Regulation of Vacuolar H\(^+\)-ATPase (V-ATPase) Reassembly by Glycolysis Flow in 6-Phosphofructo-1-kinase (PFK-1)-deficient Yeast Cells

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Yeast 6-phosphofructo-1-kinase (PFK-1) has two subunits, Pfk1p and Pfk2p. Deletion of Pfk2p alters glucose-dependent V-ATPase reassembly and vacuolar acidification (Chan, C. Y., and Parra, K. J. (2014) Yeast phosphofructokinase-1 subunit Pfk2p is necessary for pH homeostasis and glucose-dependent vacuolar ATPase reassembly. *J. Biol. Chem.* 289, 19448–19457). This study capitalized on the mechanisms suppressing vacuolar H\(^+\)-ATPase (V-ATPase) in *pfk2a* to gain new knowledge of the mechanisms underlying glucose-dependent V-ATPase regulation. Because V-ATPase is fully assembled in *pfk2Δ*, and glycolysis partially suppressed at steady state, we manipulated glycolysis and assessed its direct involvement on V-ATPase function. At steady state, the ratio of proton transport to ATP hydrolysis increased 24% after increasing the glucose concentration from 2% to 4% to enhance the glycolysis flow in *pfk2Δ*. Tighter coupling restored vacuolar pH when glucose was abundant and glycolysis operated below capacity. After readdition of glucose to glucose-deprived cells, glucose-dependent V\(_1\)\(_\text{Vo}\) reassembly was proportional to the glycolysis flow. Readdition of 2% glucose to *pfk2Δ*, which restored 62% of ethanol concentration, led to equivalent 60% V\(_1\)\(_\text{Vo}\) reassembly levels. Steady-state level of assembly (100% reassembly) was reached at 4% glucose when glycolysis reached a threshold in *pfk2Δ* (≥40% the wild-type flow). At 4% glucose, the level of Pfk1p co-immunoprecipitated with V-ATPase decreased 58% in *pfk2Δ*, suggesting that Pfk1p binding to V-ATPase may be inhibitory in the mutant. We concluded that V-ATPase activity at steady state and V-ATPase reassembly after readdition of glucose to glucose-deprived cells are controlled by the glycolysis flow. We propose a new mechanism by which glucose regulates V-ATPase catalytic activity that occurs at steady state without changing V\(_1\)\(_\text{Vo}\) assembly.

Vacuolar H\(^+\)-ATPase (V-ATPase)\(^3\) is a highly conserved ATP-driven proton pump distributed throughout the endo-

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\(^{3}\) The abbreviations used are: V-ATPase, vacuolar proton-translocating ATPase; PFK-1, 6-phosphofructo-1-kinase; BCECF-AM, 2',7'-bis(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxymethyl ester; YEP, yeast extract, peptone medium; YEPD, yeast extract, peptone, 2% dextrose medium.
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![FIGURE 1. PKF-1 activity and glycolysis are defective in pfk2Δ cells.](image)

V-ATPase function is defective in both PKF-1 deletion mutants, but it is more severely impaired in pfk2Δ (24). Glucose-dependent V1V0 reassembly is normal in pfk1Δ, but it is reduced by 40–50% in pfk2Δ. In addition, V-ATPase proton transport is partially suppressed at steady state. The cells have alkalinized vacuoles and display pH and calcium growth sensitivity, which is characteristic of yeast cells with partially defective V-ATPase activity. The mechanisms by which V-ATPase is suppressed in pfk2Δ cells are elusive. Subunit Pfk1p may regulate V-ATPase through its interaction with V1V0 in the pfk2Δ mutant. In addition, a reduction in PKF-1 function and the glycolysis flow may inhibit V-ATPase in pfk2Δ cells.

We have now investigated the interrelation between glycolysis and V-ATPase function to gain new knowledge of the mechanisms underlying glucose-dependent V-ATPase regulation. After readdition of glucose to glucose-deprived cells, V1V0 reassembly is proportional to the glycolysis flow until pfk2Δ reaches a metabolic threshold necessary to complete V1V0 reassembly. At steady state, V-ATPase also communicates with the glycolysis flow. This communication modulates proton transport to restore pH homeostasis when glucose is abundant in pfk2Δ. This is a new mechanism by which glucose regulates V-ATPase. It occurs at steady state and does not involve disassembly/reassembly.

