Microtubules Are Involved in Glucose-dependent Dissociation of the Yeast Vacuolar \([H^+]\)-ATPase in Vivo*

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The vacuolar \([H^+]\)-ATPases (V-ATPases) are composed of a peripheral \(V_1\) domain and a membrane-embedded \(V_0\) domain. Reversible dissociation of the \(V_1\) and \(V_0\) domains has been observed in both yeast and insects and has been suggested to represent a general regulatory mechanism for controlling V-ATPase activity in vivo. In yeast, dissociation of the V-ATPase is triggered by glucose depletion, but the signaling pathways that connect V-ATPase dissociation and glucose metabolism have not been identified. We have found that nocodazole, an agent that disrupts microtubules, partially blocked dissociation of the V-ATPase in response to glucose depletion in yeast. By contrast, latrunculin, an agent that disrupts actin filaments, had no effect on glucose-dependent dissociation of the V-ATPase complex. Neither nocodazole nor latrunculin blocked reassembly of the V-ATPase upon re-addition of glucose to the medium. The effect of nocodazole appears to be specifically through disruption of microtubules since glucose-dependent dissociation of the V-ATPase was not blocked by nocodazole in yeast strains bearing a mutation in tubulin that renders it resistant to nocodazole. Because nocodazole has been shown to arrest cells in the \(G_2\) phase of the cell cycle, it was of interest to determine whether nocodazole exerted its effect on dissociation of the V-ATPase through cell cycle arrest. Glucose-dependent dissociation of the V-ATPase was examined in four yeast strains bearing temperature-sensitive mutations that arrest cells in different stages of the cell cycle. Because dissociation of the V-ATPase occurred normally at both the permissive and restrictive temperatures in these mutants, the results suggest that in vivo dissociation is not dependent upon cell cycle phase.

Vacuolar \([H^+]\)-ATPases (V-ATPases)* are ATP-dependent proton pumps that are located in both intracellular compartments and the plasma membrane (1–11). They function to couple the energy of ATP hydrolysis to the active transport of protons from the cytoplasm of the cell to either the lumen of intracellular organelles or the extracellular environment. Acidification of intracellular compartments is important for a variety of basic cellular processes, including membrane traffic, zymogen activation, protein breakdown, and the coupled transport of small molecules such as neurotransmitters (1–8). Proton transport across the plasma membrane has been shown to function in pH homeostasis (9), renal acidification (10), and bone resorption (11).

The V-ATPases are multisubunit complexes composed of at least 13 subunits organized into two structural domains (1–8). The peripheral \(V_1\) domain contains eight subunits (subunits A–H) with molecular mass of 70–14 kDa and is responsible for ATP hydrolysis. The integral \(V_0\) domain is composed of five subunits (subunits a, c, c*, c**, and d) with molecular mass of 100–17 kDa and is responsible for translocation of protons across the membrane. The V-ATPases thus resemble the F-ATPases (or ATP synthases), which function in ATP synthesis (12–17). Unlike the \(F_1\) and \(F_0\) domains, however, the separate \(V_1\) and \(V_0\) domains are not capable of ATP hydrolysis or passive proton translocation under normal physiological conditions (18).

Because of the variety of functions served by V-ATPases in the cell, it is likely that the activity of V-ATPases is tightly controlled. Several mechanisms have been proposed for controlling V-ATPase activity in vivo, including reversible disulfide bond formation between conserved cysteine residues at the catalytic site (19, 20), changes in the tightness of coupling between proton transport and ATP hydrolysis (21, 22), and changes in the density of V-ATPases through selective targeting (10, 23). Among the regulatory mechanisms for which there is the most compelling evidence is reversible dissociation of the V-ATPase complex. Kane (24) has shown that in yeast, the V-ATPase undergoes dissociation into its component \(V_1\) and \(V_0\) domains in response to removal of glucose from the medium. This dissociation occurs rapidly, does not require new protein synthesis, and is rapidly reversed upon re-addition of glucose to the medium (24). Dissociation of the V-ATPase has also been shown to occur in insects during molting (25) and has been suggested to occur in mammalian cells based upon the existence of pools of free \(V_1\) and \(V_0\) domains (26, 27). Dissociation may thus represent a general mechanism for regulating V-ATPase activity.

