202A/D233A by using the D233A oligonucleotides described above. Mutations were confirmed by sequencing at the Sequencing Facility of Iowa State University, and plasmids were used to transform the vmaΔ null (vma4::URA3) yeast strain by the lithium acetate method (45). Transformants were selected on SD–Leu, pH 5, and growth mutant phenotype was assessed on SD–Leu plates buffered to pH 5 as described above, plates buffered to 7.5 (pH was adjusted with 50 mM MOPS, 50 mM MES buffer, pH 7.5), and SD–Leu plates, pH 7.5, containing 90 μM CoCl₂ (44).

**Purification of Vacular Membranes**

Vacular membranes were purified by flotation as described before (46). Six liters of yeast cells grown overnight to 1 A₅₆₀/ml in SD–Leu, pH 5, medium were harvested by centrifugation at 5,000 rpm in a GSA rotor, washed in 10 mM Tris-HCl, pH 7.5, containing 1.2 mM sorbitol, and converted to spheroplasts by the addition of zymolase 100T (0.1 units/ A₅₆₀). Spheroplasts were washed in YEPD containing 1.2 μM sorbitol, and pellets were subjected to amotic lysis by Dounce homogenization in lysis buffer (10 mM MES-Tris, pH 6.9, 0.1 mM MgCl₂, 12% Ficoll). Vacuoles were floated by two consecutive Ficoll gradients (8 and 12%), converted to vacuolar membrane vesicles by dilution in 15 mM MES, pH 7, 4.8% glycerol buffer, and stored at –80 °C.

**Whole Cell Lysates**

Wild-type and mutant cells (30 ml) were grown to midlog phase (0.6–1.0 A₅₆₀/ml SD–Leu, pH 5, medium), harvested, and resuspended in 10 mM Tris-HCl, pH 9.4, containing 10 mM dithiobritrol. After a 5-min incubation at 30 °C, cells were washed in 10 mM Tris-HCl, pH 7.5, plus 1.2 mM sorbitol (washing buffer) and converted to spheroplasts by incubation with 10 units of zymolase, rocking for 20 min at 30 °C. Spheroplasts were resuspended in washing buffer, washed twice, and treated with addition of 50 units of cracking buffer (50 mM Tris-HCl, pH 6.8, 8 mM urea, 5% SDS, 1 mM EDTA, 5% β-mercaptoethanol) prewarmed to 50 °C. Lysis was completed by incubation at 50 °C for 20 min.

**Two-dimensional Electrophoresis**

Wild-type and D233A mutant vacuolar membranes were pelleted by centrifugation and resuspended in sample buffer (9.5 mM urea, 2% Triton 100, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7 amphotely, 0.4% Bio-Lyte 3/10 amphotely) at a final concentration of 2 μg/ml. Samples were kept at room temperature for at least 15 min and loaded (10–20 μg) on top of a capillary polycrylamide gel. When wild-type and D233A mutant vesicles were combined, 10 μg of each was loaded. Vesicles were overlaid with 20 μl of overlay buffer (9 mM urea, 0.8% Bio-Lyte 5/7 amphotely, 0.2% Bio-Lyte 3/10 amphotely, 0.005% bromphenol blue) and electrophoresed overnight at 250 V in a minigel system from Bio-Rad. For second dimension electrophoresis, capillary gels were loaded onto 10% SDS-polycrylamide gels and covered with cracking buffer, and protein was separated at 150 mV. Gels were transferred to a nitrocellulose membrane and analyzed by Western blots.

**Glycerol Gradient Centrifugation**

Vacular membranes were washed twice in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA buffer. Washed vacuolar membranes were resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM dithiobritrol, 0.1% β-mercaptoethanol, 10% glycerol at a final protein concentration of 5 mg/ml and solubilized in 2% octyl-β-D-glucopyranoside. Detergent-solubilized membranes were loaded on top of a 10.5-ml 20–50% glycerol step gradient (47, 48). Gradients consisted of equal volume steps varying by 5% glycerol containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.35% octyl-β-D-glucopyranoside, and a mixture of proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 2 μg/ml chymostatin, 1 μg/ml leupeptin). Gradients were centrifuged at 48,000 rpm (200,000 × g) for 16 h in a Beckman 90Ti rotor. Fractions (0.5 ml) were collected from the bottom of the tube. An aliquot was taken from each fraction to measure its density by using a hand-held refractometer. Fractions (0.45 ml) were precipitated by the addition of trichloroacetic acid to a final concentration of 10%, and pellets were resuspended in 30 μl of cracking buffer. 10 μl of each fraction was separated in 10% gels by SDS-PAGE and analyzed using Western blots. When indicated, selected fractions were loaded onto 12% polyacryl-

amide gels, and the V-ATPase subunits were visualized by silver staining. Otherwise, fractions (0.3 ml) were precipitated, and pellets were resuspended in 10 μl of 1 x Tris-HCl, pH 7.5, to determine protein concentration as described by Bradford (49).

