The Amino-terminal Domain of the E Subunit of Vacuolar H\(^+\)-ATPase (V-ATPase) Interacts with the H Subunit and Is Required for V-ATPase Function*

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Vacuolar H\(^+\)-ATPases (V-ATPases) are highly conserved proton pumps that couple hydrolysis of cytosolic ATP to proton transport out of the cytosol. Although it is generally believed that V-ATPases transport protons by a rotary catalytic mechanism analogous to that used by F\(_{1}\)F\(_{0}\)-ATPases, the structure and subunit composition of the central or peripheral stalk of the multisubunit complex are not well understood. We searched for proteins that bind to the E subunit of V-ATPase using the yeast two-hybrid assay and identified the H subunit as an interacting partner. Physical association between the E and H subunits of V-ATPase was confirmed in vitro by precipitation assays. Deletion mapping analysis revealed that a 78-amino acid fragment at the amino terminus of the E subunit was sufficient for binding to the H subunit. Expression of the amino-terminal fragments of the E subunits from human and yeast as dominant-negative mutants resulted in dramatic decreases in bafilomycin A\(_1\)-sensitive ATP hydrolysis and proton transport activities of V-ATPase. Our data demonstrate the physiological significance of the interaction between the E and H subunits of V-ATPase and extend previous studies on the arrangement of subunits on the peripheral stalk of V-ATPase.

Vacuolar H\(^+\)-ATPases (V-ATPases)\(^*\) energize and acidify intracellular compartments of the vacuolar system of eukaryotic cells. They are essential for the normal function of secretory vesicles, the trans-Golgi network, endosomes, lysosomes, the yeast vacuole, and other intracellular membrane compartments (1, 2). In some specialized cells such as the intercalated cells of the kidney and the osteoclasts, V-ATPases reside at high levels on the plasma membrane, where they are responsible for transepithelial or cellular proton transport required for normal acid-base homeostasis and bone remodeling (2). Despite their wide range of physiological functions, V-ATPases share a highly conserved structure and common enzymatic properties that couple hydrolysis of cytosolic ATP to proton transport out of the cytosol (3). They contain two macrodomains or sectors: V\(_{1}\), a catalytic domain composed of peripheral membrane proteins, and V\(_{0}\), a transmembrane domain composed of intrinsic membrane proteins that transmits protons through the lipid bilayer (4). The V\(_{1}\) domain attaches to the V\(_{0}\) domain at the cytoplasmic face of the membrane. In Saccharomyces cerevisiae, the V\(_{1}\) domain is composed of eight distinct polypeptide chains, and the V\(_{0}\) domain contains five (4).

V-ATPases are evolutionarily related and structurally similar to F\(_{1}\)F\(_{0}\)-ATPases (F-ATPases) of bacteria, chloroplasts, and mitochondria (3). F-ATPases also have two sectors: F\(_{1}\), a peripheral attached complex composed of a catalytic head and a stalk, and F\(_{0}\), composed of intrinsic membrane subunits and a stator arm. F-ATPases have a rotary catalytic mechanism (5–7). The proton electrochemical gradient across the membrane drives translocation of protons through a pathway composed of the a and c subunits in the F\(_{0}\) sector, thought to drive rotation of a “wheel” of c subunits and consequently rotation of the attached F\(_{1}\) stalk. Rotation of the F\(_{1}\) stalk produces conformational changes in the catalytic head of F\(_{1}\), driving ATP synthesis. A stator arm, composed of long cytosolic extensions of the F\(_{0}\) subunit attached to the F\(_{1}\), holds the \(\alpha_{3}\beta_{3}\) catalytic head of F\(_{1}\) in place against the rotation of the central stalk.

