The Ras/cAMP/Protein Kinase A Pathway Regulates Glucose-dependent Assembly of the Vacuolar (H\(^+\))-ATPase in Yeast*

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Vacuolar (H\(^+\))-ATPases (V-ATPases) are ubiquitous, ATP-driven proton pumps that acidify organelles or the extracellular space. A rapid and effective mechanism for regulating V-ATPase activity involves reversible dissociation of the two functional domains of the pump, V\(_1\) and V\(_0\). This process is best characterized in yeast, where V-ATPases are reversibly disassembled in response to glucose depletion. To identify regulators that control this process in vivo, a genetic screen was performed in yeast to search for mutants that cannot disassemble their V-ATPases when grown in the absence of glucose. This screen identified IRA1 (inhibitory regulator of the Ras/cAMP pathway) and IRA2 as essential genes for regulating V-ATPase dissociation in vivo. IRA1 and IRA2 encode GTPase-activating proteins that negatively regulate Ras in nutrient-poor conditions. Down-regulation of Ras lowers cAMP levels by reducing adenylate cyclase activity. Decreased cAMP levels in turn lead to reduced activity of protein kinase A (PKA). Our results show that targeted deletion of IRA2 results in defective disassembly of the V-ATPase in response to glucose depletion, and reexpression of the gene rescues this phenotype. Glucose-dependent dissociation is also blocked in strains expressing the dominant active RAS2\(^{G19V}\) allele or in strains deficient for the regulatory subunit of PKA, both of which lead to constitutively active PKA. These results reveal a role for PKA in controlling glucose-dependent V-ATPase assembly in yeast.

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Vacuolar (H\(^+\))-ATPases (V-ATPases)\(^2\) are ATP-driven proton pumps that acidify intracellular compartments or the extracellular space (1, 2). In all eukaryotic cells, they are located on the membranes of endosomes, secretory vesicles, Golgi, and lysosomes (or vacuoles in plants and fungi), where they pump protons from the cytosol into the lumen of these organelles. The pH gradients established and maintained by V-ATPases are important for a variety of physiological processes, including coupled transport of small molecules, such as neurotransmitters (3, 4); activation of lysosomal/vacuolar acid hydrolases; processing of secreted peptides, such as insulin (5); and trafficking of endocytosed ligands or newly synthesized proteins to their appropriate cellular destinations (6). Entry of enveloped viruses, such as influenza virus, or bacterial toxins, such as diphtheria or anthrax toxin, is also dependent on acidification of intracellular compartments by V-ATPases (7). V-ATPases are also localized to the plasma membrane of certain cells, where they pump protons from the cytosol into the extracellular space. This activity is essential for normal physiological processes, such as acid/base balance by renal cells (8), pH regulation in macrophages and neutrophils (9, 10), bone resorption by osteoclasts (11, 12), and sperm maturation and storage in the epididymus (13). Certain types of cancer cells also target V-ATPases to the plasma membrane, and expression of surface V-ATPases correlates with a metastatic phenotype (14, 15).

V-ATPases are composed of two functional domains known as V\(_1\) and V\(_0\) (1, 2). The V\(_1\) domain is located in the cytosol and carries out ATP hydrolysis, whereas the V\(_0\) domain is membrane-bound and responsible for proton translocation across the lipid bilayer. The V\(_1\) domain is a 650-kDa complex composed of eight different subunits (A–H) present in one or more copies. In mammals, the V\(_0\) domain is a 260-kDa complex composed of five different subunits (a, c, c\(^{\prime}\), d, and e) present in single copies with the exception of c, which is present in multiple copies (1, 16). Yeast express an additional subunit (c\(^{\prime}\)) homologous to the c subunit (17), and mammalian V\(_0\) domains sometimes contain an accessory subunit known as Ac45 (18).

V-ATPase activity is tightly controlled. Regulating V-ATPase activity allows cells to maintain organelles at a specific pH as well as increase or decrease proton flux across particular membranes in response to specific stimuli. One primary mechanism for regulating V-ATPase activity is reversible dissociation, which involves the physical separation or assembly of the V\(_1\) and V\(_0\) domains (2, 19). This mechanism is a rapid and effective way to reduce or increase proton pumping activity and has been observed in several different organisms, including mammals, insects, and yeast. In dendritic cells of the immune system, V\(_1\) domains will assemble with V\(_0\) domains in response to maturation signals, allowing the pump to acidify organelles that function in antigen processing (20). Cultured kidney cells respond to increased glucose concentrations by assembling V\(_1\) with V\(_0\) in a manner that requires phosphatidylinositol 3-kinase activity (21). V-ATPase assembly is modulated during development in...
the tobacco hornworm gut (22), whereas in blowfly salivary gland epithelial cells, cAMP triggers assembly and proton pumping through a mechanism that may involve phosphorylation of the V1-C subunit by protein kinase A (PKA) (23, 24).