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### Fig. 1. Subunit E sequence alignment

Multisequence alignment of the yeast E subunit with homologs in two other organisms, *Drosophila melanogaster*, and human (isoform E2). Alignment was performed using the program ClustalW. Identical and conserved amino acids are highlighted in black and gray, respectively. Yeast residues mutated to alanine are indicated by arrows, and the truncation generated by site-directed mutagenesis is flanked by carets.

### Other Biochemical Methods

**ATPase assays**—ATPase activity was measured spectrophotometrically using a coupled enzymatic assay, where ATP hydrolysis was followed at 340 nm coupled to oxidation of NADH at 37 °C (50). Each reaction contained 10–30 μg of vacuolar membrane protein (or 1–1.5 g of V1Vo, purified by glycerol gradients) in the presence and absence of 1–10 μM concanamycin A (51). To estimate Kₐ and Vₘₐₓ, ATPase activity of wild-type and T202A vacuolar membranes (30 μg) was measured in the presence and absence of 1.8 μM concanamycin A at ATP concentrations of 0.005, 0.01, 0.03, 0.075, 0.1, 0.2, 0.4, 0.75, 1, 2, and 3 mM in the presence of 5 mM MgCl₂.

**Protein Assays**—Protein concentration in isolated vacuolar membranes was measured as described by Lowry (52).

**Western Blots**—Vacuolar membranes were harvested by centrifugation, solubilized in cracking buffer at 70 °C for 20 min, and subjected to SDS-PAGE in 10% acrylamide gels or two-dimensional gel electrophoresis as described above. Proteins were transferred to nitrocellulose membranes at 150 mA overnight followed by a 1–2-h transfer at 200 mA. Membranes were blocked with 2% nonfat dry milk and incubated with the monoclonal antibodies 10D7, 8B1, 13D11, and 7A2 against subunits α, A, B, and C generously donated by Dr. Patricia M. Kane (Upstate Medical University, SUNY, Syracuse, NY) and with polyclonal antibodies against subunits E and D (a kind gift from Dr. Tom Stevens, University of Oregon, Eugene, OR). IgG anti-mouse and anti-rabbit secondary antibodies were conjugated to alkaline phosphatase, and blots were developed by the addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as described above.

### RESULTS

**Subunit E Mutagenesis**—Amino acid sequence alignment of the V-ATPase subunit E from yeast, mammals, and insects shows a high degree of conservation both at the amino terminus (36% identity, residues 1–92) and carboxy terminus (39% identity, residues 186–233) (Fig. 1). In this study, we made site-directed mutations at amino acids located within these two regions of the yeast subunit E in an attempt to gain new insights into their role for V-ATPase function and assembly. Alanine substitutions were performed at polar (Ser and Thr), charged (Asp and Lys), and aromatic (Tyr) residues at the amino terminal (Ser-2, Thr-6, Thr-9, and Ser-78) and carboxyl terminal (Tyr-160, Thr-202, Lys-230, and Asp-233) half of subunit E.

**Yeast Growth Phenotype**—The effect of these mutations on V-ATPase function was first examined by growing yeast cells in buffered to pH 5 but fail to grow at pH 7.5 and in the presence of CaCl₂ (53, 54). Serial dilutions of *umaΔ* cells expressing a mutant allele of the VMA4 gene from the CEN plasmid pRS315 are shown in Fig. 2. Only the mutations S78A and T202A/D233A exhibited a *uma* growth phenotype comparable with cells lacking the VMA4 gene (*umaΔ*) (44) and cells transformed with the plasmid alone (pRS315). This phenotype indicates that the mutations S78A and T202A/D233A impaired V-ATPase function in *in vivo*. All other mutations exhibited wild-type growth under these conditions, indicating that cells retained either full or partial V-ATPase activity (55, 56).

**Characterization of Vascular Membranes**—Cellular fractionation by Ficoll gradients allows isolation of uniform sized sealed vesicles where V-ATPases are oriented outwards (46). We studied assembly of the V-ATPase complex and ATPase activity at vascular membranes purified by Ficoll gradient centrifugation from each strain. Mutants were first examined for the ability to assemble V₁V₀ complexes (Fig. 3A). Western blot analyses showed that, with the exception of the mutant S78A, mutations did not prevent V-ATPase assembly at the vacuole as indicated by the presence of V₁ (A, B, C, and D) and V₀ (α and δ) subunits in these preparations. The mutant S78A did not assemble V₁ subunits at the vacuolar membrane; subunits A, B, C, and D from V₁ were not present, although the V₀ subunits α and δ were detected at wild-type levels. We measured ATP hydrolysis in the presence and absence of the V-ATPase inhibitor concanamycin A as a means of assessing V-ATPase specific activity in these membranes (51). As shown in Table I, vacuoles from cells harboring the mutations S2A, T9A, Y160A, and R230A showed concanamycin A-sensitive ATPase activity comparable with wild-type membranes (within 20–40%). The mutations T6A, at the amino end of subunit E, and D233A, at carboxyl end, showed significantly reduced ATPase levels (by 73% or more). We referred to these mutants as inactive *in vitro*, because they did not exhibit the *uma* mutant phenotype. No ATPase activity was detected in vacuolar preparations from S78A cells, as expected from their inability to grow at neutral pH and the absence of V₁ subunits at the membranes. Vacuoles from the mutant T202A were interesting, because they showed 2-fold more specific activity than wild-type membranes. The mutants S78A, T202A, D233A, and T6A were further characterized.