Several recent studies have suggested an important link between V-ATPases and the cytoskeleton. A temperature-sensitive mutation in VMA4 was reported to cause a defect in actin distribution and bud morphology (28). In renal cells, the V-ATPase has been shown to interact with the PDZ protein NHERF, which in turn is able to bind actin-associated proteins such as ezrin (29). Finally, direct binding of V-ATPase to actin filaments has been demonstrated in osteoclasts (30). These findings have led us to investigate the possible role of interaction between the V-ATPase and cytoskeletal elements in glucose-dependent dissociation of the V-ATPase in yeast.

**EXPERIMENTAL PROCEDURES**

Materials and Yeast Strains—Leupeptin, aprotinin, and pepstatin were obtained from Roche Molecular Biochemicals. Zymolyase 100T
was from Seikagaku America, Inc. Yeast extract, dextrose, peptone, and yeast nitrogen base were from Difco. Molecular biological reagents were from New England Biolabs Inc., Promega, and Life Technologies, Inc. Monoclonal antibodies SB1-F3 against subunit A and 10D7 against subunit a, rhodamine-conjugated phalloidin, and the actin-depolymerizing agent latrunculin were obtained from Molecular Probes, Inc. Rabbit antiserum against yeast β-tubulin was a kind gift of Dr. Frank Solomon (Department of Biology, Massachusetts Institute of Technology). Cy2-labeled goat anti-rabbit antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. Nocodazole and most other chemicals were from Sigma. Saccharomyces cerevisiae strains used in this study and their genotypes are listed in Table I.

**Drug Treatment of Cells in Culture—**Yeast strains were grown in YEPD medium (yeast extract/peptone/dextrose) to mid-log phase and converted to spheroplasts. Spheroplasts were resuspended in medium with 1.2 M sorbitol. Nocodazole was added from a 5 mg/ml stock solution in Me2SO to a final concentration to 12.5 mM. Cells were incubated in the absence or presence of nocodazole for 2.5 h at 30 °C. Latrunculin was added from a 10 mM stock solution in Me2SO to a final concentration of 0.2 mM. Cells were incubated in the absence or presence of latrunculin for 15 min with shaking at 30 °C. Control cells were treated with an equal amount of Me2SO.

**In Vivo Dissociation and Reassembly of the V-ATPase in Response to Glucose Depletion and Re-addition—**Dissociation and reassembly of the yeast V-ATPase were induced by glucose depletion or re-addition to the medium (24) and was detected by Western blotting as previously described (33). Briefly, yeast cells were grown overnight to mid-log phase, and then cells were converted to spheroplasts by treatment with zymolase (24). For measurement of the effects of inhibitors on dissociation, spheroplasts were incubated in the absence or presence of inhibitors as described above and then resuspended in YEPD or YEP (yeast extract/peptone) medium containing 1.2 M sorbitol and the same concentration of inhibitors, followed by incubation for 40 min at 30 °C. For measurement of the effects of inhibitors on reassembly, spheroplasts were first incubated in YEP medium containing sorbitol for 40 min at 30 °C to induce dissociation, then incubated in the absence or presence of inhibitors as described above, and finally resuspended in YEPD or YEP medium containing sorbitol and the indicated inhibitors for 40 min at 30 °C. Spheroplasts were then solubilized with 1% C12E8 (polyoxyethylene 9-lauryl ether) in the presence of 1 mM dithiothreitol(succinimidyl propionate), and the V-ATPase was immunoprecipitated using monoclonal antibody SB1-F3 against subunit A of the V0 domain. The proteins were then separated by SDS-PAGE on 10% acrylamide gels, and Western blotting was performed using both SB1-F3 and monoclonal antibody 10D7 against the 100-kDa subunit a of the V0 domain. Blots were developed as described under “Experimental Procedures.”

