Physical Interaction between Aldolase and Vacuolar H^+-ATPase Is Essential for the Assembly and Activity of the Proton Pump*

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Vacuolar proton-translocating ATPases (V-ATPases) are a family of highly conserved proton pumps that couple hydrolysis of cytosolic ATP to proton transport out of the cytosol. Although V-ATPases are involved in a number of cellular processes, how the proton pumps are regulated under physiological conditions is not well understood. We have reported that the glycolytic enzyme aldolase mediates V-ATPase assembly and activity by physical association with the proton pump (Lu, M., Holliday, L. S., Zhang, L., Dunn, W. A., and Gluck, S. L. (2001) J. Biol. Chem. 276, 30407–30413 and Lu, M., Sautin, Y., Holliday, L. S., and Gluck, S. L. (2004) J. Biol. Chem. 279, 8732–8739). In this study, we generate aldolase mutants that lack binding to the B subunit of V-ATPase but retain normal catalytic activities. Functional analysis of the aldolase mutants shows that disruption of binding between aldolase and the B subunit of V-ATPase results in disassembly and malfunction of V-ATPase. In contrast, aldolase enzymatic activity is not required for V-ATPase assembly. Taken together, these findings strongly suggest an important role for physical association between aldolase and V-ATPase in the regulation of the proton pump.

Vacuolar proton-translocating ATPases (V-ATPases) are a family of highly conserved proton pumps that couple hydrolysis of cytosolic ATP to proton transport out of the cytosol. They reside on intracellular membranes of all eukaryotic cells and function to acidify a variety of intracellular compartments, including secretory vesicles, endosomes, lysosomes, the trans-Golgi network, and the central vacuole of yeast (1–4). Acidification of the vacuolar compartments plays an important role in a number of cellular processes, including receptor-mediated endocytosis, intracellular targeting of newly synthesized lysosomal enzymes, macromolecular processing and degradation, and coupled transport of small molecules. In mammals, V-ATPases also reside at high levels on the plasma membrane of some specialized cells such as kidney epithelial cells and osteoclasts, where they are responsible for transepithelial or cellular proton transport required for normal acid-base homeostasis of the body or bone remodeling (5). Mutations in V-ATPase subunits have been shown to cause renal tubular acidosis (6, 7) and osteopetrosis (8).

Although V-ATPases play important physiological roles in a variety of cellular processes, how the V-ATPase-directed proton transport is coupled to cellular metabolism remains poorly understood. Disassembly of V-ATPase in Saccharomyces cerevisiae was first reported to occur in the absence of extracellular glucose (9). Glucose-dependent assembly and regulation of V-ATPase was also observed in mammalian cells (10). 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 (11). The glucose-induced assembly of V-ATPase is, therefore, independent of these pathways. In a recent report, the glycolytic enzyme phosphofructokinase has been shown to interact with the “a” subunit of human V-ATPase (12). It remains to be determined whether the interaction between phosphofructose kinase and V-ATPase is physiologically relevant to V-ATPase regulation in either mammalian or yeast cells.

In a search for protein partners that interact with V-ATPase, we discovered that the glycolytic enzyme aldolase physically associated with three distinct subunits of V-ATPase (13, 14). We 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 mutant cells (14). 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 (14). As a result, ATP hydrolysis and proton transport activities of V-ATPase were also restored to normal levels by aldolase complementation (14). Taken together, these findings support the notion that the ATP-generating glycolytic pathway is directly coupled to the ATP-hydrolyzing proton pump by physical interaction between aldolase and V-ATPase.

An important issue that remains to be addressed is whether the abnormalities in V-ATPase observed in aldolase-deficient cells are caused by disruption of the aldolase-V-ATPase interaction or by attenuation of cellular metabolism medi-
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ated by aldolase as a glycolytic enzyme. In this study, we generate aldolase mutants that lack binding to the B subunit of V-ATPase but retain normal enzymatic activities. Functional analysis of the aldolase mutants shows that disruption of binding between aldolase and the B subunit of V-ATPase results in disassembly and malfunction of V-ATPase. Our data indicate that physical association between aldolase and V-ATPase is essential for V-ATPase assembly and activity.

