The Glycolytic Enzyme Aldolase Mediates Assembly, Expression, and Activity of Vacuolar H\(^+\)-ATPase*

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Ming Lu‡§, Yuri Y. Sautin¶, L. Shannon Holliday**, and Stephen L. Gluck‡

From the ‡Department of Medicine, University of California, San Francisco, California 94143 and the Departments of ¶Orthodontics, University of Florida College of Dentistry, Gainesville, Florida 32610

Vacuolar H\(^+\)-ATPases (V-ATPases) are a family of highly conserved proton pumps that couple hydrolysis of cytosolic ATP to proton transport out of the cytosol. How ATP is supplied for V-ATPase-mediated hydrolysis and for coupling of proton transport is poorly understood. We have reported that the glycolytic enzyme aldolase physically associates with V-ATPase (Lu, M., Holliday, L. S., Zhang, L., Dunn, W. A., and Gluck, S. L. (2001) J. Biol. Chem. 276, 30407–30413). Here we show that aldolase interacts with three different subunits of V-ATPase (subunits a, B, and E). The binding sites for the V-ATPase subunits on aldolase appear to be on distinct interfaces of the glycolytic enzyme. Aldolase deletion mutant cells were able to grow in medium buffered at pH 5.5 but not at pH 7.5, displaying a growth phenotype similar to that observed in V-ATPase subunit deletion mutants. Abnormalities in V-ATPase assembly and protein expression observed in aldolase deletion mutant cells could be fully rescued by aldolase complementation. The interaction between aldolase and V-ATPase increased dramatically in the presence of glucose, suggesting that aldolase may act as a glucose sensor for V-ATPase regulation. Taken together, these findings provide functional evidence that the ATP-generating glycolytic pathway is directly coupled to the ATP-hydrolyzing proton pump through physical interaction between aldolase and V-ATPase.

V-ATPase consists of two macrodomains, V\(_1\), a catalytic sector, composed of peripheral membrane proteins, and V\(_0\), a transmembrane sector, composed of intrinsic membrane proteins, that transmits protons through the lipid bilayer (4). The V\(_1\) sector attaches at the cytoplasmic face of the membrane to the V\(_0\) sector. Dissociated V\(_1\) and V\(_0\) sectors of V-ATPase in yeast are present in a dynamic equilibrium with the fully assembled proton pump (4). ATP-driven proton transport requires structural and functional coupling of the V\(_1\) and V\(_0\) sectors. The dissociated V\(_1\) sector lacks ATP hydrolytic activity, and the free V\(_0\) sector does not form an open proton pore (5). In yeast cells, the coupling of the V\(_1\) and V\(_0\) sectors is governed by glucose availability. In cells grown in glucose, the V\(_1\) and V\(_0\) sectors are assembled into V-ATPase (6). Upon glucose deprivation, V\(_1\) dissociates from V\(_0\). A panel of yeast mutants with deficiencies in the Ras-cAMP pathway, the main glucose repression/derepression pathway, and the respiratory pathway has been investigated (7). V-ATPase was found to assemble normally in all the yeast mutant strains tested, suggesting that glucose-induced assembly of V-ATPase is independent of these pathways (7).

The glycolytic pathway comprises nine enzymatic steps that convert one glucose molecule with six carbon atoms into two molecules of pyruvate, each with three carbon atoms. At the end of glycolysis, the ATP balance sheet shows a net profit of two ATP molecules/glucose molecule. For most animal cells, glycolysis is only a prelude to the citric acid cycle since the pyruvic acid quickly enters the mitochondria to be completely oxidized to CO\(_2\) and H\(_2\)O. However, in yeast such as *Saccharomyces cerevisiae*, glycolysis is the preferred metabolic pathway for ATP generation (8). Instead of being degraded in mitochondria, the pyruvate molecules generated in yeast cells stay in the cytosol and are converted into ethanol and CO\(_2\), which is then excreted. Although yeast cells can utilize a variety of carbon sources, they use up the available glucose before turning to alternative fuels (9). Addition of glucose to carbon-starved yeast cells or cells growing on a nonfermentable carbon source results in two major changes in enzymatic activities and gene expression. First, glucose represses expression of genes encoding proteins in the respiratory pathway and enzymes for utilization of alternative carbon sources. Second, glucose induces expression of genes required for glucose utilization, including genes encoding glycolytic enzymes (9).