**The Mutation S78A Destabilizes Subunit E**—Vacuolar membranes from S78A mutants resembled membranes from *umaΔ* cells (43); both contain only V₀ complexes (Fig. 3A). Because the absence of any V₁ subunit, except for subunit H (57, 58), pre-
vents assembly and targeting of the other V₁ subunits to yeast vacuoles (43, 58), we performed whole cell lysates of the S78A mutant to determine whether subunit E and/or other V₁ subunits were destabilized by the mutation. As shown in Fig. 3B, subunit E was not detected by Western blots, although very low levels were detected in other preps. V₁ subunits A, B, and D were present, indicating that the mutation S78A destabilized subunit E and prevented V₁Vo assembly.

**The Mutation T202A Generates Highly Active Complexes**—V₁Vo complexes coexist with free Vo subcomplexes at the vacuolar membranes (35–37) and the ratio V₁Vo/Vo can be changed by manipulating V₁-Vo interactions (56, 59). In order to determine whether an increased V₁Vo/Vo ratio was responsible for the enhanced ATPase activity observed in T202A membranes, we made serial dilutions of T202A vacuolar membranes. The level of V₁ subunits was estimated by Western blots and compared with wild-type membranes. Quantitative immunoblots showed that T202A membranes had about 2-fold less of subunits A, B, D, and E than wild-type and slightly reduced amounts of subunit α (Fig. 4A), suggesting that the 2-fold increased activity measured was not generated by increased amounts of V₁ complexes assembled at the vacuoles. These results were confirmed by further purifying V₁Vo and Vo complexes from the vacuoles in glycerol gradients. Detergent-solubilized T202A vacuolar membranes were fractionated in 20–50% glycerol gradients and the distribution of assembled V₁Vo and V₀ complexes analyzed by Western blots. In order to detect comparable amounts of V₁Vo and V₀ complexes, gradients for T202A were loaded with about 2-fold more vacuolar protein (450 μg) than wild-type membranes (200 μg). As shown in Fig. 5, wild-type and T202A vacuolar membranes exhibited comparable sedimentation patterns for V₁Vo and free V₀ complexes, which sediment at 36 and 24% glycerol, respectively. T202A did not exhibit more assembled V₁Vo complexes than wild-type membranes (Fig. 5), suggesting that the mutation did not perturb the balance between V₁Vo and V₀. Instead, stable V₁Vo complexes were assembled that showed enhanced ATPase activity. Peak fractions containing purified T202A mutant V₁Vo complexes exhibited on average 4-fold more concanamycin A-sensitive specific activity (4.35–5.75 μmol of Pi/min/mg) than wild type (1.12–1.37 μmol of Pi/min/mg). To gain new insights into how the T202A mutation enhanced V-ATPase activity, the kinetics of ATP hydrolysis were examined. Fig. 4B shows the initial velocity of ATP hydrolysis at ATP concentrations of 0.005–3 mM for both wild-type and T202A vacuolar membranes. The mutant T202A had 2-fold increased Vₘₐₓ (Vₘₐₓ(T202A) = 0.236 ± 0.036 units/mg; Vₘₐₓ(WT) = 0.0986 units/mg). Since levels of V₁Vo were half-reduced at the membrane, and the Vₘₐₓ of the enzyme increased by 2-fold, we concluded that the mutation T202A increased the specific activity by enhancing the turnover number of V₁Vo complexes 4-fold.

**Characterization of the Mutants T6A and D233A**—In order to better understand why vacuolar membranes from the mutants T6A and D233A were inactive, we looked for the presence of subunit H. Although inactive V₁Vo complexes can be assembled in the absence of subunit H (24, 57, 60), lack of subunit H was not the reason for T6A inactivation. However, it could account for the lost of activity in D233A membranes. Western blots showed that subunit H was present in both mutant membranes (Fig. 3C), but it was significantly lower in D233A.