Results

Stimulation of Glycolysis Triggers V-ATPase Function in pfk2Δ at Steady State—To test the hypothesis that V-ATPase function is adjusted in response to changes in the glycolysis flow, we manipulated glycolysis in pfk2Δ cells and measured V-ATPase functions. First, we measured PKF-1 activity in pfk2Δ cells to determine the extent of PKF-1 inhibition. As shown in Fig. 1A, pfk2Δ cells only express subunit Pfk1p. The enzymatic activity of PKF-1 in pfk2Δ cells was 42% lower in pfk2Δ than wild-type cells. This result is consistent with previous reports indicating that PKF-1 homomeric complexes consisting of subunit α (pfk2Δ cells) are less active than wild-type native PKF-1 hetero-octameric complexes (αβ)4 (27).

In yeast, the final product of glycolysis, pyruvate, is fermented into ethanol, the levels of which were measured in both strains at steady state (Fig. 1B). In the presence of 2% glucose, which is the standard glucose concentration in yeast growth,
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medium, the ethanol concentration was observed to be 6.6 ± 0.9 mM and 3.8 ± 0.2 mM in the wild-type and pfk2Δ strains, respectively. This result indicates that glycolysis was defective in pfk2Δ. The 42% ethanol concentration reduction is consistent with the 42% reduction of PFK-1 activity measured in pfk2Δ cells and with independent studies that reported reduced fructose-1,6-biphosphate, the 6-phosphofructo-1-kinase reaction product (27–29).

Previous characterizations of glycolysis (27) and V-ATPase pumps (24) in pfk2Δ cells were conducted in 2% glucose. Although 2% glucose is optimal for wild-type cells, it might not be optimal for PFK-1 mutants. We reasoned that the pfk2Δ glycolytic mutant would require larger concentrations of glucose to maintain a substantial glycolytic flow. The pfk2Δ cells were grown in medium containing 2% or 4% glucose, and the ethanol concentration was compared with wild-type cells (Fig. 1B). Although lower ethanol levels were detected in 2% glucose, the ethanol concentration increased in 4% glucose in pfk2Δ cells. It reached 83% of the ethanol concentration in the wild-type cells, indicating that glycolysis was stimulated.

Because the vacuolar pH is regulated by V-ATPase, vacuolar alkalinization is a direct consequence of inhibiting V-ATPase activity. The effects of stimulating glycolysis on V-ATPase proton transport were determined by measuring the vacuolar pH in vivo. We used the pH-sensitive fluorophore BCECF, which accumulates in yeast vacuoles. The cells were loaded with BCECF-AM, and the pH was measured fluorometrically according to standard calibration curves (20, 24, 31). The vacuolar pH was measured in wild-type cells and pfk2Δ after growing the cells in 2% versus 4% glucose. vma3Δ cells were used as a control because vma3Δ completely lacks V-ATPase function (32). In wild-type cells, where the V-ATPase pumps are fully active, the vacuolar lumen was acidic (pH = 5.2–5.9) at both glucose concentrations (Fig. 2A). In 2% glucose, the pfk2Δ vacuoles were alkalinized (pH 6.6), similar to the inactive V-ATPase mutant vma3Δ (pH 6.8).

In 4% glucose, the acidic pH of the vacuoles was rescued. The pfk2Δ vacuolar lumen pH in 4% glucose (pH 5.8) was almost identical to the wild-type vacuolar lumen pH in 2% glucose (pH 5.9). These results show that stimulation of glycolysis with 4% glucose stimulated V-ATPase proton transport in pfk2Δ cells at steady state. Thus, they indicate that V-ATPase proton transport is coupled to the flow of glycolysis at steady state.