In a search for protein partners that interact with V-ATPase, we discovered that the glycolytic enzyme aldolase binds directly to V-ATPase (10). In yeast cells deficient in aldolase, we observed complete disassembly and a dramatic reduction in the steady-state level of V-ATPase (10). In this report, we show that yeast mutant cells deficient in aldolase display a growth phenotype similar to that observed in V-ATPase subunit dele-
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tion mutants. V-ATPase abnormalities observed in aldolase deletion mutant cells can be rescued to normal levels by aldolase complementation. These data provide functional evidence that the glycolytic enzyme aldolase regulates V-ATPase assembly, protein expression, and activity by physical association with the proton pump.

EXPERIMENTAL PROCEDURES

Materials—The high fidelity Expand Long enzyme system was purchased from Roche Applied Science. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA). The yeast expression vector YC2/CT and polycistronic rabbit antisera against aldolase were from Invitrogen. Monoclonal antibody against the B subunit of V-ATPase (13D11) was purchased from Molecular Probes (Eugene, OR). The monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from BioGenesia (Kingston, NH). Zymolyase 100T was purchased from ICN (Costa Mesa, CA). Bafilomycin A1, 3-aminotriazole, and isopropylthiogalactoside were from Sigma.

 Yeast Two-hybrid Assays—High fidelity PCR was performed to amplify a cDNA fragment coding for 389 aa at the amino terminus that represents the cytoplasmic portion of the human a4 subunit of V-ATPase using the forward primer 5‘-ATGGCGTCTGTGTTTCGAAG-3‘ and the reverse primer 5‘-GTACGGCAGCCCAGACCTAAG-3‘. The cDNA fragment corresponds to the 100 aa at the amino terminus of the human B1 subunit of V-ATPase was amplified by PCR using the forward primer 5‘-ATGGAGATAGACAGCAGGCC-3‘ and the reverse primer 5‘-CATCGATCCCTGATGTCC-3‘. The cDNA fragment that corresponds to the 100 aa at the carboxyl terminus of the human B1 subunit of V-ATPase was amplified by PCR using the forward primer 5‘-ACACGGCAGCCCAGACCTAAG-3‘ and the reverse primer 5‘-TAAGGCGCAGCAGCCCACCC-3‘. The amplified cDNA fragments were cloned in-frame with the GAL-4 DNA binding domain on the yeast expression vector pAS2-1 (Clontech). PJ69 yeast cells harboring three reporter genes for adenine, histidine, and β-galactosidase were transformed with the cDNA constructs and spread onto 100-mm plates of minimal synthetic medium lacking tryptophan (10). Colonies of yeast cells were allowed to grow for 4 days and harvested for subsequent transformation with a human kidney cDNA expression library fused in-frame with the GAL-4 activation domain in the vector pACT2 (Clontech). After selection for growth on adenine/Leu/Try triple dropout plates, positive clones were streaked on His/Leu/Try dropout plates in the presence of 1 mM 3-aminotriazole and subsequently assayed for β-galactosidase activity. Analysis of the positive colonies was carried out as described previously (10). Yeast cells were grown overnight to stationary phase. DNA was isolated and introduced into bacteria by electroporation. DNA sequencing was carried out by incubation with BigDye Terminators and read by an automatic DNA sequencer (PE Applied Biosystems, Foster City, CA). Amino acid sequences were analyzed using the GenBank™ data base for homology to known DNA and peptide sequences.

Glutathione S-Transferase (GST) Precipitation Assays—The cytoplasmic portion of the human a4 subunit of V-ATPase was amplified by high fidelity PCR as described above using the forward primer 5‘-AAATGGCATTCCAGCTGCATACGGCAAGCTGACCTGCTGAGCC-3‘ and the reverse primer 5‘-GAGAGAATTCGTCCAAAAACTTCCTGTTGGCATTTGC-3‘. A 150-aa cDNA fragment from the amino terminus of the human B1 subunit of V-ATPase was amplified using the forward primer 5‘-ATGGGATAGACAGCAGGCC-3‘ and the reverse primer 5‘-GCTGGCCAAGCTGCATACGGCAAGCTGACCTGCTGAGCC-3‘. The amplified PCR products were purified with Qiagen gel extraction kit, digested with restriction enzymes, ligated to the expression vector pGEX-2TK (Amersham Biosciences). Clones containing the fusion constructs were identified by DNA minipreparation and restriction analysis.