Although the structure of V-ATPase remains less well defined, it is generally believed that the mechanical coupling of the V\(_{1}\) and V\(_{0}\) complexes occurs by a mechanism analogous to that of F-ATPase. Electron microscopy analysis confirms the presence of a central shaft and a peripheral stalk in bacterial and bovine V-ATPases (8, 9). The A and B subunits of V-ATPase are arrayed as a hexagon around a central stalk. The A subunit shares homology with the B subunit of F-ATPase and is the site of ATP hydrolysis. The B subunit, a homolog of the F\(_{0}\) subunit, may have a regulatory role in ATP hydrolysis. There are two isoforms of the B subunit that are encoded by different genes and have unique amino- and carboxyl-terminal sequences. The proteolipid subunits of V-ATPase are also homologous to the corresponding subunits of F-ATPase that form the proton pore. Other subunits of V-ATPase have limited or no amino acid sequence similarity to any subunits of F-ATPase. Although they are believed to function structurally in connecting the V\(_{1}\) and V\(_{0}\) domains either on the central or peripheral stalk, the subunit composition, structure, and subunit interactions of the V\(_{1}\) stalk and the putative stator arm of V-ATPases are not well understood.

Recent studies have begun to reveal some of the subunit interactions of the V-ATPase stalk. Using yeast two-hybrid assay and co-immunoprecipitation, Landolt-Marticorena et al. (10) showed that the A subunit of V-ATPase interacts with the

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H and A subunits. Arata et al. (11) showed that the B and E subunits of V-ATPase are in close proximity to each other by introducing unique cysteine residues into a cysteine-less form of the B subunit of V-ATPase and cross-linking purified vacuolar membranes. Their findings suggest that the E subunit forms part of the peripheral stalk of V-ATPase.

In this study, we report the site of interaction and physiological significance of the interaction between the V-ATPase E and H subunits. A 78-amino acid (aa) domain from the amino terminus of the E subunit of V-ATPase is required for binding to the H subunit and for maintaining proper V-ATPase function. Detection of the interaction between the E and H subunits of V-ATPase suggests that both subunits form part of the peripheral stalk of V-ATPase that anchors on V$_C$ via binding to the B subunit and on V$_o$ via binding to the a subunit.

**EXPERIMENTAL PROCEDURES**

**Materials**—The high-fidelity Expand Long enzyme system was purchased from Roche Molecular Biochemicals. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs Inc. (Beverly, MA). The yeast expression vectors YC2/CT and YES3/CT were from Invitrogen. Monoclonal antibody against the B subunit of V-ATPase (13D11) was purchased from Molecular Probes, Inc. (Eugene, OR). Monoclonal antibody against the His tag was from QIAGEN Inc. (Valencia, CA). Zymolase 100T was purchased from ICN (Costa Mesa, CA). Bafilomycin A$_1$, 3-aminotriazole, and isopropyl-1-thiogalactopyranoside were from Sigma. $^{[35S]}$Methionine was obtained from Amersham Biosciences.

**Yeast Two-hybrid Assays**—The 1.2-kb NcoI-SmaI cDNA fragment containing the entire coding region of the human E subunit of V-ATPase was isolated from a Bluescript SK recombinant plasmid and cloned in-frame into the multiple cloning site on the yeast expression vector pAS2-1 (Clontech, Palo Alto, CA). PJ69 yeast cells harboring the three reporter genes (12) were prepared by treatment with lithium acetate (LiAc). 0.1 mM 3-aminotriazole and isopropyl-$\beta$-thiogalactopyranoside were added to a final concentration of 0.2 mM and incubation was continued for 1 h. Bacteria were pelleted by centrifugation at 10,000 $\times$ g for 2 min and resuspended in ice-cold phosphate-buffered saline. Cell lysis was carried out by sonication (Sonics Dismembrator, Fisher) for 2 $\times$ 30 s. Triton X-100 was added to a final concentration of 1% to minimize aggregation of the fusion protein with bacterial proteins. Samples were centrifuged at 10,000 $\times$ g for 5 min, and the supernatants were collected, mixed with a 50% slurry of glutathione-agarose beads (Sigma) in phosphate-buffered saline, and incubated for 5 min at room temperature. The beads were collected by centrifugation, washed with ice-cold phosphate-buffered saline, and stored at 4°C in the presence of bovine serum albumin.