Subunit E assembled into V₁Vo complexes purified from cells carrying the mutation D233A migrated differently from the...
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FIG. 3. Assembly and stability of V-ATPase subunits. A, Western blot analysis of purified vacuolar membranes. Vacular vesicles were isolated from uaa4Δ cells transformed with the low copy plasmid pRS315 carrying wild-type VMA4 or VMA4 with the indicated mutation. Vacular protein (10 μg) solubilized in cracking buffer was loaded onto 10% polyacrylamide gels and separated by SDS-PAGE. Protein was transferred to nitrocellulose, and blots were probed with monoclonal antibodies against subunits a, A, B, and C and polyclonal antibodies against subunits D, E, and d. Alkaline phosphatase-conjugated goat anti-mouse (for primary monoclonal antibodies) or anti-rabbit (for primary polyclonal antibodies) secondary antibodies were added, and blots were developed by the addition of the alkaline-phosphatase substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. B, whole cell lysate of wild-type and mutant cells. uaa4Δ cells transformed with the low copy plasmid pRS315 alone, carrying wild-type VMA4 or VMA4 with the s78a or T202AD233A mutation, were grown overnight to midlog phase in SD-Leu, pH 5. A culture volume corresponding to A600 = 20–30 from each strain was removed and lysed as described under "Experimental Procedures." Proteins in the whole cell lysate (7 A600) were loaded onto a 10% polyacrylamide gel and separated by SDS-PAGE. Protein was transferred from the gels to nitrocellulose for detection of subunits A, B, D, E, and d by Western blots as described above. C, assembly of subunit H in T6A and D233A mutants. Vacular membranes from T6A, D233A, and wild-type cells were prepared as described in the legend to Fig. 3A. 10 μg of vacuolar protein was loaded per well of a 10% SDS-polyacrylamide gel, and the protein was not phosphorylated. Nevertheless, we performed two-dimensional gel analysis of wild-type and D233A vacuolar membranes, each alone (not shown) and in combination. Fig. 6B shows the migration of subunit E relative to subunits A and B visualized by Western blots. Two-dimensional gels revealed more alkaline pl for D233A than wild-type subunit E. We estimated a pl of 5.33 ± 0.03, which was 0.26 pH units higher than the pl estimated for the wild-type subunit E (5.07 ± 0.01). These results are consistent with the chemical nature of the mutation that eliminated the acidic functional group carboxylate, leaving an aliphatic methyl group at position 233. Because the phosphoryl group is a stronger acid than carboxylate, phosphorylation would have reduced the alkaline character of D233A, causing the protein to exhibit the same (or lower) pl as the wild type. These results indicate that modification by phosphorylation was not responsible for the shifted mobility.

Table I

*V*-ATPase specific activity in purified vacuolar membranes

Reported is the activity sensitive to concanamycin A inhibition. Coupled ATPase assays were performed as described under "Experimental Procedures" in the presence and absence of concanamycin A. Data are expressed as the mean ± S.E. for n separate vacuolar purifications. Relative mean values were used to express percentage of activity.

| Strain   | Activity | Percentage of activity |
|----------|----------|------------------------|
|          | μmol P/min/mg | %                     |
| Wild type | 0.18 ± 0.028 (n = 7) | 100                    |
| vma4Δ    | 0.014 ± 0.0066 (n = 2) | 8                      |
| S2A      | 0.16 ± 0.029 (n = 4)  | 89                     |
| T6A      | 0.049 ± 0.023 (n = 5) | 27                     |
| T9A      | 0.26 ± 0.054 (n = 5)  | 144                    |
| S78A     | 0.022 ± 0.0015 (n = 2) | 12                   |
| Y160A    | 0.14 ± 0.016 (n = 2)  | 78                     |
| T202A    | 0.39 ± 0.029 (n = 4)  | 216                    |
| K233A    | 0.24 ± 0.0089 (n = 5) | 133                    |
| D233A    | 0.046 ± 0.017 (n = 5) | 26                     |

The shifted migration was detected in whole cell lysates as well (Fig. 6A), suggesting that it was not generated during the time-consuming vacuolar purification protocol but that it is an inherent effect of the mutation D233A. Subunit E has been shown to interact with other cellular proteins, including RAVE, which aids glucose-dependent reassociation of V1 to V0 (17, 39, 40). Since reversible disassembly of the V-ATPase complex is a post-translational event (35) and Skp1, a component of the RAVE complex, interacts with phosphorylated proteins (61), we explored whether subunit E D233A was a phosphorylated form of the subunit. Alkaline phosphatase treatment did not change subunit E-D233A mobility (not shown), suggesting that the protein was not phosphorylated. Nevertheless, we performed two-dimensional gel analysis of wild-type and D233A vacuolar membranes, each alone (not shown) and in combination. Fig. 6B shows the migration of subunit E relative to subunits A and B visualized by Western blots. Two-dimensional gels revealed more alkaline pl for D233A than wild-type subunit E. We estimated a pl of 5.33 ± 0.03, which was 0.26 pH units higher than the pl estimated for the wild-type subunit E (5.07 ± 0.01). These results are consistent with the chemical nature of the mutation that eliminated the acidic functional group carboxylate, leaving an aliphatic methyl group at position 233. Because the phosphoryl group is a stronger acid than carboxylate, phosphorylation would have reduced the alkaline character of D233A, causing the protein to exhibit the same (or lower) pl as the wild type. These results indicate that modification by phosphorylation was not responsible for the shifted mobility.