Sensitivity to elevated pH and calcium at all temperatures is a signature of mutants that lack all V-ATPase activity (vacuolar and Golgi V-ATPase). It is known as the Vma− (vacuolar membrane ATPase) growth phenotype (33) and is only shown when over ~70% of V-ATPase function is compromised (34–36). The vma mutants grow at pH 5.0, but growth is reduced or abolished at neutral pH in the presence of high concentrations of calcium (32). The physiological basis of Vma− growth is not fully understood because the downstream consequences of inhibiting V-ATPase are extensive (33). Vacuolar alkalinization alone is not sufficient to cause the Vma− phenotype (37). However, pfk2Δ growth was fairly reduced with 2% glucose under non-permissive growth conditions at 37 °C (Fig. 2B) (24). This modest growth defect was indicative of defective vacuolar but not Golgi V-ATPase function in pfk2Δ (37).

To determine whether the Vma− growth is rescued after stimulation of glycolysis, pfk2Δ cells were plated on medium containing 100 mM CaCl2 buffered to pH 7.5 (Fig. 2B) that was supplemented with 2% glucose (left panels) versus 4% glucose (right panels). The Vma− growth defect was rescued in 4% glucose, indicating that the vacuolar V-ATPase function was restored. As expected, the wild-type strain grew under all the conditions, and vma3Δ, which completely lacks V-ATPase function (32), failed to grow under non-permissive conditions. The pfk1Δ mutant strain was examined for reference. The pfk1Δ strain lacks the PFK-1 subunit Pfk1p but has greater glycolysis flow than the pfk2Δ strain (27). The pfk1Δ cells closely mimicked the wild-type cells, as reported before (24). A normal pfk1Δ growth was consistent with the concept that vacuolar V-ATPase function is coupled to the glycolysis flow.

**FIGURE 2. Metabolic reactivation is defective in pfk2Δ cells.** A, the acidic vacuolar pH is restored in 4% glucose. Wild type, pfk2Δ, and vma3Δ cells were cultured to mid-log phase in YEP containing 2% or 4% glucose. The cells were harvested and stained with 50 μM BCECF-AM for 30 min at 30 °C. The ratio of fluorescent emission (535 nm) excited at 490 and 450 nm was measured to quantitatively assess vacuolar pH. The average fluorescence over 6 min was compared with a standard curve to generate absolute pH values. Data are presented as average pH values from three independent experiments, and error bars are standard deviation. Statistically significant differences (*, p < 0.05) were determined by two-tailed unpaired t test. B, the Vma− growth phenotype is rescued in 4% glucose. Cells were cultured to mid-log phase in 2% or 4% glucose, and 10-fold serial dilutions were stamped onto YEP plates containing 2% or 4% glucose adjusted to pH 5.0 and pH 7.5 plus 100 mM CaCl2. Cell growth was monitored for 72 h at 37 °C. Shown are representative plates of triplicates.
Under reassembly conditions (10 min after glucose readdition), the concentration of ethanol increased, indicating that glycolysis resumed. Although addition of 2% glucose increased ethanol concentrations to 4.1 ± 0.2 mM and 2.9 ± 0.2 mM in the wild-type and pfk2Δ strains, respectively, steady state levels were not reached. These results also showed that 2% glucose resumed glycolysis in the two strains, but to a lesser extent in pfk2Δ. The pfk2Δ mutant, when 2% glucose was readded, mimicked the wild-type cells deprived of glucose, suggesting that insufficient glycolytic flow upon reactivation is responsible for the V:\n\nVvo reassembly defects in pfk2Δ.

Addition of glucose after nutrient limitation is known to trigger a rapid increase of NADH/NAD\(^+\) that inhibits the glycolytic enzyme triose phosphate dehydrogenase (39) and results in a transient peak in NADH levels (42). To monitor the rate of metabolic reactivation when glucose was readded, NADH was measured by autofluorescence. Readdition of 2% glucose stimulated NADH synthesis in wild-type and pfk2Δ cells (Fig. 3B). However, the NADH synthesis rate was reduced by 70% in pfk2Δ cells. These results are consistent with the ethanol measurements, indicating that glucose-mediated metabolic reactivation was defective in pfk2Δ.