The subunit of V-ATPase from a human kidney cDNA expression library was amplified by high-fidelity PCR using the forward primer 5'-GCATGAAATTTGACACAAATGATCGTCCGACG-3' and the reverse primer 5'-ACACAAGGTCGCTCAGGCGGTCCGCG-3'. After restriction enzyme digestion with EcoRI and HindIII, the cDNA fragment was cloned in-frame into the B subunit and on V$_o$ via binding to the a subunit via the GST fusion vector pGEX-2TK (Amersham Biosciences). Clones containing the fusion construct were identified by DNA mini-preparation and restriction enzyme digestion and verified by DNA sequencing as described (14).

**Deletion Mapping Analysis**—Deletion mutants of the E and H subunits of V-ATPase were made by PCR using the high-fidelity Expand Long enzyme system and oligonucleotide primers as follows: wild-type Ea, 5'-ATTTCCATGCTGTCCTCAGGAGCTGTCGAC-3'; wild-type Eb, 5'-ATATTGCCCTCTGGCAGACACGATCGTAC-3'; wild-type Ha, 5'-ATATTGCCCTCTGGCAGACACGATCGTAC-3'; wild-type Hb, 5'-ATATTGCCCTCTGGCAGACACGATCGTAC-3'. Amplified cDNA was ligated into the pAS2-1 and pGEX-2TK expression vectors, respectively. The derived DNA sequences were analyzed by searching the GenBank$^{29}$EBI Data Bank for homology to known DNA and peptide sequences.

**Glutathione S-Transferase (GST) Precipitation Assays**—The entire coding region of the human V-ATPase E subunit cDNA was amplified by high-fidelity PCR as described above using the forward primer 5'-AATTGGACTGCCGATCTGCTGACGGTGACGTCGAC-3' and the reverse primer 5'-GAGAAGAGTCTGACCAACTCTCTGTTGAGCTGTCGAC-3'. The PCR-amplified product was purified with a QIAEX II kit (Qiagen Inc.), digested with the restriction enzymes BclII and EcoRI (Invitrogen) using the manufacturer's buffer solution, purified again with the QIAEX II kit, and cloned in-frame into the GST expression vector pGEX-2TK (Amersham Biosciences). Clones containing the fusion construct were identified by DNA mini-preparation and restriction enzyme digestion and verified by DNA sequencing as described (14). Bacterial colonies were inoculated in LB medium containing ampicillin (50 $\mu$g/ml) for 15 h at 20°C in a shaking incubator, harvested by centrifugation at 10,000 $\times$ g for 10 min, and resuspended in ice-cold phosphate-buffered saline. Cell lysis was carried out by sonication (Sonics Dismembrator, Fisher) for 2 $\times$ 30 s. Triton X-100 was added to a final concentration of 1% to minimize aggregation of the fusion protein with bacterial proteins. Samples were centrifuged at 10,000 $\times$ g for 5 min, and the supernatants were collected, mixed with a 50% slurry of glutathione-agarose beads (Sigma) in phosphate-buffered saline, and incubated for 5 min at room temperature. The beads were collected by centrifugation, washed with ice-cold phosphate-buffered saline, and stored at 4°C in the presence of bovine serum albumin.

**Immunoprecipitation**—Yeast cells were grown in supplemented minimal medium lacking methionine, harvested at centrifugation 4°C for 10 min, resuspended by incubation with 50 mM DTT for 10 min, and pelleted by centrifugation at 100,000 $\times$ g for 1 h. The pellets were washed in ice-cold phosphate-buffered saline, and resuspended in ice-cold phosphate-buffered saline. Samples were incubated with 20 $\mu$g of anti-HA affinity beads (Sigma) for 2 h at 4°C with gentle mixing. The beads were recovered by centrifugation, washed five times with Nonidet P-40 buffer (150 mM NaCl, 1% Nonidet P-40, and 50 mM Tris (pH 8.3) at 4°C for 30 min at 4°C with shaking. The beads were recovered by centrifugation, washed five times with Nonidet P-40 buffer, resuspended in Laemmli sample loading buffer (2% SDS, 10% glycerol, 100 mM diithiothreitol, 1% bromophen blue), and boiled for 3 min. After centrifugation, the supernatants were collected and applied to SDS-polyacrylamide gels for analysis by SDS-PAGE.
Interaction between V-ATPase E and H Subunits