The Double Mutation D233A/T202A Is Detrimental for Function and Assembly—We showed that alanine substitution of Thr-202 and Asp-233 separately generated distinctive V1Vo complexes harboring the mutation D233A.

V1Vo complexes harboring the mutation D233A.

V1Vo complexes harboring the mutation D233A.

The shift in mobility was detected in whole cell lysates as well (Fig. 6A), suggesting that it was not generated during the time-consuming vacuolar purification protocol but that it is an inherent effect of the mutation D233A. Subunit E has been shown to interact with other cellular proteins, including RAVE, which aids glucose-dependent reassociation of V1 to V0 (17, 39, 40). Since reversible disassembly of the V-ATPase complex is a post-translational event (35) and Skp1, a component of the RAVE complex, interacts with phosphorylated proteins (61), we explored whether subunit E D233A was a phosphorylated form of the subunit. Alkaline phosphatase treatment did not change subunit E-D233A mobility (not shown), suggesting that the protein was not phosphorylated. Nevertheless, we performed two-dimensional gel analysis of wild-type and D233A vacuolar membranes, each alone (not shown) and in combination. Fig. 6B shows the migration of subunit E relative to subunits A and B visualized by Western blots. Two-dimensional gels revealed more alkaline pl for D233A than wild-type subunit E. We estimated a pl of 5.33 ± 0.03, which was 0.26 pH units higher than the pl estimated for the wild-type subunit E (5.07 ± 0.01). These results are consistent with the chemical nature of the mutation that eliminated the acidic functional group carboxylate, leaving an aliphatic methyl group at position 233. Because the phosphoryl group is a stronger acid than carboxylate, phosphorylation would have reduced the alkaline character of D233A, causing the protein to exhibit the same (or lower) pl as the wild type. These results indicate that modification by phosphorylation was not responsible for the shifted mobility.

The Double Mutation D233A/T202A Is Detrimental for Function and Assembly—We showed that alanine substitution of Thr-202 and Asp-233 separately generated distinctive V1Vo complexes. Both strains exhibited wild-type growth (Fig. 2), but V1Vo from T202A cells was more active than wild type, and V1Vo from D233A was less active (Table I). We generated the double mutant T202A/D233A and examined the effect of this mutation on V-ATPase assembly and function. Combined, these mutations had a detrimental effect on V-ATPase function. T202A/D233A showed slower growth than wild-type cells at pH 5 and exhibited the uaa4Δ mutant growth phenotype; cells were unable to grow at neutral pH and in the presence of CaCl2. As expected from the cell growth characteristics, D233A/T202A had significantly less ATPase activity than the wild-type enzyme. The activity was reduced by more than 90% in vacuolar membranes (Fig. 7) and glycerol gradient fractions containing V1Vo complexes (D233A/T202A = 0.10 μmol of P/min/mg versus WT = 1.37 μmol of P/min/mg). Although subunit E mobility resembled the wild type, significantly lower
levels of subunits E, D, and C were detected (Figs. 5 and 7). V₁ and V₀ subunits were present and assembled into V₁V₀ (Fig. 5), but mutant D233A/T202A V₁V₀ complexes sedimented at lower density (34% glycerol) than wild-type (36% glycerol) (Fig. 5), showing that V-ATPase complexes were structurally different.

Carboxyl End Truncation of Subunit E—We further investigated the role of the carboxyl-terminal region of subunit E by truncating the last five residues (Arg-229, Lys-230, Phe-231, Phe-232, and Asp-233). Cells expressing a carboxyl end truncated subunit E (229–233/H9004) did not have the vma mutant phenotype; cells exhibited wild-type growth at neutral pH and in the presence of CaCl₂ (Fig. 2). V₁V₀ assembly was normal in this strain, but it was unexpected that vacuolar membranes retained wild-type levels of concanamycin A-sensitive ATPase activity (Fig. 7), because the mutant D233A was inactive (Table I). These observations showed that a single amino acid substitution at position 233 was more harmful than the removal of the last five residues of subunit E. We concluded that residues 229–233 of subunit E are not essential for V-ATPase assembly and function, and the significance of this outcome is discussed below.