EXPERIMENTAL PROCEDURES

Materials—The Taq DNA polymerase was purchased from Promega (Madison, WI). The high-fidelity Expand Long enzyme system was from Roche Applied Science. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA). The bacterial expression vector, pET33b, was from Novagen (San Diego, CA). The yeast expression vector, YES3/CT, was from Invitrogen. Monoclonal antibodies 10D7, 8B1, and 13D11 against the a, A, and B subunits of V-ATPase, respectively, were purchased from Molecular Probes (Eugene, OR). Zymolyase 100T was purchased from ICN (Costa Mesa, CA). Acridine orange, bafilomycin A1, fructose 1,6-bisphosphate, glycerol phosphate dehydrogenase, NADH, and triose phosphate isomerase were from Sigma.

Deletion Mapping Analysis—cDNA fragments coding for successive deletion mutants from the C terminus of human aldolase B were amplified by high fidelity PCR. A cDNA fragment coding for the full-length human aldolase B (364 aa) was amplified, using the forward primer 5’-ATGGCCCCACCGATTTCAG-3’ and the reverse primer 5’-CTAGTAGGTATAGCAAGCTGTG-3’. A 304-aa cDNA fragment at the N terminus of human aldolase B was amplified, using the forward primer 5’-ATGGCCCCACCGATTTCAG-3’ and the reverse primer 5’-GGCCCGTCCATAAGAGAA-3’. A 244-aa cDNA fragment at the N terminus of human aldolase B was amplified, using the forward primer 5’-ATGGCCCAACCGATTTCAG-3’ and the reverse primer 5’-ATGATACGTCTTGGTGCAGGCATG-3’. A 184-aa cDNA fragment at the N terminus of human aldolase B was amplified, using the forward primer 5’-ATGGCCCAACCGATTTCAG-3’ and the reverse primer 5’-AGGTACCAGTCCATTCTGCTGAC-3’.

PCR-based Random Mutagenesis—Error-prone PCR was carried out to make single amino acid mutations (substitutions) in the candidate binding sites. The procedure relies on manganese-induced mis-insertion of nucleotides by Taq polymerase as well as reduced concentration of each dNTP and increased number of PCR cycles to decrease the fidelity of PCR amplification (15). Over 50% of the mutants were found to carry single base substitution. A cDNA fragment corresponding to the last 66 aa at the C terminus of human aldolase B was amplified, using the forward primer 5’-AGTTTCTCTTATGGACGG-3’ and the reverse primer 5’-ATGGGTGTTGAACAAATCTTAAAGAG-3’.

Site-directed Mutagenesis—The human aldolase B non-catalytic mutant, R303W (16), was generated by two-step PCR amplification. Initially, two overlapping cDNA sub-fragments

TABLE 1

Physical association of human aldolase B mutants to the a, B, and E subunits of V-ATPase

| Aldolase B mutant | pAS2-a | pAS2-B | pAS2-E |
|------------------|--------|--------|--------|
| A318E            | +      | -      | +      |
| R303W            | +      | +      | +      |

FIGURE 1. The A318E aldolase mutant does not interact with the B subunit of V-ATPase. The GST-B subunit fusion protein was purified using glutathione-agarose beads and incubated with equal amounts of 35S-labeled wild-type and mutant aldolase B, respectively. After washing, proteins bound to the beads were recovered and analyzed by SDS-PAGE and autoradiography.
harboring the R303W point mutation were amplified by high fidelity PCR. The overlapping cDNA fragments were denatured, annealed, and used as template for the second amplification.

**TABLE 2**
The aldolase non-binding mutants retain normal enzymatic activity

One unit of aldolase activity corresponds to the conversion of 1 μmol of fructose 1,6-diphosphate to dihydroxyacetone phosphate and glyceraldehydes 3-phosphate per min at pH 7.4 at 25 °C.

|                          | unit/mg protein |
|--------------------------|-----------------|
| Yeast wild-type aldolase | 9.7 ± 0.4       |
| Yeast aldolase mutant (K323M) | 9.3 ± 0.3     |
| Human wild-type aldolase B | 11.4 ± 0.6     |
| Human aldolase B mutant (A318E) | 11.8 ± 0.7    |

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The yeast aldolase non-catalytic mutant, H108A (17), was made by amplifying two overlapping sub-fragments. After denaturing and annealing, the cDNA was amplified by a second PCR reaction, using the forward primer 5'-ATGGGTGTGAACAAATCTTA-3' and the reverse primer 5'-TAAAGTGTAGTGTACGGA-3'. The human and yeast non-catalytic aldolase mutants were cloned into the yeast expression vector, YES3/CT, and transformed into yeast cells for functional analysis.