We measured metabolic reactivation after adding 4% glucose. Fig. 3, A and B, shows the level of ethanol and NADH formation that resulted from the addition of 4% glucose to pfk2Δ after a brief glucose depletion period. The concentration of ethanol reached a steady-state level of 6 mM ± 0.6 mM and 3.8 ± 0.7 mM for the wild type and pfk2Δ, respectively. The rate of NADH synthesis in wild-type and pfk2Δ cells increased by 15%. It reached up to 46% in pfk2Δ cells, which was a larger increase than that observed upon addition of 2% glucose. Thus, doubling the concentration of glucose from 2% to 4% stimulated glucose-dependent metabolic reactivation by about 50% in pfk2Δ, as indicated by ethanol levels and the NADH formation rate. Next, we exposed pfk2Δ to 2% and 4% glucose to manipulate glycolysis and directly establish the role of glycolysis in V-ATPase assembly and activity.

4% Glucose Rescues Glucose-dependent Reassembly and Vacuolar Acidification—If V1\n\nVvo reassembly is governed by the glycolysis flow, we anticipated reassembly levels to increase after stimulating glycolysis in pfk2Δ cells. Fig. 4A shows the extent of V1\n\nVvo reassembly in pfk2Δ after addition of 4% glucose. Reassembly levels were measured using biosynthetically \(^{35}\)S-radiolabeled cells in pulse-chase experiments as described under “Experimental Procedures.” The radiolabeled cells were chased in YEP medium containing 2% glucose for 20 min (steady-state condition, +G), YEP medium without glucose for 10 min (disassembly condition, −G), and after readdition of varied concentrations of glucose (0.1–4%) for an additional 10 min (reassembly conditions, ±G). After the chases, the V-ATPase complexes were immunoprecipitated under nondenaturing conditions with anti-V1 subunit B to immunoprecipitate V1 and V1\n\nVvo versus anti-Vo subunit a, which can only immunoprecipitate V1 when it is disassembled from V1.

About 80% of the V1\n\nVvo complexes disassembled upon glucose depletion (Fig. 4A). After glucose readdition, reassembly was proportional to the concentration of glucose added. However, pfk2Δ required greater concentrations of glucose to reach
wild-type levels of reassembly. In the wild-type cells, 50% reassembly was detected after addition of 0.3% glucose; 100% reassembly was reached with 0.5% glucose. In contrast, addition of 0.3% glucose did not trigger significant reassembly in pfk2Δ cells. A gradual pH drop was detected in vma2Δ at 3 min after V-ATPase reactivation (acidification) was complete in wild-type and pfk2Δ cells (Fig. 4B). This observation further indicated that glucose-mediated vacuolar acidification was V-ATPase-dependent in pfk2Δ cells.

In wild-type cells cultured in 4% glucose, ATP hydrolysis and proton transport activities increased by 21% and 17%, respectively, compared with those grown in 2% glucose (Fig. 5A). In contrast, ATP hydrolysis was unchanged in pfk2Δ membranes from cells cultured in 2% versus 4% glucose, which was 64–67% of the wild-type ATP hydrolysis. However, proton transport significantly increased (by 30%) when pfk2Δ cells were grown in 4% glucose from that observed in 2% glucose. As a result, the proton transport/ATP hydrolysis ratio increased 24% in the pfk2Δ mutant (from 0.63 to 0.83). Western blotting analyses of vacuolar membrane fractions showed comparable levels of V1 subunit A and subunit B in 2% glucose and 4% glucose (Fig. 5B). Based on these results, we concluded that the V-ATPase pumps fine-tuned their catalytic activity in 4% glucose, which enabled pfk2Δ cells to restore vacuolar pH homeostasis.

Proton Transport Is Increased at Vacuolar Membranes—The rates of proton transport and ATP hydrolysis were measured in purified vacuolar membranes from pfk2Δ cells. V-ATPase activity was determined in the presence and absence of the specific V-ATPase inhibitor concanamycin A, and the ratio of proton pumping to ATP hydrolysis was used as a means of estimating the coupling efficiency of the enzyme when glycolysis was stimulated in pfk2Δ cells.