Purification of the fusion proteins was performed as described previously (10). Briefly bacterial colonies were inoculated into LB medium containing ampicillin and grown for 16 h at 37 °C in a shaking incubator. The cultures were diluted 1:10 and grown for 3 h, isopropylthiogalactoside was added to a final concentration of 0.2 mM, and incubation was continued for 1 h. Bacteria were pelleted by centrifugation at 10,000 × g for 2 min and resuspended in ice-cold phosphate-buffered saline and centrifuged again with QIAEX II beads, and cloned in-frame into the GST expression vector pGEX-2TK (Amersham Biosciences). Clones containing the antigen-agarose beads (Sigma) in phosphate-buffered saline and incubated for 5 min at room temperature. The beads were recovered by centrifugation and incubated with 35S-labeled aldolase in Nonidet P-40 buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris, pH 8) at 4 °C for 3 h with shaking. The beads were recovered by centrifugation, washed five times in Nonidet P-40 buffer, resuspended in Laemmli sample loading buffer (2% SDS, 10% glycerol, 10 mM dithiothreitol, and 0.001% bromphenol blue) and boiled for 3 min. After centrifugation, the supernatants were collected and applied to SDS-polyacylamide gels. The gels were dried for autoradiography analysis.

Immunoprecipitation and Immunoblotting—Yeast cells were grown in supplemented minimal medium lacking methionine, harvested by centrifugation at 1000 × g for 5 min, resuspended in pretreatment buffer (0% Triton, pH 9.0, 10 mM dithiothreitol), and incubated for 5 min. Spheroplasts were generated by treatment with zymolyase 100T (ICN) for 20 min in SPC buffer (1% glucose, 1% sorbitol, 50 mM K2HPO4, 16 mM citric acid, pH 5.8), labeled with [35S]methionine for 60 min, lysed in solubilization buffer (10 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA, 2 mM dithiothreitol, 1% polyoxyethylene-9-lauryl ether) for 15 min and incubated with the anti-B subunit antibody 13D11 (Molecular Probes) for 60 min. After another 60-min incubation with protein A-agarose beads, the immunoprecipitates were collected by centrifugation, washed three times, and incubated for 5 min at 70 °C in 50 μL of cracking buffer (50 mM Tris-HCl, pH 7.0, 8% s urea, 5% SDS, 5% β-mercaptoethanol) for SDS-polyacrylamide gel electrophoresis and autoradiography analysis. Quantitation of the V0 and V1 subunits was performed using a densitometer.

Vacuolar membrane vesicles were prepared from midlog phase yeast cells as described previously (11). Western blotting was carried out by separating V-ATPase immunoprecipitates on 10% polyacrylamide gels as described previously (10) using a Bio-Rad mini gel apparatus and transferring electrophoretically to Hybond nitrocellulose membranes (Amersham Biosciences) using a Trans-Blot apparatus (Bio-Rad). The membranes were incubated to block nonspecific binding in 5% nonfat dry milk in TTBS (10 mM Tris, pH 8.0, 500 mM NaCl, and 0.05% Tween 20) with gentle agitation for 1 h at room temperature. Polyclonal rabbit antisera against aldolase was diluted 1:1000 in TTBS and applied to the membranes for 1 h. After washing with TTBS, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG in TTBS for 1 h, washed five times in TTBS, and incubated with SuperSignal West Dura Substrate working solution (Pierce) according to the manufacturer’s instructions.

ATPase and Proton Translocating Activities—Bafilomycin A1-sensitive ATP hydrolysis of vacuolar membranes was assayed by measuring the production of inorganic phosphate as described previously (11). Briefly vacuolar membrane vesicles in ATPase buffer (150 mM NaCl, 2 mM MgCl2, 1 mM vanadate, 1 mM azide, pH 6.75) were preincubated for 15 min at room temperature in the presence and absence of bafilomycin A1. The reaction was initiated by addition of ATP in a final concentration of 3 mM and stopped by addition of trichloroacetic acid. The samples were extracted with chloroform to remove lipid and detergent. After centrifugation, the upper aqueous phase was transferred to clean test tubes and incubated with buffers containing ascorbic acid and ammonium molybdate. The concentration of inorganic phosphate was measured by a spectrophotometer at 700 nm and converted to rate of ATP hydrolysis.