**Western Blotting Analysis**—The aldolase mutants derived from site-directed mutagenesis were expressed in wild-type yeast cells or in yeast cells deficient in aldolase. The levels of expression of these mutant proteins were determined by Western blotting as previously described (13).

**Aldolase Activity Assay**—The aldolase mutant proteins were expressed in bacteria, purified, and examined for their enzymatic activities. The coupled enzyme assay was used to determine aldolase enzymatic activity as previously described (13). Briefly, aldolase mutant proteins were incubated in Tris-HCl buffer (pH 8) containing fructose 1,6-bisphosphate, NADH, glycerol phosphat dehydrogenase, and triose phosphate isomerase. Aldolase enzymatic activity was determined by measuring NADH oxidation at 340 nm using a spectrophotometer.

**Immunoprecipitation**—To determine whether the aldolase mutants affect V-ATPase assembly, we carried out immunoprecipitation analysis as described previously (13). Briefly, yeast cells were grown in mid-log phase in the presence of glucose and harvested. Spheroplasts were generated by treatment with Zymolyase, lysed with solubilization buffer containing 1% C12E9, and incubated with anti-B subunit antibody, followed by addition of protein G-agarose beads. The immunoprecipitates were analyzed by Western blotting with the anti-a subunit antibody. Quantification of the signals was performed using a densitometer (13).

**Purification of Yeast Vacuolar Membrane Vesicles**—Yeast vacuolar membrane vesicles were prepared as previously described (18).
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A

Wild-type A318E Vector
Subunit a
Heavy chain

B

Wild-type K323M
Subunit a
Heavy chain

I

Vector A318E K323M
Aldolase

C

[Graph showing relative activity (%)]

D

[Graph showing relative assembly (%)]

J

[Graph showing relative interaction (%)]

E

[Graph showing relative ATP hydrolysis (%)]

F

[Graph showing relative ATP hydrolysis (%)]

G

[Graph showing relative proton transport (%)]

H

[Graph showing relative proton transport (%)]
Briefly, yeast cells were grown to mid-log phase, harvested, and treated with Zymolyase. The spheroplasts were resuspended, homogenized in a loosely fitting Dounce homogenizer, and centrifuged in a swinging bucket rotor. The vacuoles free from contaminating lipid granules and other membranous organelles were collected. Vacular membrane vesicles were prepared by diluting the purified vacuoles.

**ATP Hydrolytic Activity**—To determine whether the aldolase mutants affect V-ATPase activity, ATP hydrolytic assay was carried out. Bafilomycin-sensitive ATP hydrolysis of V-ATPase was assayed by measuring the production of inorganic phosphate as previously described (18).

**The Proton Transport Activity of V-ATPase**—To determine whether the aldolase mutants affect V-ATPase-driven proton transport, we determined the rate of proton transport in yeast cells expressing the mutant proteins as we previously described (18). Vacuolar membrane vesicles were incubated with acridine orange and ATP. Proton transport was measured by tracking the rate of quenching using a fluorescence spectrometer with excitation at 493 nm and emission at 545 nm as previously described (18).

**RESULTS**

*Generation of Aldolase Mutants That Lack Binding to the B Subunit of V-ATPase*—By using the yeast two-hybrid and GST precipitation assays, we initially identified the interaction between the glycolytic enzyme aldolase and the E subunit of human V-ATPase (13). This initial observation was further extended to include the a and B subunits of human V-ATPase that also interact with aldolase (14). Among the three highly homologous aldolase isoforms in mammals, the human aldolase B isoform-specific enzymatic properties (16, 20–21). Subsequent analysis also showed that the mutant retained its binding to the a and E subunits of human V-ATPase (Table 1). A GST precipitation assay was also carried out to confirm the lack of binding to the B subunit of human V-ATPase (Table 1). The wild-type aldolase B was detected to associate with the B subunit of V-ATPase (Fig. 1). In contrast, no binding was observed between the A318E aldolase mutant and the B subunit of V-ATPase (Fig. 1).