When reassembly occurs (3, 21), the V1/Vo reassembly level was somewhat higher in 4% glucose (Fig. 4A), and the wild-type vacuole pH was slightly more acidic.

Glucose-induced acidification was defective in pfk2Δ in response to 2% glucose even though 60% of the V1/Vo complexes reassembled in the glycolytic mutant (Fig. 4A). However, V-ATPase proton transport resumed after 4% glucose readdition. Reactivation of V-ATPase proton transport was indicated by the rapid acidification of the vacuolar lumen. The vma2Δ strain that cannot assemble V1/Vo complexes (43) was used as a negative control because vma2Δ completely lacks V-ATPase activity and vacuolar buffering capacity (2). As expected, the net vacuole pH was considerably more alkaline in vma2Δ than in wild-type and pfk2Δ cells. A gradual pH drop was detected in vma2Δ at 3 min after V-ATPase reactivation (acidification) was complete in wild-type and pfk2Δ cells (Fig. 4B). This observation further indicated that glucose-mediated vacuolar acidification was V-ATPase-dependent in pfk2Δ cells.

The V1/Vo complexes reassemble after reassembly, which acidifies the vacuolar lumen and restores membrane gradients and secondary transport systems (20). To determine whether reassembly restored V-ATPase proton transport, we measured the vacuolar pH after readdition of 4% glucose to pfk2Δ. Vacuolar acidification was detected within 1–2 min of glucose readdition to the wild-type cells (Fig. 4B), which is within the timescale when reassembly occurs (3, 21). The V1/Vo reassembly level was somewhat higher in 4% glucose (Fig. 4A), and the wild-type vacuole pH was slightly more acidic.

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FIGURE 5. Proton transport is increased at vacuolar membranes from \( pfk2\Delta \) cells cultured in 4% glucose. A, V-ATPase coupling efficiency is increased in 4% glucose. Vacular membrane fractions from wild-type and \( pfk2\Delta \) cells were purified by density centrifugation. ATP hydrolysis (left panel) was assayed spectrophotometrically in the presence and absence of the V-ATPase inhibitor concanamycin A (100 nm) by using an enzymatic coupled assay that measures NADH oxidation at 340 nm. The average wild-type specific activity in 2% glucose for the concanamycin A-sensitive ATP hydrolysis was 3.0 μmol of ATP/min/mg of total vacuolar protein. ATP-dependent proton transport (right panel) was measured by fluorescence quenching of 1 μM 9-aminomethylene-2-methoxyacidine (excitation, 410 nm; emission, 490 nm) upon addition of 0.5 mM ATP/1 mM MgSO\(_4\) to 5 μg of total protein in vacuolar membrane vesicles. Initial velocities were calculated for 15 s following MgATP addition. The average wild-type slope was –1117.47 fluorescence units/15 s. Data represent six independent vacuolar preparps. Statistically significant differences (*, \( p < 0.05 \); **, \( p < 0.01 \); ns, not significant) were as compared with the wild type in the presence of 2% glucose and determined by two-tailed unpaired t test. B, V-ATPase assembly is comparable in 4% glucose and 2% glucose. Vacular membrane vesicles were purified from \( pfk2\Delta \) and wild-type cells cultured overnight in 2% glucose or 4% glucose. Membrane protein (1 μg total membrane protein/well) was separated by SDS-PAGE in 10% gels. Protein markers are 150, 100, 75, and 50 kDa. A representative gel is shown (top panel). Gels from two independent experiments were scanned using a Bio-Rad ChemiDoc XRS +, and data were analyzed using Multi Gauge V3.0 and GraphPad Prism 5 software. Data were expressed as fold increase Pfk1p:V1 subunit ratio ± S.D. relative to the wild type (bottom panel).

in \( pfk2\Delta \), which was suggestive of lower Pfk1p-V-ATPase binding affinity in this mutant.

Discussion

This study capitalized on the cellular mechanisms suppressing V-ATPase function in \( pfk2\Delta \) to gain new knowledge of the mechanisms underlying glucose-dependent V-ATPase regulation. We took advantage of the fact that V-ATPase is fully assembled in \( pfk2\Delta \) cells at steady state and glycolysis partially suppressed to manipulate the glycolysis flow and assess its direct involvement on V-ATPase function.