RESULTS

Detection of the Interaction between the E and H Subunits of V-ATPase—We previously cloned the gene coding for the E subunit of V-ATPase (18) and made monoclonal antibodies against its protein product (19). To search for protein binding partners of the E subunit, we performed yeast two-hybrid assays using the E subunit as the bait, selecting from a human kidney cDNA expression library. One of the first cDNA clones identified as interacting with the E subunit was the H subunit of V-ATPase (18). This finding is consistent with a previous report indicating that the E subunit could be cross-linked to the H subunit of V-ATPase from bovine clathrin-coated vesicles (20).

In a yeast two-hybrid assay, true positive colonies express selectable reporter genes only when the bait fusion protein interacts with the target fusion protein. False-positive colonies carry plasmids that do not encode hybrid proteins interacting directly with the bait protein and express reporter genes by other mechanisms (21). The use of three different reporter genes (ADE2, HIS3, and lacZ) under the control of different promoters eliminates many false-positive colonies, particularly from proteins that do not bind to the bait fusion protein, but instead interact directly with promoter sequences flanking the Gal4-binding site or with proteins bound to the flanking sequences (12). Nevertheless, putative true positive clones should be independently tested for binding to the bait protein.

To confirm the interaction between the E and H subunits of V-ATPase, we performed precipitation assays with GST-E subunit fusion proteins. The coding sequence for the E subunit was ligated in-frame to the coding sequence for GST in a bacterial expression vector. The GST-E subunit fusion protein and the H subunit were expressed in bacteria, and the fusion proteins were purified using glutathione beads and incubated with the [35S]-labeled H subunit, respectively. After washing, proteins bound to the beads were recovered and analyzed by SDS-PAGE and autoradiography. Physical interaction was detected between the GST-E subunit fusion protein and the H subunit. No binding was observed between the H subunit and the GST native protein.
interaction with the H subunit (Fig. 2, second lane). Subsequent deletion mapping analysis of the amino terminus of the E subunit revealed a region of 78 aa (aa 1−78), E-N1, as the binding domain required for interaction with the H subunit (Fig. 2A). As indicated by β-galactosidase activity, the interaction between E-N1 and the H subunit generated target gene activation at a level similar to that observed with the full-length E and H subunits. Additional deletions in the 78-aa region, E-N11 and E-N12, failed to bind to the H subunit (Fig. 2A). Two overlapping deletion mutants covering the entire coding region of the H subunit were also prepared. Neither H subunit fragment showed interaction with the E subunit, suggesting that the entire H subunit might be required to fold to a particular configuration for interaction with the E subunit.

We used GST precipitation assays to examine the H subunit-binding domain at the amino terminus of the E subunit in greater detail. A construct was prepared expressing a chimeric protein consisting of the 78-aa fragment at the amino terminus of the E subunit in tandem with the GST protein. The fusion protein was purified using glutathione beads and incubated with the H subunit labeled with [35S]methionine. After extensive washing, the glutathione beads were boiled in sample buffer, and the supernatant was analyzed by SDS-PAGE and autoradiography. Binding of the labeled H subunit to the GST-E subunit fusion protein was evident in the precipitation assay (Fig. 1, first lane). In contrast, no binding to GST was detected (Fig. 1, second lane).