To examine whether the A318E mutant retains aldolase enzymatic activity, we added a His tag on the mutant and expressed it in bacteria. The mutant protein was induced for expression by isopropyl 1-thio-β-D-galactopyranoside and purified using nickel beads. Aldolase enzymatic activity was measured using the coupled enzyme assay as described (14). No major difference in aldolase enzymatic activity was detected between the aldolase B mutant and the wild-type aldolase B protein (Table 2), indicating that the aldolase enzymatic activity was not impaired by the A318E point mutation.

By using a similar experimental approach, we carried out random mutagenesis in the corresponding region of the yeast aldolase (aa 293–359). One yeast aldolase mutant, K323M, was identified for lack of binding to the B subunit of yeast V-ATPase. The K323M mutant protein was subsequently expressed in bacteria, purified, and assayed for enzymatic activity. Normal aldolase enzymatic activity was observed (Table 2), indicating minimum impact of the K323M point mutation on aldolase enzymatic activity.

**The Effects of Human and Yeast Non-binding Aldolase Mutants on V-ATPase Assembly and Activity**—Previous studies show that V-ATPase remains completely disassembled, is
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dramatically reduced in expression, and displays minimum proton transport activity in yeast cells deficient in aldolase (13). The abnormalities in V-ATPase can be fully restored by aldolase complementation (14). Because the mammalian and yeast aldolases are functionally interchangeable (22) and V-ATPases are conserved between human and yeast (1), we examined the effects of human and yeast non-binding aldolase mutants on V-ATPase assembly and activity in yeast cells deficient in aldolase. Transformed yeast cells with the aldolase mutants were plated out on dropout plates for selection of colonies harboring the human or yeast aldolase mutant. The selected yeast colonies were cultured, lysed, and analyzed for expression of the mutant proteins by Western blotting using antibodies against the human or yeast aldolase, respectively. The levels of mutant protein expression were found to be 2- to 3-fold over that of the wild-type aldolase (not shown). By co-immunoprecipitation analysis, complementation of aldolase-deficient yeast with wild-type human aldolase B restored V-ATPase assembly (Fig. 2A), bafilomycin A₁-sensitive ATPase activity (Fig. 2C), and proton transport activity (Fig. 2E). In contrast, transformation of the aldolase-deficient yeast with the human aldolase B A318E mutant failed to rescue the defects in V-ATPase assembly observed in yeast cells deficient in aldolase (Fig. 2A). Consistent with the effect on V-ATPase assembly, transformation with the aldolase B A318E mutant failed to rescue bafilomycin A₁-sensitive ATP hydrolytic activity (Fig. 2C) and proton transport activity (Fig. 2E). Similarly, we examined the effect of transformation of aldolase-deficient yeast either with the wild-type yeast aldolase, or with the non-binding K323M mutant, on complementation of V-ATPase assembly and activity. As shown in Fig. 2, expression of the wild-type yeast aldolase, but not the B subunit binding-defective K323M mutant, restored V-ATPase assembly (Fig. 2B), bafilomycin A₁-sensitive ATP hydrolytic activity (Fig. 2D), and proton transport activity (Fig. 2F).

To independently confirm our observation that the binding between aldolase and the B subunit of V-ATPase is essential for the assembly and activity of the proton pump, we expressed the human and yeast aldolase mutants in wild-type yeast cells. V-ATPase assembly, bafilomycin A₁-sensitive ATPase activity, and proton transport were examined. With overexpression of the non-binding aldolase B mutant, A318E, immunoprecipitation and activity studies showed a 70% reduction in V-ATPase assembly (Fig. 3, A and C), 66% reduction in V-ATPase-mediated ATP hydrolytic activity (Fig. 3E), and 72% reduction in proton transport activity (Fig. 3G). A dramatic decrease in V-ATPase assembly was also observed in wild-type yeast cells expressing the yeast aldolase mutant, K323M (Fig. 3, B and D). As expected, V-ATPase-mediated ATP hydrolytic and proton transport activities were also dramatically reduced (Fig. 3, F and H). Our data, therefore, indicate that physical association between aldolase and V-ATPase is essential for the assembly and activity of the proton pump.