DISCUSSION

Mutational analysis of the yeast V-ATPase structural genes has been critical to understanding V-ATPase function (1–3). These studies revealed the role of the V₁ subunits A and B for catalysis (55, 62–65) and the V₀ proteolipids subunits c, c’, and c” for proton transport (66–69) and have provided information on V-ATPase structure and its versatile cellular roles (1–3). Here we reported a mutagenesis study of subunit E and offered new insights into the functions and structural organization of the subunit E within the V-ATPase complex. Similar to subunit C of the peripheral stalk (56), the function of subunit E was considerably resistant to mutation; most mutants complemented the growth phenotype of vma4/H9004 cells. However, only the mutations S2A, T9A, Y160A, and K230A did not have any obvious mechanistic effect on catalysis.

At the amino terminus, we made point mutations of Ser-2, Thr-6, and Thr-9. T6A had an effect on the enzyme function. Subunit E-T6A sustained V₁V₀ assembly, but the enzyme lacked V-ATPase activity in purified vacuolar membranes. Although this outcome was surprising because T6A mutants complemented the growth phenotype of vmaΔ mutants, Table I shows that T6A mutant pumps retained sufficient residual levels of activity to sustain wild-type growth. V-ATPase mutants can lose up to 70–75% of their function and still retain wild-type growth characteristics (55, 56). In yeast (vma13Δ cells) (57, 58) and the bovine enzyme (16), inactive V₁V₀ complexes can be assembled in the absence of subunit H, and Lu et al. (70) showed that the amino end of subunit E (residues 1–83) interacts with subunit H in a two-hybrid system and in vitro. We examined whether the inhibitory effect of the mutation was caused by dissociation of subunit H in vitro during the process of vacuolar purification. Wild-type levels of subunit H were detected in vacuolar membranes isolated from T6A cells (Fig.
charsky  conserved, and both residues may be essential for G-E dimer formation within the peripheral stalk (74). Functional parallels may exist between Ala-79 of the b dimer of F1 (73) and has a specialized function at the membranes. Quantitative immunoblots of purified vacuolar membranes as well as glycerol gradient fractionation showed reduced levels of both V1V0 and V0 (Figs. 4A and 5). These observations suggest that cells adjusted the amount of V-ATPase subunits at the membrane in order to compensate for the enhanced activity of the enzyme, perhaps to prevent unnecessary ATP hydrolysis. Hyperactive mutants of the V-ATPase complex are unusual, and when these mutations are at peripheral stalk subunits, V1 and V0 interactions are generally affected. For example, mutations at subunit G enhanced ATPase activity, because V1 disassembly was partially reduced, increasing the amount of V1 sectors assembled at the membranes (59). Curtia et al. (56) found mutations at the carboxyl end of subunit C, which increased the kcat of the enzyme, but V1V0 complexes were less stable. Recent crystallographic structures of subunit C predict that some of these residues interact with the A3B3 hexamer (75). The mutant T202A exhibited a 2-fold increase of Vmax and about 2-fold less V0 assembly at the membranes, indicating that the mutation increased the kcat of the enzyme by a factor of 4. As expected, specific activity measured in peak fractions containing T202A mutant V1V0 complexes purified by gradients was 4-fold higher than the wild-type. To our knowledge, this is the first point mutation described in an accessory V-ATPase subunit that exhibited enhanced ATP hydrolytic activity while allowing stable assembly of V1V0 complexes. A mechanistic effect on catalysis that increases ATP hydrolysis could have occurred if the mutation T202A generated structural changes at the A3B3 hexamer. Communication between peripheral stalk subunits and the nucleotide binding sites was previously described. Treatment of the clathrin-coated vesicle enzyme with cysteine, which modifies Cys-254 in subunit A, results in dissociation of the A3B3 hexamer. AMP-PNP binding generates structural changes on subunit E (76, 77). Direct interactions between subunit E and the external surface of subunit B have been shown in yeast (28, 29). Cysteine cross-linking experiments using an energy-minimized model of the subunit B based on the crystal structure of the mitochondrial F1-ATPase showed that subunit E is in close proximity of Ala-15, Lys-45, and the residues Thr-202 is also highly conserved, and vacuolar membranes from the mutant T202A exhibited twice more concanamycin A-sensitive ATPase activity than wild-type membranes. This is the only hyperactive mutant we observed, and our results indicated that T202A enhanced activity by a catalytic effect of the mutation rather than by hyperassembly of V1V0 at the membranes.