A 78-aa Amino-terminal Fragment of the E Subunit Interacts with the H Subunit—To identify domains in the E and H subunits required for the interaction, we carried out deletion mapping analysis. Two overlapping cDNA subfragments were amplified by high-fidelity PCR from the coding region of the E subunit, designated E-N (aa 1−148) and E-C (aa 132−226) (Fig. 2A), and fused in-frame with the Gal4 DNA-binding domain in the two-hybrid vector pAS2-1. The resulting fusion plasmids were cotransformed into PJ69 cells with the pACT2 vector containing the H subunit and selected for cell growth on dropout plates. Colonies were observed from cells harboring E-N, indicating that the interaction domain in the E subunit required for interaction with the H subunit resides in the amino terminus of the E subunit from aa 1 to 148. In contrast, no growth of yeast cells was detected on dropout plates when transformed with E-C. Subsequent deletion mapping analysis of the amino terminus of the E subunit revealed a region of 78 aa (aa 1−78), E-N1, as the binding domain required for interaction with the H subunit (Fig. 2A). As indicated by β-galactosidase activity, the interaction between E-N1 and the H subunit generated target gene activation at a level similar to that observed with the full-length E and H subunits. Additional deletions in the 78-aa region, E-N11 and E-N12, failed to bind to the H subunit (Fig. 2A). Two overlapping deletion mutants covering the entire coding region of the H subunit were also prepared. Neither H subunit fragment showed interaction with the E subunit, suggesting that the entire H subunit might be required to fold to a particular configuration for interaction with the E subunit.

We used GST precipitation assays to examine the H subunit-binding domain at the amino terminus of the E subunit in greater detail. A construct was prepared expressing a chimeric protein consisting of the 78-aa fragment at the amino terminus of the E subunit in tandem with the GST protein. The fusion protein was purified using glutathione beads and incubated with the H subunit labeled with [35S]methionine. As shown in Fig. 2B, the H subunit bound directly to the fusion protein containing the 78-aa amino-terminal fragment of the E subunit.

The Amino-terminal Domain of the E Subunit Is Required for ATP Hydrolysis and Proton-translocating Activities—To investigate the significance of the interaction between the V-ATPase E and H subunits, we examined the physiological effects of expression of the amino-terminal domain of the E subunit in yeast. The 78-aa fragment at the amino terminus of the human E subunit and the corresponding fragment of the yeast E subunit were separately cloned into the yeast expression vector
Discussion

V-ATPases and F-ATPases share the common structural elements of a peripheral catalytic head attached to a stalk domain to an intrinsic membrane domain for proton translocation. In each, the catalytic head is composed of a hexagon of three heterodimers of homologous subunits, and proteolipid subunits compose an essential part of the proton translocation domain. On the basis of these observations, it is believed that V-ATPases operate by a rotary mechanism analogous to that of F-ATPases. According to the rotary mechanism, ATP hydrolysis in the catalytic head drives rotation of a central stalk that is tightly linked to a ring of proteolipid subunits, each of which has a proton-binding acidic residue in the hydrophobic core. Rotation of the proteolipid ring is thought to induce conformational changes in individual proteolipid subunits as they pass across another membrane subunit, driving proton transport across the membrane.

Subunits of V-ATPase other than those in the catalytic head and the proteolipids have little or no sequence similarity to subunits of F-ATPases, suggesting that their function may be
The physiological significance of the interaction. First, the labeled H subunit protein bound to a GST-E subunit fusion protein, but not to GST alone (Fig. 1). Second, deletion mapping analysis showed that the 78-aa fragment at the amino terminus of the E subunit was responsible for binding to the H subunit (Fig. 2). Third, when the 78-aa fragment was expressed in yeast cells, dramatic decreases in ATP hydrolysis and proton transport activities were observed in purified vacuolar membrane vesicles, indicating disruption of V-ATPase function (Fig. 4). Taken together, our results support the notion that the interaction between the E and H subunits is physiologically relevant to V-ATPase structure and function.