To examine how the aldolase mutants acted in a dominant negative manner on V-ATPase assembly and activity, we expressed the non-binding aldolase mutants in wild-type yeast cells. The expression levels of the V5-tagged exogenous aldolase mutant proteins were determined to be 2- to 3-fold over the endogenous aldolase expression (data not shown). Immunoprecipitation analysis was subsequently carried out using the antibody against the B subunit of V-ATPase. The immunoprecipitates were probed by Western blotting using antibodies against the yeast aldolase. The binding between aldolase and assembled V-ATPase in cells expressing the human and yeast non-binding aldolase mutants was reduced by 60 and 67%, respectively (Fig. 3, I–J). These data indicate that overexpression of the non-binding aldolase competitively inhibits binding of the endogenous yeast aldolase to V-ATPase.

Glucose addition and removal promotes assembly and disassembly, respectively, of the yeast V-ATPase. In principle, aldolase could act predominantly by bringing the V-ATPase into approximation with a glycolytic complex. Incorporation of the non-binding aldolase mutants (and therefore exclusion of the endogenous aldolase) might explain the dominant-negative effects on the assembly and activity of the proton pump. To address this possibility, we examined the effects of glucose addition and removal on wild-type yeast cells overexpressing the wild-type aldolase gene. Immunoprecipitation analysis showed normal assembly of V-ATPase even in the absence of glucose (Fig. 4), suggesting that overexpression of aldolase overcomes the normal sensitivity to glucose deprivation.

Aldolase Enzymatic Activity Is Not Required for V-ATPase Assembly—The preceding experiments showed that glycolytic activity is not required for V-ATPase assembly if aldolase is overexpressed. To further investigate whether the enzymatic activity of aldolase is required for V-ATPase assembly, we generated the enzymatically inactive human aldolase B mutant, R303W, by PCR-based site-directed mutagenesis and cloned in the yeast expression vector, pYES3/CT. The R303W aldolase B mutant, found in a patient with hereditary fructose intolerance, was previously shown to have minimum aldolase enzymatic activity (16). We examined binding of the R303W mutant to the a, B, and E subunits of V-ATPase by using the yeast two-hybrid assay. The R303W point mutation in the catalytic domain of aldolase B exerted no discernible effect on the binding of aldolase B to each of the three V-ATPase subunits (Table 1).

![FIGURE 4. Overexpression of yeast aldolase overcomes the effect of glucose removal on V-ATPase assembly. Wild-type yeast cells expressing wild-type yeast aldolase or the empty vector were cultured in YPD medium. Equal numbers of cells were incubated with culture medium in the presence or absence of glucose for 30 min. The cells were lysed and immunoprecipitated with the anti-B subunit antibody 13D11. The immunoprecipitates were analyzed by Western blotting using antibodies against the a subunit of V-ATPase.](http://www.jbc.org/)

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Table 1.
When the V5-tagged R303W mutant was expressed in wild-type yeast cells, its abundance was found to be 2- to 3-fold over that of the endogenous aldolase. Immunoprecipitation analysis showed normal assembly of V-ATPase in yeast expressing the aldolase B R303W mutant (Fig. 5A), suggesting that the catalytic activity of aldolase is not required for V-ATPase assembly.

Similarly, the bafilomycin A1-sensitive ATP hydrolytic and proton transport activities appeared to be normal in cells expressing the R303W mutant (Fig. 5, C and E). Moreover, we generated the catalytically inactive mutant of the yeast aldolase, H108A (17), by PCR-based site-directed mutagenesis and cloned it in the yeast expression vector, pYES3/CT. The V5-tagged H108A mutant was expressed in wild-type yeast cells, and its expression level was found to be twice as much as that of the endogenous aldolase. Assembly of V-ATPase in cells expressing the H108A mutant was identical to that in wild-type yeast cells (Fig. 5B).