V-ATPase regulation by reversible dissociation is best characterized in yeast (2). Here, V-ATPases are disassembled in response to glucose depletion and reassembled when glucose (or another rapidly fermented carbon source) is added back to the media (25). This process occurs within minutes, does not require new protein synthesis, and is not affected by disruption of the major signaling pathways known to respond to nutritional changes (26). Interestingly, the glycolytic enzyme aldolase binds V-ATPase subunits and may play a role in stabilizing the assembled pump (27, 28). Point mutations in aldolase that disrupt its interaction with the V-ATPase without affecting glycolytic activity result in a disassembled pump, and overexpressing aldolase blocks V-ATPase dissociation upon glucose removal (29). It also appears that assembly and disassembly are independently controlled processes, because intact microtubules are required for disassembly but not reassembly (30), and a heterotrimeric complex known as RAVE functions in assembly but not disassembly (31, 32). Although reversible dissociation has been well studied in yeast, it is not known how signals regarding nutritional state are transmitted to the V-ATPase to regulate its assembly.

In an effort to understand how V-ATPase activity is controlled in cells in response to stimuli, we performed a genetic screen in yeast to identify gene products required for down-regulating V-ATPase activity through disassembly of the V1 and V0 domains. Because assembly or disassembly occurs rapidly in response to specific stimuli, we hypothesized that a signaling molecule or pathway controls this process. This screen identified the Ras GTPase-activating proteins (GAPs) IRA1 and IRA2 as essential factors for regulating V-ATPase disassembly in yeast in response to glucose depletion. We show that expressing the dominant active RAS2val19 allele or deleting the PKA regulatory subunit BCK1 results in a similar phenotype, suggesting that PKA mediates the effects of Ras on V-ATPase assembly.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies**—G418, phenylmethylsulfonyl fluoride, dithiothreitol (DTT), raffinose, and Protein A-Sepharose were purchased from Sigma. Selection medium was purchased from General Electric, and signal was detected using Eastman Kodak Co. BioMax Light film. Monoclonal antibodies 10D7 (anti-a) and 8B1 (anti-A) were purchased from Invitrogen. Peroxidase-conjugated monoclonal antibody 3F10 (anti-HA) was purchased from Roche Applied Science.

**Yeast Strains and Media**—YPH500 (MATα, ura3-52, lys2-801amber, ade2-101his3Δ200, his3Δ200, leu2-Δ1) (33) was maintained in YPD (1% yeast extract, 2% bacto-peptone, 2% glucose). ira2Δ, ras1Δ, ras2Δ, and bcy1Δ deletion strains (34) and their parental strain BY4742 (MATα, his3Δ1, leu2-Δ0, lys2-Δ0, ura3-Δ0) were purchased from Open Biosystems and maintained in YPD (parental strain) or YPD with 0.2 mg/ml G418 (deletion strains). IRA2 rescue strains were generated by cloning IRA2 plus its endogenous promoter into the plasmid pRS316 (33) and then transforming this plasmid into the ira2Δ strain. Transformants were selected and maintained on −ura-cil, 0.2 mg/ml G418 plates. To clone IRA2 plus endogenous promoter, genomic DNA was isolated from BY4742 and used to amplify the IRA2 open reading frame plus 500 flanking base pairs upstream and downstream. The following primers were used for the PCR: forward, 5′-AACCAAAGGCTGCTCTCATAAAGGG-3′; reverse, 5′-AAGACTGATCTCAGAATTATGTT-3′. The resulting PCR product was cloned into the plasmid pCR-XL-TOPO using the TOPO XL PCR cloning kit (Invitrogen), analyzed by sequencing, and then cloned into pRS316 using the Spel and Xhol restriction sites. Yeast expressing RAS2val19 were generated by transforming BY4742 with plasmid pPHY453. pPHY453 contains the RAS2val19 allele cloned into pRS415 (35) and was a kind gift from Paul Herman. Yeast expressing wild type or R25L G subunit were generated by transforming yeast strain SF838-5A (vma10Δ::ura3) with plasmid pRS315 containing N-Myc-tagged VMA10 wild type or R25L (36). The vma10Δ yeast strain and VMA10 plasmids were kind gifts from Patricia Kane. The HA-tagged aldolase strain was purchased from Open Biosystems and maintained in YPD. This strain carries a randomly inserted, in-frame 3× HA tag within the aldolase open reading frame in a Y800 background (MATa leu2-Δ98 cry1Δ/ MATα leu2-Δ98 CRY1, ade2-101 HIS3/ade2-101 his3Δ200, ura3-52 can1Δ/ura3-52 CAN1, lys2-801/lys2-801, CYS2/ CYP2, trp1-1/TRP1 Cir′ carrying pGAL-cre (amp, ori, CEN, LEU2)) (37, 38). To express RAS2val19 in this strain, RAS2val19 was moved from pPHY453 into pRS316 using HindIII and XhoI sites, and the resulting plasmid was transformed into the HA-aldolase strain. Transformants were selected and maintained on −uracil plates.