The identification of interaction between the E and H subunits of V-ATPase extends previous studies on the arrangement of subunits on the peripheral stalk of V-ATPase. The a subunit (Vph1p) of V-ATPase has no homologous counterpart in F-ATPase and is an integral part of the V0 domain. The cytosolic amino terminus of the a subunit has been shown to bind to the H subunit (10). Further deletion mapping analysis showed that a truncated form of the H subunit (aa 160–478) with a deletion of the 159 aa at the amino terminus retains binding to the a subunit (10). It has been proposed that the interaction between the a and H subunits represents the point of contact between V0 and V1 (10). By introducing unique cysteine residues into a cysteine-less form of the B subunit of V-ATPase and performing cross-linking on purified vacuolar membranes, Arata et al. (11) showed that the B subunit is in close proximity to the E subunit on the outer surface of the V1 domain, indicating that the E subunit likely forms part of the peripheral stalk of V-ATPase. The E subunit can be cross-linked with cysteine residues introduced near the top of the B subunit and in a region closer to the membrane, but not within the A3B3 catalytic hexamer.

Although it has yet to be determined whether the B and E subunits interact directly, the data from this and previous studies (10, 11) indicate that the peripheral stalk of V-ATPase is composed of the a, E, and H subunits. The discovery of interaction between the E and H subunits of V-ATPase in this study provides a critical missing link that suggests a plausible structure for the peripheral stalk of V-ATPase. From the catalytic head, the B subunit provides an anchor on the peripheral stalk probably through interaction with the E subunit. In turn, the E subunit binds to the H subunit, which anchors on the V0 domain through interaction with the a subunit. It is unlikely, however, that the V-ATPase peripheral stalk subunits are arrayed linearly. For example, the a subunit of V-ATPase, a component of the peripheral stalk, not only interacts with the H subunit, but also directly interacts with the A subunit of the catalytic head (10). Further work will be needed to determine whether additional subunits of V-ATPase reside on the peripheral stalk and to identify the interacting surfaces on the various subunits.

The E subunit physically associates not only with the H subunit, but also with regulatory protein molecules that are not V-ATPase subunits. The glycolytic enzyme aldolase interacts in vitro and in vivo with the E subunit of V-ATPase (13). Upon immunofluorescent staining, aldolase co-localizes extensively with V-ATPase both in the kidney proximal tubule and in the osteoclast (13). In osteoclasts, cells that are essential for bone remodeling, aldolase undergoes a subcellular redistribution from cytoplasmic vesicles to the ruffled membrane together with V-ATPase following osteoclast activation (13). In yeast, deletion of the aldolase gene results in a profound reduction in cellular V-ATPase content and complete disassembly of V1 from V0 (13). Our recent data show that normal levels of V-ATPase content and assembly can be restored by aldolase.
complementation.2 Taken together, these data indicate a direct interaction between glycolysis, an ATP- and proton-generating pathway, and the E subunit of V-ATPase, an ATP-hydrolyzing and hydroxyl-generating proton pump.

The glycolytic pathway and the RAVE complex have recently been shown to play a role in the expression and assembly of V-ATPase (13, 24). Aldolase- and RAV1-deficient cells have a similar phenotype, failing to form intact V-ATPase in response to glucose. It remains to be determined whether the glycolytic pathway, and the RAVE complex act coordinately in glucose-induced assembly and disassembly of V-ATPase. Several other associated proteins interacting with V-ATPase have been identified, including the Na+/H+ exchanger regulatory factor (25), the platelet-derived growth factor receptor (26), αβ1 integrin (27), the human immunodeficiency virus protein Nef (28), and the human Papillomavirus protein E5 (26).

In summary, we have demonstrated that the amino-terminal domain of the V-ATPase E subunit interacts directly with the H subunit. Disruption of the E-H subunit interaction results in a marked decrease in both ATP hydrolysis and proton transport activities of V-ATPase. Our findings are consistent with the assignment of the E and H subunits to the peripheral stalk of V-ATPase, which is required for maintaining proper V-ATPase activity in response to proteins interacting with V-ATPase. Elucidation of the physiological significance of these protein-protein interactions is likely to further advance our understanding of this fundamental proton pump.

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