At steady state, the ratio of proton transport to ATP hydrolysis increases in response to high glucose levels in \( pfk2\Delta \). Enhanced V-ATPase proton transport restores vacuolar pH homeostasis. It likely allows cells to preserve energy when glycolysis is suboptimal and glucose abundant (4% glucose). One importance of these findings is that they revealed V-ATPase elasticity of coupling as a new mechanism of how glucose regulates V-ATPase pumps without changing the V1V0 assembly state. This study also showed that, under V1V0 reassembly conditions, the level of glucose-induced reassembly directly corresponds to the glycolysis flow in \( pfk2\Delta \) cells. V1V0 reassembly is complete after the rate of glycolysis reaches a threshold (40–46% of the wild type in 2% glucose) when metabolism resumes after glucose is readded to glucose-deprived cells.

The Glycolysis Flow Communicates with V-ATPase and Regulates Its Activity at Steady State—At steady state, in 2% glucose, the ethanol concentration is significantly reduced (by 42%) in \( pfk2\Delta \) cells. This level of glycolytic reduction mimics the level of PFK-1 activity reduction (42%) (Fig. 1A), as expected, because PFK-1 catalyzes the second limiting step reaction of the pathway.

V-ATPase proton transport is suppressed in 2% glucose in \( pfk2\Delta \). Consequently, the vacuolar lumen is alkalinized (Fig. 2A). This alteration in V-ATPase function is not due to V1V0 disassembly or catalytic defects because \( pfk2\Delta \) cells cultured in 2% glucose display wild-type levels of V1V0 complexes at the membrane and express catalytically competent wild-type V-ATPase pumps (24). Rather, vacuolar V-ATPase function is inhibited \( \text{in vivo} \) in \( pfk2\Delta \) cells cultured in 2% glucose. This study indicates that V-ATPase activity is suppressed in \( pfk2\Delta \) because glycolysis is suppressed. Regulation of V-ATPase activity at steady state by the glycolysis flow has not been reported before.

V-ATPase activity is remarkably sensitive to changes in the glycolysis flow. Addition of 4% glucose restores 83% of wild-type ethanol concentration at steady state (Fig. 1B). This signif-
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icant stimulation of glycolysis also restores the vacuolar acidic pH (Fig. 2A). This result is indicative of a causal correlation between the glycolysis flow and V-ATPase activity. It is evidence that glucose regulates V-ATPase activity at steady state. Accordingly, the PFK-1 subunit deletion mutant pfkΔ, which has milder glycolysis defects than pfkA (27), has milder V-ATPase alterations (24). Thus, V-ATPase proton transport is adjusted in response to the glycolytic flow at steady state.

V-ATPase Adjusts Catalytic Activity in Response to Glucose

When Glycolysis Is Suboptimal—V-ATPase catalytic coupling is tighter in pfkΔ cells in 4% glucose (Fig. 5A) when glycolysis operates below capacity at steady state. The proton transport/ATPase ratio increases by 24%, indicating that V-ATPase is more efficient (transports more protons per ATP hydrolyzed), probably to help cells to preserve energy. Importantly, these findings indicate that ATP hydrolysis and proton transport by V-ATPase pumps may not always be optimally coupled in vivo.

Using pfkΔ cells, this study showed that glucose can control V-ATPase activity without changing the V-ATPase assembly state. Until now, glucose-dependent regulation of yeast V-ATPase has been via V1V0 disassembly and reassembly in response to glucose depletion and readdition, respectively (3). Although the ability of V-ATPase to change coupling of ATP hydrolysis and proton pumping has been described before (44), the involvement of glucose and glycolysis has not been reported. Increased efficiency of coupling was observed in some genetic subunit a (47, 48) and a non-homologous region of the V1 subunit Pfk2p. However, the PFK-1 subunit Pfk1p may play an inhibitory role in pfkΔ cells. The level of Pfk1p that co-immunoprecipitates with V-ATPase subunits is significantly reduced in pfkΔ cells in 4% glucose (Fig. 6). This decrease can be explained if Pfk1p is recruited to support greater glycolytic demands in 4% glucose, after which V-ATPase proton transport is enhanced. Whether binding of subunit Pfk1p to V-ATPase is inhibitory is an interesting possibility that will be addressed in future studies.