Thr-202 is also highly conserved, and vacuolar membranes from the mutant T202A exhibited twice more concanamycin A-sensitive ATPase activity than wild-type membranes. This is the only hyperactive mutant we observed, and our results indicated that T202A enhanced activity by a catalytic effect of the mutation rather than by hyperassembly of V1V0 at the membranes. Quantitative immunoblots of purified vacuolar membranes as well as glycerol gradient fractionation showed reduced levels of both V1V0 and V0 (Figs. 4A and 5). These observations suggest that cells adjusted the amount of V-ATPase subunits at the membrane in order to compensate for the enhanced activity of the enzyme, perhaps to prevent unnecessary ATP hydrolysis. Hyperactive mutants of the V-ATPase complex are unusual, and when these mutations are at peripheral stalk subunits, V1 and V0 interactions are generally affected. For example, mutations at subunit G enhanced ATPase activity, because V1 disassembly was partially reduced, increasing the amount of V1 sectors assembled at the membranes (59). Curtia et al. (56) found mutations at the carboxyl end of subunit C, which increased the kcat of the enzyme, but V1V0 complexes were less stable. Recent crystallographic structures of subunit C predict that some of these residues interact with the A3B3 hexamer (75). The mutant T202A exhibited a 2-fold increase of Vmax and about 2-fold less V0 assembly at the membranes, indicating that the mutation increased the kcat of the enzyme by a factor of 4. As expected, specific activity measured in peak fractions containing T202A mutant V1V0 complexes purified by gradients was 4-fold higher than the wild-type. To our knowledge, this is the first point mutation described in an accessory V-ATPase subunit that exhibited enhanced ATP hydrolytic activity while allowing stable assembly of V1V0 complexes. A mechanistic effect on catalysis that increases ATP hydrolysis could have occurred if the mutation T202A generated structural changes at the A3B3 hexamer. Communication between peripheral stalk subunits and the nucleotide binding sites was previously described. Treatment of the clathrin-coated vesicle enzyme with cysteine, which modifies Cys-254 in subunit A, results in dissociation of the A3B3 hexamer. AMP-PNP binding generates structural changes on subunit E (76, 77). Direct interactions between subunit E and the external surface of subunit B have been shown in yeast (28, 29). Cysteine cross-linking experiments using an energy-minimized model of the subunit B based on the crystal structure of the mitochondrial F1-ATPase showed that subunit E is in close proximity of Ala-15, Lys-45, Glu-106, Asp-199, Glu-494, and Thr-501 of subunit B. Interactions between Thr-202 of subunit E and one or more residues at the surface of subunit B could be affected by the mutation and structural changes transmitted to the catalytic sites. Residues like Asp-199 of subunit B, which are closer to the ATP-binding site (28, 29), would be good candidates to interact with Thr-202 of subunit E. Although we consistently observed little or none of the 75-kDa proteolytic product of the a subunit commonly detected by the monoclonal antibody 10D7 in immunoblottings (Fig. 5) of this mutant, it is not known at this time whether this protection contributed to the enhanced catalysis of the mutant T202A.

Sequence alignment of subunit E showed 82% conservation between residues 195 and 233 of the carboxyl end (34% iden-
Site-directed Mutagenesis of the V-ATPase Subunit E

**Fig. 6. Analysis of the mutant D233A by whole cell lysate and two-dimensional gel electrophoresis.** A. pRS315 containing wild-type VMA4 or VMA4 with the D233A mutation in vma4Δ cells was grown overnight to midlog phase in SD–Leu, pH 5. Whole cell lysates were performed and analyzed by Western blots as described in the legend to Fig. 3B. B. Vacuolar protein from wild-type (10 μg) and D233A mutant membranes (10 μg) were combined and loaded onto the same capillary gel (first dimension). Proteins were electrophoresed for 16 h followed by separation on SDS-PAGE using 10% polyacrylamide (second dimension) as described under “Experimental Procedures.” Proteins were transferred to nitrocellulose, and blots were probed with monoclonal antibodies against subunits A and B and polyclonal antibodies against subunits E and D (not shown). pI values for mutant (5.07 ± 0.01) and wild-type subunit E (pI = 5.33 ± 0.03) were estimated by measuring subunit E migration (first dimension) relative to subunits B (pI = 4.79) and D (pI = 5.71).

**Fig. 7. V1V0 assembly and ATPase activity in vacuolar membranes from the mutants T202A/D233A and 229–233A.** Vacuolar vesicles from vma4Δ cells harboring wild-type VMA4, vma4-D233A, T202A, or vma4-229–233A in the plasmid pRS315 were purified by Ficoll gradient centrifugations as described under “Experimental Procedures.” V-ATPase assembly was analyzed by Western blots as described in the legend to Fig. 3 and V-ATPase specific activity measured in the presence and absence of the V-ATPase inhibitor concanamycin A. Three separate vacuum purifications were used to calculate specific activity of each mutant membrane (μmol of P/min/mg ± S.E.): 229–233A = 0.188 ± 7.0 × 10⁻⁴, T202A/D233A = 0.0154 ± 0.0144. Relative mean values were used to express percentage of activity as indicated in Table 1.