Proton transport activity was measured by ATP-dependent quenching of acridine orange using a PerkinElmer Life Sciences fluorescence spectrometer with excitation at 493 nm and emission at 545 nm (11). Purified vacuolar membrane vesicles were resuspended in 25 mM Tris, pH 7.2, 25 mM KCl, 5 mM MgCl2, 0.5 mM acridine orange, and 5 mM MgCl2 in the presence and absence of bafilomycin A1. ATP was added at a final concentration of 5 mM to initiate transport.

RESULTS

The Glycolytic Enzyme Aldolase Interacts with Three Distinct Subunits of V-ATPase—We previously detected interaction between aldolase and the E subunit of V-ATPase (10). To search for protein molecules that interact with other subunits of V-ATPase, we carried out yeast two-hybrid assays using the a and B subunits of V-ATPase as baits, respectively. Subunit a is an integral membrane protein possessing an amino-terminal hydrophilic domain and a carboxyl-terminal hydrophobic domain containing multiple membrane-spanning segments (12–15). The 389 aa at the amino terminus that represent the cytoplasmic portion of the a4 subunit was cloned as the bait.
The catalytic "head" of V-ATPase consists of three pairs of A and B subunits arrayed as a hexagon. The A subunit is the site of ATP hydrolysis, and the B subunit may have a regulatory role (16, 17). The central portion of the B subunit is believed to be required for binding to the A subunit. To maximize chances for identification of protein molecules that may regulate V-ATPase but are not elements of the core structure of the enzyme, we cloned two regions, 100 aa each, from the amino and carboxyl termini of the B subunit as baits. Screening a human aldolase cDNA and clones with deletions up to 107 aa from the amino terminus of aldolase were found to grow on culture plates buffered at both pH 5.5 and pH 7.5 (Fig. 2B), displaying a growth phenotype similar to that observed in the V-ATPase subunit deletion mutants. In contrast, mutant cells deficient in the glycolytic enzyme glucose-6-phosphate isomerase were found to grow on culture plates buffered at both pH 5.5 and pH 7.5 (Fig. 2B), consistent with our previous observation that the assembly and expression of V-ATPase were much less affected in these mutant cells (10). These results support the notion that aldolase deficiency results in V-ATPase malfunction by disrupting the physical association between aldolase and V-ATPase.

**TABLE I**

Aldolase interacts with the α, β, and E subunits of V-ATPase on distinct interfaces

| Aldolase deletion mutants | pAS2-a | pAS2-B-N | pAS2-B-C | pAS2-E |
|---------------------------|--------|----------|----------|--------|
| Aldolase-(1–364)           | +      | +        |          | +      |
| Aldolase-(108–364)         | +      | +        |          | +      |
| Aldolase-(121–364)         | -      | -        |          | -      |
| Aldolase-(126–364)         | -      | -        |          | -      |
| Aldolase-(163–364)         | -      | -        |          | -      |
| Aldolase-(198–364)         | +      | -        |          | -      |

Simultaneous interactions of aldolase with the α, β, and E subunits of V-ATPase were further confirmed by in vitro binding assays (Fig. 1, E–H). In the absence of aldolase, no binding was detected among V-ATPase subunits (Fig. 1, F–H), suggesting that the interactions occur directly through aldolase. Our findings that aldolase binds to both the V₁ and V₀ subunits of V-ATPase support the notion that aldolase may act as a nucleus in assembly of V₁ and V₀ sectors into the V-ATPase holoenzyme.