This study showed that at least one mechanism by which PFK-1 modulates V-ATPase is via the glycolysis flow. The RAS/cAMP/PKA signaling pathway, which has been linked to glucose-dependent V1V0 disassembly and reassembly, is also intertwined with PFK-1. Activation of the RAS/cAMP/PKA pathway enhances the glycolysis flow because PKA stimulates formation of fructose-2,6-bisphosphate, the most potent activator of PFK-1. Thus, RAS/cAMP/PKA could control V-ATPase assembly via glycolysis (16).

We cannot eliminate the possibility that glucose-dependent pH changes could also regulate V-ATPase. Cytoplasm alkalization is glucose concentration-dependent and correlates to the level of V1V0 activity. Similar to the activating effect ΔpH has on the Fo subunit of the evolutionary related F-ATPase (34), binding of protons to the membrane-bound V0 subunit a of the V-ATPase could prime V1V0 proton transport. In Arabidopsis,
the V-ATPase coupling ratios are sensitive to the cytosol and vacuolar pH (55). In the lemon fruit, variable coupling and pH-dependent slip regulate the V-ATPase pump (56).

Clearly, the scope of glucose-dependent V-ATPase regulation is more complex than initially anticipated. It extends beyond V-V\textsubscript{a} disassembly and/or reassembly and is intimately linked to the metabolic state of a cell, specifically the glycolysis flow. The finding that V-ATPase changes catalytic efficiency when the cellular demands for membrane transport increase (4% glucose) and glycolytic ATP production operates below capacity can have implications in human health, particularly for distal renal tubular acidosis (25), viral infections (57), and the metabolic switch in cancers (58, 59), where glucose-dependent regulation of V-ATPase is conserved.

**Experimental Procedures**

**Materials and Strains**—The Tran\textsuperscript{[35S]}-label was from MP Biomedicals (Santa Ana, CA). The antibodies anti-Myc, anti-phosphoglycerate kinase 1 (22C5D8), and anti-V-ATPase (pfk2) primers to amplify the genes with 5\textsuperscript{X}m-photoschyzochlorophylnylhydrazone. For steady-state analyses, the cells were deprived of glucose on ice for 10 min, and the vacuolar pH was monitored 20 min after 2% or 4% glucose readjustment (final concentration).

**Immunoprecipitations**—Pulse-chase experiments were conducted at the indicated glucose concentrations and times following protocols described before (21). The V-ATPase complexes were immunoprecipitated from whole cell lysates with the monoclonal antibodies 13D11 (anti-V\textsubscript{1} subunit B) and 10D7 (anti-V\textsubscript{a} subunit a, Vph1p isoform) and the protein separated by SDS-PAGE (13% acrylamide gels). The gels were dried, scanned in a Fuji scanner (FLA-5100), and analyzed using Multi Gauge and GraphPad Prism 5 software as described previously (24). The proportion of V\textsubscript{a} assembled into V\textsubscript{1}V\textsubscript{a}, determined by comparing the amount of V\textsubscript{a} subunit a immunoprecipitated with 13D11 with the total amount of V\textsubscript{a} subunit a immunoprecipitated with both antibodies. For non-radiolabeled immunoprecipitations, the 13D11 antibody was used to immunoprecipitate V-ATPase from whole cell lysates (61). Protein was separated by SDS-PAGE in 10% gels and analyzed by Western blotting using the monoclonal antibodies 8B1 (anti-V1 subunit) and 13D77 (anti-V\textsubscript{a} subunit B) and yeast PFK-1 polyclonal antibodies. The nitrocellulose membranes were blotted with horseradish peroxidase secondary antibody and scanned using a Bio-Rad ChemiDoc XRS+, and then the intensity of protein bands was quantified using Multi Gauge and GraphPad Prism 5 software.