Although highly conserved in yeast, mammals, and insects, the last five residues of subunit E are not conserved in plants. We showed that truncation of the last five amino acids (229–233Δ) and alanine substitution of Lys-230 (K230A) did not affect the enzyme assembly and activity, suggesting that these residues are not essential for function of the yeast V-ATPase. However, the mutant D233A showed a different phenotype. D233A resembled the mutant T6A at the amino end because both were inactive for ATP hydrolytic activity in vitro but did not exhibit mutant growth characteristics. Partially active V1V0 complexes, which sustained wild-type growth, could be assembled in D233A membranes. The mutation could have destabilized E-H interactions, and further inactivation in vitro produced during vacuolar purification. Western blots showed significantly reduced levels of subunit H in D233A vacuolar membranes (Fig. 3C). The carboxyl end of subunit E did not interact with subunit H by two-hybrid assay (70), but it could be because the carboxyl end is further folded into a particular configuration. A functional folding was probably retained upon deletion of residues 229–233; but substituting Asp-233 with alanine disrupted it. Our findings could be explained if structural changes generated by the mutation D233A were transmitted to the A3B3 hexamer, to subunit H, or if the carboxyl end of subunit E directly interacts with H.

Although it was intriguing that the mutation D233A changed the migration characteristics of subunit E in polyacrylamide gels, the aberrant migration generated by the mutation had no a major effect of V1V0 assembly, because detergent-solubilized V1V0 complexes from D233A and wild-type membranes exhibited the same fractionation pattern in sucrose gradients. Two-dimensional gels and phosphatase treatment indicated that subunit E was not phosphorylated. A shifted migration has been described for subunit E in plants (76, 77), where an intramolecular disulfide bridge formed between conserved cysteine residues helps in regulating the V-ATPase activity. However, there are not cysteine residues in the yeast subunit E, and SDS-PAGE as well as two-dimensional gel analysis suggested that the mutation D233A instead generated an altered structure with SDS resistant to extreme denaturing treatment, which affected the way subunit E migrated in the gels.

Unlike mutations on the peripheral stalk subunits C (56) and G (59), we found that most mutations on the peripheral stalk subunit E did not cause specific loss of other subunits at the vacuolar membranes. Only S78A (described above) and the double mutant T202A/D233A showed major assembly defects. Whole cell lysates showed that D233A/T202A cells expressed stable subunit E and other V1 subunits (Fig. 3B), indicating...
that this mutation destabilized V₁ and V₀ interactions rather than subunit levels. V₁V₀ assembled at the membranes of T202A/D233A cells were structurally different from wild-type complexes, perhaps because they lacked more than one subunit. V₁V₀ fractionated at a different glycerol density (Fig. 5), and binding of subunit E to the complex was weakened. Fractions containing gradient-purified T202A/D233A complexes had only 7% of the wild-type specific activity and significantly reduced levels of subunits E, C, and D, suggesting that the double mutation destabilized the organization of the peripheral subunits at the vacuoles. Subunit C readily dissociates from V₁ when V₁V₀ reversibly disassembles (35, 38, 78), and its dissociation, which destabilizes V₁V₀ interactions (56), could explain the increased level of free V₀ detected in T202A/D233A membranes (Fig. 5). Defective assembly of V₁ probably accounts for the abnormal growth and inactivation in the double mutant, which had only background measurable activity comparable with vma4Δ mutant membranes (Table I).

New insights into the topology of the subunit E within the V-ATPase complex are emerging. The amino end of subunit E is probably closer to V₀ at the membrane because it interacts with subunit H (70), which localizes at the interface between V₁ and V₀ as shown by genetics studies (30) and two-dimensional electron microscopy analysis (16). Our results are consistent with a model where subunit E interacts with subunits G and H. A dimer involving subunits E and G may include the amino-terminal end of G (Tyr-46) (59) and residues surrounding Ser-78 in subunit E. Secondary structure predictions based on subunit E amino acid sequence analysis indicate that subunit E is highly α-helical. However, if subunit E forms a continuous helix that extends from V₁ to V₀, it would extend about 2-fold the distance between the top of V₁ and the membrane (14). Subunit E could have additional folding at its carboxy-terminal half. Our results support the idea that a more complex structure may reside at the carboxy-terminal region, which could possibly interact with subunit H. Although the carboxy-terminal domain of subunit E is highly conserved, no other functional information about this region is available, and our study opens new vistas about the function of the peripheral stalk subunit E during catalysis. For instance, subunit E could mediate cellular changes to the catalytic sites upon binding to regulatory proteins like RAVE, which interact with subunit E as a means of regulating V-ATPase function. We are investigating the effect of these mutations on V₁V₀ reversible disassembly, and future experiments will study RAVE-V₁ interactions in these and other mutations of subunit E.

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Mutational Analysis of the Stator Subunit E of the Yeast V-ATPase
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