**Genetic Screen**—YPH500 was mutagenized using the mTn3 (LEU2 lacZ) transposon library (39), a kind gift from Michael Snyder. Yeast were transformed with NotI-digested library DNA, and the resulting mutants were selected on −leucine, 2% raffinose plates and analyzed visually for red color. Approximately 30,000 mutants were screened, and 104 mutants that appeared red were selected, subcloned, and preserved as frozen stocks. To identify the location of the transposon in the selected mutants, yeast were transformed with pRSQ2-URA3, a kind gift from Michael Snyder, and selected on −leucine−uracil plates. Yeast genomic DNA was isolated, digested with EcoRI, ligated, and transformed into Escherichia coli. Recovered plasmids were sequenced using a primer specific for a sequence in the insertion, 5′-GCTGCAAGGCATTAGTGG-3′.
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Glucose-dependent Dissociation—Yeast cells were grown in selection media to an absorbance at 600 nm of 0.75–0.9, and then two or three aliquots of yeast cells equivalent to 10 optical density units were taken for the assay. Yeast were converted to spheroplasts by incubating in Tris/DTT buffer (0.1 M Tris-HCl, pH 9.4, 10 mM DTT) for 10 min at 30 °C, rinsing once in Spheroplast buffer (YPD with 0.1 M MES-Tris, pH 7.5, 0.7 M sorbitol, 2 mM DTT), and then incubating in Spheroplast buffer containing 0.3 mg of Zymolyase 100T for 40 min at 30 °C. Yeast were then rinsed twice in YPD/0.7 M sorbitol (1% yeast extract, 2% bacto-peptone, 2% glucose, 0.7 M sorbitol) or YEP/1.2 M sorbitol (1% yeast extract, 2% bacto-peptone, 1.2 M sorbitol) and then incubated in YPD/0.7 M sorbitol or YEP/1.2 M sorbitol for 30 min. For reassociation experiments, yeast were incubated in YEP/1.2 M sorbitol for 30 min followed by YPD/0.7 M sorbitol for 30 min. Yeast were then lysed in Lysis buffer (1× PBS, pH 7.4, 1% polyoxyethylene 9-lauryl ether, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride), and cell lysate was then incubated for 1 h at 4 °C in Lysis buffer containing 25 mM dithiobis(succinimidyl)propionate, preincubated with Protein A-Sepharose for 30 min at 4 °C, and then incubated with the monoclonal antibody 8B1 (anti-A) at 1:500 overnight at 4 °C. Antibodies and bound protein were isolated with Protein A-Sepharose and separated by SDS-PAGE. After transferring membranes were isolated by centrifugation on two consecutive Ficoll gradients (12 and 8%) and diluted in transport buffer (15 mM MES-Tris, pH 7, 4.8% glycerol).