Aldolase Deletion Mutant Cells Display a Growth Phenotype Similar to That Observed in the V-ATPase Subunit Deletion Mutants—Previous studies showed that deletion of individual c, B, or E subunits of V-ATPase disrupted V-ATPase assembly and resulted in undetectable levels of ATP hydrolytic and proton transport activities (18, 19). Yeast cells lacking the c, B, or E subunit of V-ATPase, however, were able to grow on culture plates buffered at pH 5.5 but not at pH 7.5, most likely due to abnormalities in cytosolic calcium homeostasis resulting in a lethal phenotype at high extracellular pH (20, 21). To investigate the relationship between aldolase and V-ATPase activity, we examined the growth pattern of a yeast mutant strain deficient in aldolase (22). The aldolase deletion mutant strain, fba1, expressed undetectable levels of aldolase protein (Fig. 2A) and displayed aldolase enzymatic activity below 2% of that observed in wild-type cells (22). The aldolase mutant cells were able to grow on culture plates buffered at pH 5.5 but not at pH 7.5 (Fig. 2B), displaying a growth phenotype similar to that observed in the V-ATPase subunit deletion mutants. These results support the notion that aldolase deficiency results in V-ATPase malfunction by disrupting the physical association between aldolase and V-ATPase.

**Restoration of V-ATPase Activity to Normal Levels by Aldolase Complementation—**Since aldolase physically interacts with V-ATPase in vivo and deletion of the aldolase gene results in complete disassembly and a 3-fold reduction in the steady-state level of V-ATPase (10), we examined whether the V-ATPase abnormalities observed in aldolase deletion mutant cells could be restored to normal levels by aldolase complementation. The open reading frame coding for the yeast aldolase gene was amplified by high fidelity PCR. The amplified cDNA fragment was verified by DNA sequencing and subsequently cloned into the expression vector pYC2/CT (Invitrogen), which is designed for inducible expression of recombinant proteins in S. cerevisiae. The vector contains the galactose-inducible GAL-1 promoter and harbors the CEN6 origin for non-integrative centromeric maintenance and low copy replication of the plasmid (generally one to two copies per cell). Aldolase deletion mutant cells were transformed with the aldolase-containing expression vector. The transformed cells were spread onto 100-mm culture plates containing 0.2% glucose and 2% galactose. Since the presence of glucose inhibits the growth of aldolase deletion mutant cells (22), aldolase-expressing colonies were selected by growth on glucose-containing plates and confirmed by Western blotting. The aldolase-expressing cells were subsequently examined for V-ATPase assembly and expression. Normal levels of assembly and protein expression of V-ATPase were observed as measured by immunoprecipitation using an antibody against the B subunit of V-ATPase, 13D11. Our data, therefore, demonstrate restoration of V-ATPase assembly and protein expression to normal levels in aldolase deletion mutant cells by aldolase complementation.

To examine whether V-ATPase activity was also restored, we prepared vacuolar membrane vesicles as described previously (11) and measured ATP hydrolytic activity that is sensitive to the specific inhibitor of V-ATPase, bafilomycin A₁. ATP hydrolysis of vacuolar membrane vesicles was assayed by measuring the production of inorganic phosphate (11). As shown in Fig. 3A, aldolase complementation restored ATP hydrolysis to normal levels in the aldolase deletion mutant cells. The proton transport activity of V-ATPase was also measured by tracking the ATP-dependent quenching of acridine orange using a fluorescence spectrometer with excitation at 493 nm and emission at 545 nm (11). As expected, aldolase complementation restored V-ATPase proton transport activity to normal levels (Fig. 3B).