**NADH Autofluorescence**—NADH was monitored as described by Poulsen et al. (42) with the following modifications. The cells were grown overnight to an optical density of 0.6–1.0 A\textsubscript{600}/ml in YEPD, harvested, and cells with an optical density of 100 A\textsubscript{600} were resuspended in 50 mm potassium phosphate buffer (pH 6.8) up to a density of 10% by weight. The cells were starved of glucose by incubation in the same phosphate buffer for 3 h on a rotary shaker at 30°C and placed on ice for 10 min. The NADH fluorescence intensity (excitation at 366 nm, emission at 450 nm) was measured without glucose for 60 s and then continuously recorded for an additional 90 s with readout of 2% glucose or 4% glucose at 30°C in a FluoroMax 4 spectrofluorometer (Horiba Jobin Yvon Inc.).

**Ethanol Concentration**—For steady-state analyses, the cells were cultured overnight to an optical density of 0.6–1.0 A\textsubscript{600}/ml in YEPD (pH 5.0) medium (wild-type and pfk2Δ) containing 2% or 4% final glucose concentration. Cells with a total of 2.0 optical density A\textsubscript{600}/ml per condition were harvested and then converted to spheroplasts by zymolase treatment (35). The spheroplasts were incubated at 30°C for 10 min in YEP adjusted to pH 5.0 with 50 mm succinic acid/50 mm sodium phosphate containing 2% or 4% glucose plus 1.2 m sorbitol. The ethanol concentration was measured using an ethanol assay kit (ab65343, Abcam) according to the instructions of the manufacturer. For glucose-depletion and readjustment analyses, the cells were grown in medium containing 2% glucose overnight and converted to spheroplasts as described above, and then the
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spheroplasts were incubated in YEP medium with 2% or 4% glucose for 20 min, in YEP for 10 min, and in YEP for 10 min, followed by addition of 2% glucose, 4% glucose, or 2% 2-deoxy-glucose for 10 min.

PKF-1 Enzymatic Activity—Wild-type and pFk2A cells were cultured overnight to an optical density of 0.6–1.0 A_600/ml in YEPD (pH 5.0) medium. Cells were converted to spheroplasts and lysed in 15 mM MES-Tris (pH 6.9) containing 5% glycerol at a final concentration of 1.0 optical density A_600/μl. PKF-1 activity was measured spectrophotometrically at 37 °C using the coupled enzyme assay of Lotscher et al. (62). Whole cell lysates (20.0 optical density A_600) was added to the enzymatic assay mixture (25 mM Tris (pH 6.9), 2 mM ATP, 5 mM MgCl_2, 2 mM phosphoenolpyruvate, 30 units/ml pyruvate kinase, 30 units/ml l-lactic dehydrogenase, and 0.5 mM NADH), and the reaction was started by addition of 5 mM fructose-6-phosphate. NADH oxidation was monitored spectrophotometrically at 340 nm for 5 min. One unit of PKF-1 activity is defined as 1 μmol fructose 1,6-bisphosphate formed/min in an optical density of 20 A_600.

Other Methods—Vacuolar membranes fractions were purified by Ficoll density gradient centrifugation as described before (35, 48, 61). Protein concentration was measured by the Bradford assay (63). ATP hydrolysis was measured by monitoring NADH oxidation spectrophotometrically (62) using 5% glycerol at a final concentration of 1.0 optical density A_600/μl. PFK-1 activity was measured spectrophotometrically at 37 °C using the coupled enzyme assay of Lotscher et al. (62). Whole cell lysates (20.0 optical density A_600) was added to the enzymatic assay mixture (25 mM Tris (pH 6.9), 2 mM ATP, 5 mM MgCl_2, 2 mM phosphoenolpyruvate, 30 units/ml pyruvate kinase, 30 units/ml l-lactic dehydrogenase, and 0.5 mM NADH), and the reaction was started by addition of 5 mM fructose-6-phosphate. NADH oxidation was monitored spectrophotometrically at 340 nm for 5 min. One unit of PKF-1 activity is defined as 1 μmol fructose 1,6-bisphosphate formed/min in an optical density of 20 A_600.

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