Similarly, the bafilomycin A1-sensitive ATP hydrolytic and proton transport activities were also unaffected in cells expressing the H108A mutant (Fig. 5, D and F). Thus, studies with both human and yeast aldolase show that B subunit binding, but not the enzymatic activity of aldolase, is required for V-ATPase assembly and function.

To eliminate the possibility that endogenous yeast aldolase was responsible for V-ATPase assembly in cells expressing catalytically inactive aldolase, we expressed either wild-type human aldolase B or the non-catalytic R303W mutant in aldolase-deficient yeast cells and examined V-ATPase assembly by coimmunoprecipitation assay. Expression of the non-catalytic mutant resulted in normal levels of V-ATPase assembly (Fig. 6A). Similarly, expression of the non-catalytic yeast H108A aldolase produced V-ATPase assembly equal to that in cells expressing wild-type yeast aldolase (Fig. 6B). These data demonstrate that the B subunit binding of aldolase, but not its enzymatic activity, is essential for normal V-ATPase assembly and strongly suggest that the mechanism for V-ATPase disassembly with glucose removal is dissipation of a complex of V-ATPase with aldolase.

**DISCUSSION**

Recent studies from our laboratory show that the ATP-generating glycolytic pathway is directly coupled to the ATP-hydrolyzng proton pump by protein-protein interactions (13, 14). In this study, we examined whether physical association...
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FIGURE 6. V-ATPase assembly is independent of the aldolase enzymatic activity. A, yeast cells deficient in aldolase were transformed with the pYES3/CT plasmid harboring the non-catalytic human aldolase B mutant, R303W. The transformed cells were cultured, lysed, and immunoprecipitated with the anti-B subunit antibody, 13D11. The immunoprecipitates were probed with the anti-a subunit antibody, 10D7. B, yeast cells deficient in aldolase were transformed with the pYES3/CT plasmid harboring the non-catalytic yeast aldolase mutant, H108A. Immunoprecipitation analysis of V-ATPase assembly was carried out as described in A.

between aldolase and V-ATPase was essential for the assembly and activity of the proton pump. We initially generated human and yeast aldolase mutants that failed to bind the B subunit of V-ATPase by random mutagenesis. It was anticipated that a single mutation would disrupt a specific interaction between aldolase and V-ATPase with minimum impact on the overall protein structure, so that the mutant is more likely to retain aldolase enzymatic activity and binding sites for other V-ATPase subunits. As expected, the aldolase mutants were observed to retain normal enzymatic activities (Table 2) and binding to the a and E subunits of V-ATPase (Table 1). When the aldolase mutants were introduced and expressed in aldolase-deficient cells, abnormalities in V-ATPase assembly and activity persisted (Fig. 2), indicating a failure in rescuing the V-ATPase defects. Furthermore, we expressed the human and yeast non-binding aldolase mutants in wild-type yeast cells and observed dramatic decreases in V-ATPase assembly and activity (Fig. 3). In contrast, the aldolase enzymatic activity appeared to be not essential for V-ATPase assembly (Figs. 5 and 6). Thus, our data strongly suggest an important role for physical association between aldolase and V-ATPase in the regulation of assembly and activity of the proton pump.

Although aldolase enzymatic activity is clearly not essential for V-ATPase assembly (Figs. 5 and 6), more detailed analysis is required to determine whether proper V-ATPase function depends on glycolysis-derived ATP. It is conceivable that disruption of energy flow of the glycolytic pathway may cut off ATP supply to the ATP-hydrolyzing proton pump, leading to malfunction of V-ATPase. Among the naturally occurring mutants of human aldolase B found in patients with hereditary fructose intolerance, three missense mutations (W147R, R303W, and A337V) have been characterized (16). They all retained the proper protein structure of aldolase but displayed greatly impaired enzymatic activity (16). These findings implicate that aldolase enzymatic activity is required for V-ATPase function. Our preliminary analysis of one of the three mutants (R303W), however, showed that V-ATPase assembly and activity appeared to be normal (Figs. 5 and 6). The seemingly paradoxical data may well be explained by the nature of the in vitro assays carried out with yeast cells. Because exogenous ATP was added in the ATPase assay, the effect of the R303W mutant on V-ATPase activity may be masked. Taken together, our data suggest that the aldolase enzymatic activity is not required for the assembly but may be required for the activity of the proton pump.