Isolation of Vacular Membrane Vesicles—Vacular membrane vesicles were isolated as described in our previous study (40). Briefly, 1 liter of yeast cells was grown overnight at 30 °C to an absorbance at 600 nm of 1.2–1.4 in selective media, rinsed once with water, and then converted to spheroplasts by incubating in Tris/DTT buffer for 20 min at 30 °C, rinsing once in Spheroplast buffer, and then incubating in Spheroplast buffer containing 2 mg of Zymolyase 100T for 60–90 min at 30 °C. The resulting spheroplasts were osmotically lysed, and vacuolar membranes were isolated by centrifugation on two consecutive Ficoll gradients (12 and 8%) and diluted in transport buffer (15 mM MES-Tris, pH 7, 4.8% glycerol).

Other Methods—Protein concentrations were determined by the Lowry method (41). ATPase activity was measured using a continuous spectrophotometric assay in the presence or absence of 1 μM concanamycin A (42, 43). 3 μg of vacuolar membrane vesicles were diluted into assay mixture containing 50 mM NaCl, 30 mM KCl, 20 mM HEPES, 0.2 mM EGTA, 10% glycerol, 1 mM ATP, 2 mM MgSO4, 1.5 mM phosphoenolpyruvate, 0.25 mg/ml NADH, 20 μg/ml pyruvate kinase, and 20 μg/ml lactate dehydrogenase, and the absorbance change at 340 nm was measured at 30 °C. Proton transport was measured by ATP-dependent fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine in the presence or absence of 1 μM concanamycin A using a PerkinElmer Life Sciences LS-5 spectrofluorometer (44). 3 μg of vacuolar membrane vesicles were dilute into assay mixture containing 50 mM NaCl, 30 mM KCl, 20 mM HEPES, 0.2 mM EGTA, 10% glycerol, and 1 μM 9-amino-6-chloro-2-methoxyacridine. Fluorescence quenching was measured following the addition of 1 mM ATP and 2 mM MgSO4.

RESULTS

Yeast Genetic Screen to Identify V-ATPase Regulators—To identify factors involved in V-ATPase dissociation in vivo, a genetic screen was performed in yeast to look for mutants that cannot dissociate their V-ATPases in response to glucose depletion. To perform the initial screen, we used the adenine-deficient Saccharomyces cerevisiae strain YPH500, which produces a red pigment derived from an intermediate in adenine biosynthesis. In cells containing a functional V-ATPase, this pigment accumulates in the acidic vacuole, turning the colony red. By contrast, in cells lacking functional V-ATPase, no pigment accumulates in the vacuole, and the colony appears white. Because glucose promotes V-ATPase assembly in yeast, YPH500 colonies normally appear red when plated on glucose. When V-ATPases are triggered to disassemble by plating on poorly fermented carbon sources, such as raffinose, the red pigment does not accumulate, and the colonies appear white (Fig. 1A). We used this property to screen for mutants that could not disassemble their V-ATPases in nutrient-poor conditions, because such mutants would appear red on raffinose plates. To perform the screen, a transposon library (39) was used to mutagenize the YPH500 genome, followed by plating of mutant cells on raffinose and selection of colonies that appeared red. From 30,000 colonies screened, 104 mutant colonies were isolated and confirmed as remaining red on raffinose plates.

Mutants that were positive by this initial screen were then tested using a secondary screen designed to directly measure V-ATPase assembly in response to glucose depletion. The yeast cell wall was removed, and the resulting spheroplasts were cultured in the presence or absence of glucose and then lysed. An antibody directed against the A subunit of the V1 domain was used to immunoprecipitate both the V1 domain and the V1/V0 holoenzyme (Fig. 1B). The immunoprecipitated proteins were separated by SDS-PAGE, and Western blotting was performed using antibodies directed against subunit A (as a measure of the amount of V0 immunoprecipitated) and subunit A (as a measure of the amount of V1). Dissociation appears as a reduction in the amount of V0 immunoprecipitated with V1. Mutants that did not show a decrease in assembly of the V-ATPase in response to glucose removal were scored as being defective in vivo dissociation. Of the 104 mutants isolated in the initial screen, 57 have been tested using the secondary screen. Of these, nine mutants showed defective dissociation in this assay.