**RESULTS**

Recent studies have suggested that there may exist interactions between the V-ATPase and cytoskeletal elements (28–30). Because dissociation of the V-ATPase has been proposed to represent an important mechanism of regulating V-ATPase activity in vivo (24, 25), we wished to determine whether interactions with the cytoskeleton might play a role in dissociation of the V-ATPase complex. Dissociation of the V-ATPase in yeast was induced by incubation of spheroplasts in glucose-free medium (24) and was detected by solubilization of the V-ATPase with detergent and immunoprecipitation with an antibody directed against subunit A of the V0 domain (34). Western blotting was then performed using antibodies against both subunit A and the 100-kDa subunit a of the V0 domain.
Glucose

Nocodazole

Latrunculin

subunit a

subunit A

FIG. 2. Effect of nocodazole and latrunculin on glucose-dependent reassembly of the yeast V-ATPase. Wild-type cells (YPH499) were grown to mid-log phase and converted to spheroplasts as described under “Experimental Procedures.” Spheroplasts were incubated for 40 min at 30 °C in YEP medium without glucose to induce dissociation of the V-ATPase. Spheroplasts were then incubated in the absence or presence of 40 μM nocodazole for 2.5 h or of 0.2 mM latrunculin for 15 min at 30 °C, followed by incubation for 40 min at 30 °C in YEP medium with or without glucose and in the continued presence or absence of the corresponding inhibitor. Spheroplasts were solubilized with detergent; the V-ATPase was immunoprecipitated with the anti-subunit A antibody; and Western blotting was performed using antibodies against both subunits A and a as described in the legend to Fig. 1.

(35). Dissociation appeared as a reduction in the amount of the 100-kDa subunit a immunoprecipitated with the antibody directed against subunit A, as previously described (35).

As shown in Fig. 1, preincubation of spheroplasts with the actin filament-disrupting agent latrunculin (40) had no effect on dissociation of the V-ATPase in response to glucose depletion. By contrast, preincubation with the microtubule-disrupting agent nocodazole (41) dramatically reduced the amount of glucose-dependent dissociation observed. To determine the effects of these agents on in vivo reassembly of the V-ATPase, spheroplasts were first incubated in glucose-free medium to induce dissociation; incubation was continued in the absence or presence of the inhibitors; and finally, glucose was added back to the medium to induce reassociation of V1 and V0 domains. The assembly status of the V-ATPase was assessed as described above. As can be seen from the data in Fig. 2, neither latrunculin nor nocodazole blocked reassembly of the V-ATPase upon re-addition of glucose. These results suggest that in vivo dissociation, but not reassembly, of the V-ATPase is dependent upon the presence of intact microtubules, but that neither process requires intact actin filaments.

To test the effects of latrunculin and nocodazole on the cytoskeleton in yeast, cells were treated with either latrunculin or nocodazole, and the actin and microtubular networks were visualized using staining with rhodamine-conjugated phalloidin or a polyclonal antibody against β-tubulin, respectively. As shown in Fig. 3, although latrunculin completely disrupted the actin cytoskeleton in yeast, nocodazole had almost no effect on actin. By contrast, treatment with nocodazole completely disrupted the microtubular network, whereas latrunculin had relatively little effect on microtubules (Fig. 4). These results suggest that nocodazole is affecting dissociation of the V-ATPase through disruption of microtubules, rather than through a more general disruption of the cytoskeleton.

FIG. 3. Effect of nocodazole and latrunculin treatment on actin staining in yeast. Wild-type yeast cells (YPH499) were grown to mid-log phase and then incubated with 2% MeSO for 2.5 h (control), 0.2 mM latrunculin for 15 min, or 40 μM nocodazole for 2.5 h. After incubation, the cells were fixed with formaldehyde, and actin filaments were labeled with rhodamine-conjugated phalloidin as described under “Experimental Procedures.”