Increasing evidence suggests that glycolysis-derived ATP is preferentially used in rapid biological processes. First, glycolysis-derived ATP is required in cardiac myocytes for preventing ATP-sensitive K+ channels from opening and thereby maintaining cellular membrane potential (23). Second, fast twitch fibers of skeletal muscle utilize ATP produced by glycolysis, whereas slow twitch fibers largely use ATP synthesized by mitochondria (24). Third, glycolytic enzyme complexes have been observed in retina and neural postsynaptic density, suggesting that glycolysis may be involved in local ATP generation (25, 26). Finally, glycolysis has been demonstrated to be essential for glutamate accumulation into synaptic vesicles and for sustaining normal synaptic transmission (27). Ikemoto et al. (27) propose that glycolysis-derived ATP is utilized by the V-ATPase proton pump to generate an electrochemical proton gradient across the membrane of synaptic vesicles, providing the driving force for glutamate transport into synaptic vesicles. We have shown that the glycolytic enzyme aldolase mediates assembly and activity of V-ATPase (13, 14). The importance of aldolase-mediated regulation of V-ATPase is further supported by observations that glycolysis-catalyzed ATP generation and V-ATPase-mediated proton transport are two ancient biological processes that dated as far back as to Archaebacteria, e.g. Pyrococcus horikoshii (28). All these studies support the notion that rapid, energy-consuming cellular processes rely on glycolysis-derived ATP and that mitochondria may not be sufficient or close enough to meet the ATP requirement for these processes.

Glucose-dependent assembly of V-ATPase was initially reported to occur in yeast cells (9). Subsequent analysis in mammalian cells demonstrated that assembly and activity of V-ATPase was also glucose-dependent (10). In this study, by using yeast cells as a model system, we observed that both the human and yeast aldolase mutants act in a similar manner in regulating V-ATPase assembly and activity. These findings suggest a highly conserved mechanism for metabolic regulation of V-ATPase by direct coupling of glycolysis to the proton pump.
We previously reported that aldolase physically associates with the a, B, and E subunits of V-ATPase (14). Co-immunoprecipitation analysis showed that in vivo interaction between aldolase and V-ATPase in yeast cells increased dramatically in the presence of glucose (14). These findings suggest that aldolase may act as a glucose sensor and mediate V-ATPase assembly. In this study, we demonstrate the importance of aldolase binding to the B subunit of V-ATPase in regulating V-ATPase assembly and activity. Furthermore, aldolase may work in concert with other regulatory proteins for V-ATPase assembly and activity. Furthermore, aldolase-E subunit interactions are also required for V-ATPase assembly by binding to multiple subunits of V-ATPase. Disruption of a single interaction between aldolase and V-ATPase may render the aldolase-V-ATPase complex unstable, preventing the formation of assembled and functional holoenzyme.

In summary, our findings show that physical association between aldolase and the B subunit of V-ATPase is important for the proton pump function. Aldolase has been shown to bind the a, B, and E subunits of V-ATPase simultaneously (14). It remains to be determined whether the aldolase-a subunit and aldolase-B subunit interactions are also required for V-ATPase assembly and activity. Furthermore, aldolase may work in concert with other regulatory proteins for V-ATPase assembly and activity. For instance, aldolase associates with fully assembled V-ATPase on the vacuolar membrane but not with the free V1 or V0 sectors (14). In contrast, the RAVE complex binds to the V1 domain whenever it is present in the cytosol but not to V0 or fully assembled V-ATPase (29). Aldolase and RAVE appear to bind V-ATPase in a mutually exclusive manner. It is, therefore, likely that the RAVE complex is recruited to interact with V1 released from the vacuolar membrane upon glucose deprivation and that, when extracellular glucose is restored, the V1-RAVE complex rapidly dissociates to release V1 for V-ATPase re-assembly.

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14. Rave complex rapidly dissociates to release V1 for V-ATPase re-assembly.
Physical Interaction between Aldolase and Vacuolar H\(^+\)-ATPase Is Essential for the Assembly and Activity of the Proton Pump

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