IRA2 Is Required for V-ATPase Regulation by Disassembly—One mutant identified twice in the primary screen and confirmed using the secondary screen is disrupted in the IRA2 gene. IRA2 gene is a Ras GAP that stimulates the intrinsic GTPase activity of Ras proteins to convert them from the active, GTP-bound form to the inactive, GDP-bound form (45–47). In S. cerevisiae, GTP-bound Ras activates adenylate cyclase, leading to increased cAMP levels and PKA activity (48). This pathway is
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**FIGURE 1. Strategy for genetic screen to identify gene products involved in regulating glucose-dependent dissociation of the V-ATPase.** The adenine-deficient yeast strain YPH500 was used for the screen. A, red pigment accumulates in the vacuole of these yeast in a manner that depends on V-ATPase activity. When grown on glucose, colonies appear red (○), indicating functional V-ATPase (left). Insertional mutagenesis was performed on YPH500, followed by plating on media containing raffinose, a poorly fermented carbon source known to trigger V-ATPase disassembly. When grown on raffinose, most colonies appeared white (□), indicating low V-ATPase activity due to dissociation of the pump (right). Mutants that appeared red on raffinose-containing media were selected as candidates for defective V-ATPase disassembly and were subject to a secondary screen designed to directly evaluate V-ATPase assembly state. B, a glucose-dependent dissociation assay was used for the secondary screen. Spheroplasts were incubated in the presence or absence of glucose followed by lysis in the presence of the detergent polyoxyethylene 9-lauryl ether to solubilize the V-ATPase. Antibodies that specifically recognize the A subunit of the V1 domain were used to immunoprecipitate the V1 domain and the V1V0 holoenzyme. Assembly was measured by performing a Western blot on immunoprecipitated complexes using an antibody that specifically recognizes the A subunit of the V1 domain. An antibody that recognizes subunit A of the V0 domain was used as a loading control. Glucose-dependent dissociation is reflected as a decrease in the amount of a subunit immunoprecipitated with the A subunit antibody. Mutants that did not show such a decrease were identified as strains with defective V-ATPase regulation and were selected for further study.

**FIGURE 2. IRA2 is essential for glucose-dependent V-ATPase dissociation.** Wild type, *ira2Δ*, and the *ira2Δ* yeast strain reexpressing wild type IRA2 on the plasmid pRS316 were tested for glucose-dependent dissociation, as described in the legend to Fig. 1A. The *ira2Δ* strain showed similar levels of a subunit immunoprecipitated with the antibody directed against subunit A in the presence or absence of glucose, indicating defective dissociation of the pump in nutrient-limiting conditions. Reexpressing IRA2 in the *ira2Δ* strains (rescue) restored the dissociation phenotype to that observed in the wild type strain.

*IRA2* is required for V-ATPase regulation, glucose-dependent dissociation was tested in a yeast strain in which the *IRA2* gene had been replaced with the kanamycin resistance gene (34). Although glucose removal triggered normal disassembly of the V1 domain from the V0 domain in the parental strain expressing wild type *IRA2*, the *ira2Δ* strain showed a dramatic reduction in V-ATPase regulation by disassembly (Fig. 2). Introduction of a plasmid containing the wild type *IRA2* gene into the *ira2Δ* strain restored the wild type phenotype. We conclude from these results that *IRA2* is essential for V-ATPase disassembly in yeast in response to glucose depletion.

Loss of *IRA2* Does Not Affect V-ATPase Stability or Activity—One possible way in which mutations could lead to reduced dissociation of the V-ATPase in response to glucose depletion is to increase the inherent stability of the V-ATPase complex. Certain point mutations in the G subunit of the yeast V-ATPase result in such increased stability and reduced dissociation following glucose removal (36). These G subunit mutations are characterized by increased levels of V1 subunits on isolated vacuolar membranes (resulting from the increased stability of the complex). To test whether loss of *IRA2* leads to a more stable V-ATPase complex, we performed Western blots on vacuolar membranes isolated from wild type, *ira2Δ*, or rescue strains maintained in the presence of glucose. Increased stability is reflected by more intense V1 subunit staining on isolated vacuoles. No difference in the levels of V1 or V0 subunits was observed among these three strains (Fig. 3A), indicating that stability of the pump is not affected by loss of *IRA2*.

To verify that increased stability can be measured using this method, we analyzed yeast strains expressing an R25L point mutation in the V-ATPase G subunit. This point mutation has previously been reported to confer increased stability to the pump (36). Consistent with previous results, an increase in stability was observed in the R25L strain relative to wild type as evidenced by an increase in V1 staining relative to V0 (Fig. 3B).
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We next tested whether loss of IRA2 leads to altered V-ATPase activity. In some cases, reduced activity can lead to defective dissociation (26), so a reduction in activity is another possible way in which in vivo dissociation could be inhibited. ATPase and proton pumping assays were performed on vacuolar membranes in the presence or absence of the specific V-ATPase inhibitor concanamycin A. No significant difference in concanamycin-sensitive ATP hydrolysis and proton transport activity was observed among the three strains, indicating that the phenotype observed in ira2Δ mutants upon glucose depletion is not due to increased stability of the assembled pump. 