**Up-regulation of Aldolase-V-ATPase Interaction in the Presence of Glucose—**Based on its interaction with the V₁ domain of V-ATPase and genetic manipulation analysis, the RAVE com-
Aldolase interacts with three distinct subunits of V-ATPase. A, the GST-a, GST-B, and GST-E subunit fusion proteins, along with the GST native protein, were purified using glutathione-agarose beads and incubated with $^{35}$S-labeled aldolase, respectively. After washing, proteins bound to the beads were recovered and analyzed by SDS-PAGE and autoradiography. Aldolase was detected to associate with all three fusion proteins. In contrast, no binding was observed between aldolase and the GST native protein. B, the truncated protein with a deletion of 107 aa from the amino terminus of aldolase bound to the GST-a and GST-B subunit fusion proteins but not to the GST-E subunit fusion protein. C, the truncated protein with a deletion of 121 aa from the amino terminus of aldolase bound to the GST-a subunit fusion only. D, the truncated protein containing the 110 aa from the amino terminus of aldolase was incubated with the GST-E subunit fusion protein. No binding was detected. E, His-tagged aldolase bound to nickel beads was incubated simultaneously with the MBP-a subunit fusion protein (82 kDa), the GST-B subunit fusion protein (40 kDa), and the GST-E subunit fusion protein (60 kDa). After washing, bound proteins were recovered and probed by Western blotting using antibodies against MBP, GST, and the E subunit of V-ATPase, respectively. F, the MBP-a subunit fusion protein bound to amylose resin was incubated simultaneously with the GST-B subunit fusion protein and the GST-E subunit fusion protein in the presence or absence of His-tagged aldolase (as indicated). The bound proteins were recovered and probed by Western blotting using antibodies against the His tag, MBP, and the E subunit of V-ATPase, respectively. G, the GST-B subunit fusion protein bound to glutathione beads was incubated simultaneously with the MBP-a subunit fusion protein and the His-tagged E subunit protein in the presence or absence of His-tagged aldolase. The bound proteins were recovered and probed by Western blotting using antibodies against the His tag, MBP, and the E subunit of V-ATPase, respectively. H, His-tagged E subunit was cleaved with thrombin and incubated with the E11 monoclonal antibody followed by addition of protein A beads. The MBP-a subunit fusion protein and the GST-B subunit fusion protein were added in the presence or absence of His-tagged aldolase. After incubation and washing, the bound proteins were recovered and probed by Western blotting using antibodies against the His tag, MBP, and the GST protein, respectively.
plex was proposed to play a role in glucose-dependent assembly of V-ATPase (23). Further studies, however, demonstrated that the interaction between RAVE and V1 was not glucose-sensitive, consistent with a constitutive rather than a regulatory role for the RAVE complex in V-ATPase assembly (24). We examined the interaction between aldolase and V-ATPase in vivo. Wild-type yeast cells were incubated with culture medium in the presence and absence of glucose. Vacuolar membrane vesicles were prepared as described previously (11). Fully assembled V-ATPase holoenzyme on the vacuolar membrane was purified by immunoprecipitation using the 13D11 antibody against the B subunit of V-ATPase. The purified V-ATPase preparation was probed using anti-aldolase antibodies. As shown in Fig. 4, a dramatic increase in interaction was observed between aldolase and fully assembled V-ATPase in the presence of glucose. This increase in interaction between aldolase and assembled V-ATPase was reversible upon glucose depletion followed by glucose readdition (Fig. 4A), consistent with the notion that aldolase plays a regulatory role in V-ATPase assembly, expression, and activity in a glucose-dependent manner.

To examine whether aldolase binds to free V1 and V0 sectors, yeast cell lysates were prepared in the presence and absence of glucose, immunoprecipitated using the 13D11 antibody against the B subunit of V-ATPase, and analyzed by Western blotting using anti-aldolase antibodies. Minimum levels of aldolase were detected in the absence of glucose (Fig. 4B), suggesting that aldolase does not bind to the free V1 sector. We also prepared vacuolar membrane vesicles in the absence of glucose and carried out immunoprecipitation using the 10D7 antibody that recognizes its epitope on the free V0 sector. The immunoprecipitates were probed using anti-aldolase antibodies. No aldolase was detected (Fig. 4C), suggesting that aldolase does not bind to the free V0 sector.

The Glycolytic Enzyme GAPDH Is Physically Associated with the Aldolase-V-ATPase Complex—Glycolytic enzyme complexes have been observed in retina and neural postsynaptic density (25, 26), suggesting that they may be involved in local ATP generation. Such complexes may improve the efficiency of sequential metabolic reactions. To determine whether glycolytic enzymes other than aldolase are complexed with V-ATPase, we
carried out immunoprecipitation using anti-V-ATPase antibodies. The V-ATPase immunoprecipitates were probed by Western blot using antibodies against aldolase, subunit a on V₀, and subunit A on V₁. The interaction between aldolase and assembled V-ATPase was increased by 8-fold in the presence of glucose as quantitated by a densitometer. B, to examine possible interaction between aldolase and the V₁ sector, equal numbers of wild-type yeast cells were incubated in YEP medium either with glucose (+) or without glucose (−). The cells were lysed and immunoprecipitated using the 13D11 antibody against the B subunit of V-ATPase. The immunoprecipitates were analyzed by Western blot using antibodies against aldolase and the a, A, and B subunits of V-ATPase, respectively. C, to examine whether aldolase binds to the free V₀ sector, yeast cells were incubated in YEP medium in the absence of glucose. Immunoprecipitation was carried out using the 10D7 antibody that recognizes its epitope on the free V₀ sector only. The immunoprecipitates were probed using antibodies against aldolase and the a, A, and B subunits of V-ATPase, respectively.