To further test whether the inhibitory effect of nocodazole is due to disruption of microtubules, glucose-dependent dissociation of the V-ATPase was measured in two yeast strains bearing mutations in tubulin that result in resistance to nocodazole. DBY7051 and DBY8154 were selected for resistance to the structurally related compound benomyl (31), but the mutation in the TUB2 gene present in these strains also results in resistance of growth to nocodazole (data not shown). As shown in Fig. 5, glucose-dependent dissociation of the V-ATPase was unaffected by nocodazole in both the DBY7051 and DBY8154 strains. These results suggest that nocodazole exerts its effect on dissociation of the V-ATPase through disruption of microtubules, rather than through some nonspecific target.

Nocodazole inhibits mitosis and arrests yeast cells in the G2 phase of the cell cycle through disruption of microtubules involved in formation of the spindle body (42). It was therefore possible that nocodazole inhibited dissociation of the V-ATPase indirectly by blocking progression of cells through the cell cycle. To test this possibility, glucose-dependent dissociation was examined in yeast strains bearing temperature-sensitive mutations in CDC genes that result in arrest of cells in different stages of the cell cycle. Mutations in the CDC2, CDC8, CDC15, and CDC28 genes result in arrest of yeast cells at the nonpermissive temperature in the G2, S, M, and G1 phases of the cell cycle, respectively (32). Yeast cells were grown to mid-log phase, incubated at either the permissive (25 °C) or nonpermissive (37 °C) temperature for 4 h, converted to spheroplasts, and then incubated in either the absence or presence of glucose at the same temperature as the original incubation for an additional 40 min. The V-ATPase was solubilized and immunoprecipitated, and assembly was assessed as described above. As shown in Fig. 6, glucose-dependent dissociation of the V-ATPase was nearly identical at the permissive and nonpermissive temperatures in all four yeast strains, although somewhat reduced immunoprecipitation of subunit A was observed in the strains bearing a mutation in CDC9 incubated in the absence of glucose at both temperatures. These results suggest that nocodazole does not inhibit dissociation of the V-ATPase in yeast by blocking progression of cells through the cell cycle. The results also indicate that glucose-dependent dissociation of the V-ATPase can occur at any stage of the cell cycle.

Disruption of microtubules has been shown to lead to frag-
mentation of the central vacuole in yeast (42), whereas glucose depletion leads to coalescence of vacuolar membrane vesicles into a single large vacuole (38). This suggests that the assembly status of the V-ATPase may be sensitive to vacuolar morphology and that nocodazole inhibits dissociation by preventing formation of a single vacuole on glucose depletion. To test this idea, assembly of the V-ATPase was measured in response to exposure of cells to two other stress conditions shown to lead to formation of a single large vacuole, namely ethanol shock and nitrogen starvation (43, 44). As shown in Fig. 7, neither of these conditions led to dissociation of the V-ATPase complex. Incubation of spheroplasts in medium lacking nitrogen for up to 3 h also induces no V-ATPase dissociation (data not shown). These results indicate that although formation of a single large vacuole in yeast may be a necessary condition for dissociation of the V-ATPase to occur, it is not a sufficient condition.

Because mutations that lead to inhibition of V-ATPase activity have been shown to inhibit dissociation of the V-ATPase in response to glucose depletion (45, 46), it was possible that nocodazole might inhibit dissociation through some direct effect on the activity of the V-ATPase. To test this, concanamycycin-sensitive ATPase activity and ATP-dependent proton transport were examined in vacuoles isolated from spheroplasts incubated in the absence or presence of nocodazole. As shown in Table II, no differences in either ATP hydrolysis or proton transport were observed following treatment with nocodazole, suggesting that nocodazole does not block dissociation by irreversibly inhibiting V-ATPase activity.

To test whether the dependence of in vivo dissociation on microtubules resulted from some direct interaction of the V-ATPase with tubulin, spheroplasts were incubated in the absence or presence of glucose and the absence or presence of nocodazole; the V-ATPase was immunoprecipitated using the antibody against subunit A, and Western blotting was performed using both antibodies against subunits A and β-tubulin. The results of these experiments indicate either that the V-ATPase does not directly associate with microtubules or, if it does, that this association does not persist following cell disruption or vacuole isolation.