IRA2—the homolog IRA1 is required for V-ATPase disassembly upon glucose removal. A glucose-dependent dissociation assay was performed on strains containing wild type IRA1 or IRA1 with a transposon-mediated insertion as described in the legend to Fig. 1. Levels of subunit a do not decrease upon glucose removal in yeast with a disrupted IRA1 gene, indicating defective glucose-dependent V-ATPase dissociation.

Constitutively Active Ras2 Blocks V-ATPase Dissociation—Because Ira proteins are negative regulators of Ras, we next tested whether Ras plays a role in controlling V-ATPase assembly. To test the effects of overactive Ras on V-ATPase regulation, we analyzed glucose-dependent dissociation in a strain expressing RAS2val19, a dominant active mutant with greatly reduced GTPase activity (48). Interestingly, V-ATPase dissociation was blocked in strains expressing RAS2val19 (Fig. 6), consistent with the reduced dissociation in the ira deletion strains.

We next wished to determine the effect of reducing Ras activity on V-ATPase dissociation. Ras is encoded by two genes in S.
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![FIGURE 6. Effects of Ras mutations on reversible dissociation. Wild type, ras1Δ, ras2Δ, and wild type yeast expressing RAS2val19 on the plasmid pRS415 were tested for glucose-dependent dissociation as described in the legend to Fig. 1. Reassembly of the pump was tested by incubating the cells in the absence of glucose, followed by the presence of glucose, before lysing the cells for immunoprecipitation (indicated as -/+). Reversible dissociation occurred normally in ras1Δ and ras2Δ strains but was blocked in yeast expressing RAS2val19.](image)

![FIGURE 7. Constitutively active PKA blocks V-ATPase dissociation. Yeast deficient for the PKA regulatory subunit (bcy1Δ) were assayed for glucose-dependent dissociation as described in the legend to Fig. 1B. bcy1Δ strains show no decrease in a subunit levels in the absence of glucose, indicating that PKA promotes V-ATPase assembly and/or inhibits disassembly.](image)

cerevisiae, RAS1 and RAS2. Strains lacking either RAS1 or RAS2 are viable, but deletion of both isoforms in the same strain is lethal (48). When we tested glucose-dependent dissociation in yeast deficient for either RAS1 or RAS2, we found that V-ATPases dissociated normally in these strains (Fig. 6). Adding glucose back to the media led to reassembly of the pump in wild type, ras1Δ, and ras2Δ strains but had no effect on yeast expressing RAS2val19 (Fig. 6). We conclude from these results that Ras plays a role in regulating V-ATPase disassembly in yeast in response to glucose removal and that Ras1 and Ras2 can complement each other in this function.

**PKA Regulates V-ATPase Assembly**—In yeast, Ras stimulates adenylyl cyclase, leading to increasing cAMP levels (48). Strains with activated Ras (through deletion of Ira proteins or expression of RAS2val19) display elevated levels of cAMP and are phenotypically similar to strains with activated PKA. Therefore, we sought to determine whether PKA regulates V-ATPases by testing the effects of increased PKA activity on glucose-dependent dissociation. PKA is a tetramer composed of two catalytic subunits and two regulatory subunits (48). cAMP activates PKA by binding the regulatory subunits, allowing the catalytic subunits to phosphorylate downstream substrates. Yeast with a deletion in the gene that encodes the regulatory subunits, BCY1, have constitutively active PKA that is no longer cAMP-dependent (50). When we tested for glucose-dependent V-ATPase dissociation in a bcy1Δ strain, we found that V-ATPases remain assembled upon glucose removal (Fig. 7). These data indicate that activated PKA causes V-ATPases to assemble and/or blocks V-ATPase disassembly, leading to increased V-ATPase activity. In addition, these data show that PKA mediates the effects of overactive Ras signaling (whether through decreased GAP activity or expression of dominant active Ras2) on V-ATPase assembly.