**FIG. 4.** Aldolase mediates V-ATPase assembly in response to glucose. A, equal numbers of wild-type yeast cells were incubated in YEP medium either with glucose (+) or without glucose (−) or initially without glucose for 20 min followed by readdition of glucose (+/−). Yeast vacuoles were purified, and immunoprecipitation was carried out using the monoclonal antibody 13D11 that recognizes the B subunit of V-ATPase (Molecular Probes). The V-ATPase immunoprecipitates were subsequently analyzed by Western blot using antibodies against aldolase, subunit a on V₀, and subunit A on V₁. The interaction between aldolase and assembled V-ATPase was increased by 8-fold in the presence of glucose as quantitated by a densitometer.

**DISCUSSION**

V-ATPases hydrolyze ATP and couple the energy derived from ATP hydrolysis to proton transport. Although V-ATPases play important physiological roles in a variety of cellular processes, how ATP is supplied to V-ATPase for coupling of proton transport is poorly understood. To reveal mechanism(s) underlying regulation of V-ATPases under physiological conditions is of paramount importance as this may provide us with much knowledge about the molecular basis for metabolic control of proton transport. The V-ATPase of the yeast *S. cerevisiae* has proven to be an excellent model for molecular genetic analysis of V-ATPases. First, manipulation of the yeast genome is relatively straightforward. Deletion mutant (knock-out) strains for virtually every yeast gene are commercially available. Second, shuttle expression vectors harboring various selectable markers can be used to introduce genes of interest into yeast cells with ease. Third, yeast cells can be cultured in large numbers, and V-ATPase can be purified on a routine basis for biochemical and physiological assays.

Assembly and disassembly of V-ATPase in *S. cerevisiae* were first reported to occur in the presence and absence of extracellular glucose (6). However, V-ATPase was found to assemble normally in a panel of yeast mutants with deficiencies in the known glucose-sensing pathways, including the Ras-cAMP, Snf1p, protein kinase C, and Rts1p pathways (7). Glucose-induced assembly of V-ATPase is, therefore, independent of these pathways. In a search for protein partners that interact with V-ATPase, we discovered that the glycolytic enzyme aldolase binds directly to V-ATPase (10). The interaction between aldolase and V-ATPase was further verified by *in vitro* and *in vivo* binding assays (10). Deletion of the aldolase gene in yeast is associated with complete disassembly and a 3-fold reduction in the steady-state level of V-ATPase (10). V-ATPase assembly and protein levels were also examined in a yeast deletion mutant strain deficient in glucose 6-phosphate isomer-
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...the glucose-6-phosphate isomerase gene (10). Normal levels of V-ATPase expression were observed in the glucose-6-phosphate isomerase deletion mutant cells (10). These observations suggest that the V-ATPase abnormalities in the aldolase deletion mutant cells are specific to disruption of the aldolase-V-ATPase interaction.

In this study, we initially examined the growth pattern of yeast mutant cells deficient in aldolase and observed a growth phenotype similar to that previously reported in the V-ATPase subunit deletion mutants (Fig. 2B). Our data suggest that aldolase deficiency in yeast cells results in V-ATPase malfunction by disrupting the physical association between aldolase and V-ATPase. Furthermore, when the wild-type aldolase gene was introduced and expressed in aldolase deletion mutant cells, abnormalities in V-ATPase assembly and protein expression were restored to normal levels. As a result, ATP hydrolysis and proton transport activities of V-ATPase were also restored to normal levels by aldolase complementation (Fig. 3). Thus, we provide functional evidence that aldolase mediates assembly, expression, and activity of V-ATPase in yeast cells. In a recent report, another glycolytic enzyme, phosphofructokinase, has been shown to interact with the α subunit of human V-ATPase (27). It remains to be determined whether the physical interaction between phosphofructokinase and V-ATPase is physiologically relevant to V-ATPase regulation in either mammalian or yeast cells.