**Constitutively Active Ras2 Affects the Aldolase/V-ATPase Interaction**—To begin to explore the mechanisms by which PKA promotes V-ATPase assembly, we determined the effects of increased Ras signaling on the aldolase/V-ATPase interaction. Previous studies show that the glycolytic enzyme aldolase interacts with the V-ATPase and suggest that this interaction stabilizes the pump in its assembled state (27–29). Glucose removal disrupts the aldolase/V-ATPase interaction. We hypothesized that overactive Ras signaling would block the dissociation of aldolase upon glucose removal. Yeast expressing HA-tagged aldolase were transformed with RAS2val19, and the amount of aldolase immunoprecipitated with V1 was measured. The amount of aldolase associated with the V-ATPase decreased upon glucose removal in the HA-aldolase strain, confirming previous reports (Fig. 8A) (28). Interestingly, the expression of RAS2val19 resulted in decreased levels of aldolase bound to the V-ATPase even in the presence of glucose (Fig. 8A). Furthermore, no decrease in aldolase was observed upon glucose removal. The amount of bound aldolase in RAS2val19-expressing strains in either the presence or absence of glucose was about the same as in wild type strains in the absence of glucose. As before, V-ATPase assembly decreased upon glucose removal in the HA-aldolase strain but remained unchanged with the expression of RAS2val19 (Fig. 8B). Thus, Ras signaling leads to increased V-ATPase assembly but decreased aldolase binding.

**DISCUSSION**

Regulation of V-ATPase activity by reversible dissociation is well characterized in yeast, but the signals that transmit information regarding nutritional status to the V-ATPase are unknown. By screening for mutants defective in V-ATPase disassembly upon glucose removal, we have identified the glucose-sensitive Ras/cAMP/PKA pathway as playing an essential role in this process. We showed that V-ATPases remain assembled in the absence of glucose when PKA is stimulated through several methods, including reduction of Ras-GAP activity, expression of constitutively active Ras2, and deletion of the PKA regulatory subunit. This represents the first identification of signaling molecules involved in regulation of V-ATPase activity in yeast and marks a significant advance in our understanding of the factors that control V-ATPase assembly and activity in response to nutrient depletion.

PKA has been shown to play a role in V-ATPase assembly in other model systems. Exposure of insect epithelial cells to...
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FIGURE 8. Activated Ras2 affects the aldolase/V-ATPase interaction. A, yeast expressing HA-tagged aldolase and Ras2<sup>val19</sup> on the plasmid pRS316 were converted to spheroplasts, incubated in the presence or absence of glucose and then lysed. V-ATPases were immunoprecipitated using an antibody that recognizes the A subunit, and V-ATPase-associated aldolase was detected by performing a Western blot on immunoprecipitated proteins using an anti-HA antibody, with the anti-A antibody as a loading control. Glucose removal led to a decrease in associated aldolase in the presence of glucose, with no change in the amount of bound aldolase upon glucose removal. B, yeast expressing HA-tagged aldolase were assayed for glucose-dependent V-ATPase dissociation, as described in the legend to Fig. 1B. Consistent with results shown in Fig. 6 for a different yeast strain, dissociation was normal in the HA-aldolase strain but was blocked upon expression of Ras2<sup>val19</sup>.</p>

CAMP results in massive translocation of V<sub>i</sub> domains to the apical membrane and proton pumping across the cell surface (23, 51). This effect is blocked by PKA inhibitors. In addition, the catalytic (but not regulatory) subunit of PKA translocates to the apical membrane when these cells are treated with the CAMP analog 8-CPT-CAMP (52). Interestingly, phosphorylation of the V-ATPase C subunit by PKA also occurs concomitantly with these translocation events (24). Although the C subunit is not phosphorylated in the assembled holoenzyme, it is possible that transient phosphorylation of subunit C by PKA may serve as a signal for assembly in these cells. It will be important to identify the downstream PKA substrate in yeast cells and characterize how phosphorylation by PKA leads to V-ATPase assembly. Preliminary experiments in which the yeast V-ATPase was immunoprecipitated with antibodies against subunit A followed by immunoblot with an antibody directed against a phosphopeptide corresponding to the PKA recognition site have detected no difference in the presence or absence of glucose (data not shown). Because V-ATPase dissociation in yeast occurs only on specific membranes (i.e. vacuolar but not Golgi (53)), it will also be of interest to determine whether activated PKA localizes to specific membranes in yeast.