We previously reported the interaction between aldolase and the E subunit of V-ATPase (10). In this study, we show two other subunits of V-ATPase (subunits a and B) also interact with aldolase. Thus, aldolase physically associates with three different subunits of V-ATPase (Table I). Deletions mapping analysis revealed that distinct domains exist on aldolase for interactions with the α, β, and E subunits of V-ATPase (Table I). Although the sites and determinants of aldolase-V-ATPase interaction under physiological conditions are yet to be determined, GST precipitation assays show that aldolase binds well to purified α, β, and E subunits of V-ATPase in vitro (Fig. 1). Since free V₁ and V₀ sectors exist in a dynamic equilibrium with fully assembled V-ATPase (4), it is plausible that aldolase may mediate V-ATPase assembly by binding simultaneously to free V₁ and V₀ sectors. A dramatic increase in interaction between aldolase and V-ATPase was observed in the presence of glucose (Fig. 4). These findings further support the notion that aldolase acts as a glucose sensor in mediating V-ATPase assembly.

Deletion of the aldolase gene in yeast cells results in both disassembly of V-ATPase and a reduction in V-ATPase proteins (10). These abnormalities can be restored by aldolase complementation (Fig. 3). Thus, our data support the notion that aldolase regulates V-ATPase assembly and protein expression through physical association with V-ATPase. Glucose deprivation, however, leads to disassembly of V-ATPase but shows no adverse effect on the steady-state levels of V-ATPase (6). Upon readdition of glucose, the disassembled V₁ and V₀ domains become fully assembled to normal levels (6). This suggests that disassembly of V-ATPase does not necessarily provide the signal for reduction in V-ATPase proteins; rather assembly and protein expression of V-ATPase are regulated by two distinct mechanisms.

The RAVE complex binds to the V₁ domain whenever it is present in the cytosol but not to V₀ or fully assembled V-ATPase (24). In the absence of glucose, the interaction between RAVE and V₁ increases (24). It has been suggested that the RAVE complex is recruited to interact with V₁ released from the vacuolar membrane by glucose deprivation and that, when extracellular glucose is restored, the V₁-RAVE complex rapidly dissociates to release the bound V₁ for V-ATPase reassembly (24). However, in the deletion mutant of the α subunit where the V₁ domain is properly assembled but remains entirely cytosolic, the levels of Rav1 and Rav2 do not change through glucose deprivation and readdition. Thus, the interaction between RAVE and V-ATPase is not glucose-sensitive; instead RAVE binds to V₁ whenever it is present in the cytosol (24). In contrast, we show that the interaction between aldolase and V-ATPase was increased dramatically in the presence of glucose (Fig. 4). It is not clear whether aldolase and the RAVE complex associate with free V₁ sectors in the same or different protein complexes. If aldolase, RAVE, and V₁ are found in the same protein complex, it may suggest that both aldolase and RAVE are required for V-ATPase assembly. Aldolase may act as the regulator for V-ATPase assembly because its expression is induced by glucose (9) and the interaction between aldolase and V-ATPase increases dramatically in the presence of glucose (Fig. 4). Alternatively, if aldolase, RAVE, and V₁ exist in different complexes, it would indicate that aldolase and RAVE may bind to V₁ in a mutually exclusive manner, which would support the notion that V₁ is released from its association with RAVE upon glucose addition and then binds aldolase for reassembly with V₀ into the V-ATPase holoenzyme.

In summary, we have demonstrated that the glycolytic enzyme aldolase interacts with three distinct subunits of V-ATPase. Aldolase deletion mutant cells display a growth phenotype similar to that observed in V-ATPase subunit deletion mutants. The V-ATPase abnormalities observed in aldolase deletion mutant cells can be restored to normal levels by aldolase complementation. Taken together, these findings support the notion that the ATP-generating glycolytic pathway is directly coupled to the ATP-hydrolyzing proton pump (Fig. 6). Since glycolysis also generates protons, coupling the V-ATPase to glycolysis may provide a means for titrating in situ cytosolic hydroxyl ions generated as a consequence of proton transport into the vacuole, effectively coupling proton transport to pyruvate generation, thus preventing the formation of an unphysiological alkaline pH layer near the pump (Fig. 6). The aldolase-V-ATPase interaction may have important implications for human disease. In patients with hereditary fructose intolerance, caused by mutations in aldolase B (28), infusion of fructose induces a profound increase in urinary pH (29) that may be a consequence of disruption of coupling of glycolysis to the V-ATPase and reduced proton secretion by the renal proximal tubule.

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