V-ATPases in dendritic cells of the immune system assemble and acidify digestive organelles in response to maturation signals (20), but the proteins that transmit such signals to the V-ATPase have not yet been identified. CAMP and PKA are known to play roles in the process of dendritic cell maturation (54, 55), but whether they mediate V-ATPase assembly during this process is not known.

PKA can regulate V-ATPase activity through mechanisms that do not involve reversible dissociation but rather trafficking of fully assembled enzymes. CAMP-activated PKA stimulates V-ATPase trafficking in rat epididymal cells to increase V-ATPase activity at the plasma membrane, where proton extrusion by the V-ATPase aids in sperm maturation and storage (56). Treatment of macrophages with interleukin-1 leads to increased proton pumping by V-ATPases across the plasma membrane to assist in cytosolic pH regulation (57). PKA is involved in this process, but whether V-ATPase activity increases through trafficking or assembly is not known.

Previous studies have explored whether cAMP mediates the effects of glucose on V-ATPase assembly in yeast (26). The addition of exogenous cAMP to yeast deficient for PDE2, the primary phosphodiesterase that cleaves CAMP, was reported to have no effect on V-ATPase assembly. It is possible that the level of activation of PKA obtained by the addition of exogenous CAMP was not as high as that achieved in the present studies by disruption of IRA1 or IRA2, expression of Ras2<sup>val19</sup>, or deletion of the PKA regulatory subunit. Quantitation of the levels of activated PKA obtained under the previous experimental conditions may help to resolve this discrepancy.

Disruption of either IRA1 or IRA2 resulted in the same phenotype with respect to V-ATPase disassembly, suggesting that that endogenous levels of either protein are insufficient to mediate V-ATPase disassembly upon glucose removal. This observation is common for other irαΔ phenotypes and may be due to the fact that deletion of either gene results in elevated Ras-GTP and CAMP levels (46, 47, 49, 58).

Yeast harboring certain point mutations in the V-ATPase G subunit (V<sub>i</sub>) display the same regulation defect as those with elevated Ras signaling (36). However, these G subunit mutations also lead to an increase in the inherent stability and activity of the pump, whereas the enzymatic properties of V-ATPases in irαΔ mutants are normal. The defects in V-ATPase disassembly in irαΔ mutants are therefore most likely due to dysfunctional regulation of the pump rather than a change in the enzyme itself.

The glycolytic enzyme aldolase binds assembled V-ATPase complexes and appears to play a role in stabilizing the pump (27, 28). Yeast overexpressing aldolase exhibit the same phenotype as those with constitutively active PKA, with defective disassembly of the pump upon glucose depletion (29). The Ras/CAMP/PKA pathway is known to mediate the response of yeast to glucose through activation of glycolytic enzymes (48). Therefore, it is not surprising that overactive Ras affected the aldolase/V-ATPase interaction, although the underlying mechanisms are unclear. Aldolase has one putative PKA recognition site, but there are no reports of PKA-dependent phosphorylation of the enzyme. As noted above, preliminary attempts to detect a change in phosphorylated proteins immunoprecipitated with an antibody against the V-ATPase A subunit showed
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no difference in the presence or absence of glucose. Other effectors may therefore be involved in transmitting the signals from PKA to the V-ATPase to affect assembly. Furthermore, in RAS2val19-expressing cells, V-ATPase assembly increased relative to wild type, whereas the levels of bound aldolase decreased, implying that Ras2 may promote V-ATPase assembly through a mechanism that does not depend on aldolase binding. Whether aldolase performs a V-ATPase stabilization role in mammalian cells has not been tested, although it is interesting to note that patients with certain aldolase mutations have the same clinical outcome as those expressing mutant forms of the renal-specific V-ATPase, namely renal tubular acidosis (59).

In conclusion, we have shown that regulation of V-ATPase assembly and activity in yeast in response to glucose is controlled by the Ras/cAMP/PKA pathway. This represents the first identification of signaling molecules responsible for controlling V-ATPase activity in yeast and allows for further work aimed at testing whether PKA regulates V-ATPase assembly in other systems and characterizing the mechanisms by which PKA accomplishes this. V-ATPases are key participants in a variety of normal and disease processes in cells, and a better understanding of the mechanisms that regulate V-ATPase activity in response to stimuli can aid in the development of effective and specific therapeutics.

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