**DISCUSSION**

Although dissociation of the V-ATPase complex has been shown to represent an important mechanism of regulating activity in both yeast (24) and insects (25), the mechanism by which dissociation is controlled remains unknown. In yeast, dissociation of the V-ATPase in response to glucose depletion has been shown to require catalytic activity of the enzyme (45, 46). On the other hand, many of the signaling pathways that are activated upon removal of glucose from the medium are not involved in dissociation of the V-ATPase. These include the Ras-cAMP pathway, the Snf1p kinase-regulated pathway, the protein kinase C-dependent pathway, and the protein phosphatase 2A-dependent stress response pathway (45). In addition, the assembly status of the V-ATPase does not appear to be controlled by the level of glucose 6-phosphate in the cell, the earliest intracellular metabolite of glucose, as is the case for at least some of the other processes controlled by glucose in yeast.
Rather, V-ATPase assembly is dependent upon the presence and further metabolism of rapidly fermentable carbon sources, including glucose, fructose, and mannose (45).

Dissociation of the V-ATPase in response to glucose depletion has been suggested to represent a mechanism of conserving cellular stores of ATP (24). In fact, there appears to exist a considerable surplus of V-ATPase activity in the cell that is not essential to maintain a wild-type growth phenotype (i.e., growth at pH 7.5) since mutants possessing as little as 20% of wild-type V-ATPase activity still show normal growth at pH 7.5 (47, 48). Thus, cells may retain sufficient capacity to acidify their intracellular compartments even upon glucose depletion. A transient decrease in cellular ATP levels has been measured in yeast upon glucose depletion and has been suggested to serve as a possible trigger in the dissociation process (45). However, V-ATPase assembly cannot be directly tracking ATP levels in the cells since ATP concentrations are restored within 20 min of glucose removal, whereas the V-ATPase remains dissociated (45).

We demonstrate in this study that dissociation of the V-ATPase in response to glucose depletion is dependent upon intact microtubules, but not on actin filaments. This result was somewhat unexpected given previous reports of V-ATPase association with actin (30) and actin-associated proteins such as the ezrin-binding protein NHE-RF (29). The latter protein has been shown to bind to the B1 isoform of the V-ATPase in renal B-type intercalated cells, and this interaction has been suggested to be involved in the selective targeting of V-ATPases to the apical or basolateral membrane in these cells. Moreover, direct interaction between the V-ATPase and actin has been demonstrated in osteoclasts (30), cells that have the capacity to target V-ATPases to the plasma membrane (11). Thus, actin/V-ATPase interactions may be important only in cells that target V-ATPases to the cell surface. Since V-ATPases are restricted to intracellular compartments in yeast, interactions between V-ATPases and actin filaments may not exist or may be less important than in mammalian cells.

It is interesting that although dissociation of the V-ATPase in yeast requires intact microtubules, reassembly of the V-ATPase upon re-addition of glucose does not require microtubules. This suggests that dissociation and reassembly of the V-ATPase may represent independently controlled processes. It is possible that microtubules may be involved in movement of dissociated V1 domains away from the vacuole or in movement of as yet unidentified signaling molecules to the vacuole. In fact, many signaling processes are dependent upon an intact microtubular network (49). Polarized delivery of intact V-ATPases in renal intercalated cells has also been shown to depend upon an intact microtubular network (50), but this result most likely represents the dependence of vesicular movement on microtubules. In contrast to the reported association of the V-ATPase with actin in osteoclasts (30), no direct association of tubulin with the V-ATPase could be detected in yeast (Fig. 8).
Effect of nocodazole on ATPase activity and proton transport by the yeast V-ATPase in isolated vacuoles

Wild-type yeast cells (YPH499) were grown to mid-log phase and converted to spheroplasts as described under “Experimental Procedures.” Spheroplasts were then incubated in YPD medium in the absence or presence of 40 μM nocodazole for 2.5 h, followed by lysis and isolation of vacuoles as described under “Experimental Procedures.” ATPase activity and ATP-dependent proton transport were measured for isolated vacuoles in the absence and presence of 1 μM concanamycin A using a coupled spectrophotometric assay and uptake of acridine orange, respectively. Values are expressed relative to activities measured for control vacuoles in the absence of both nocodazole treatment and concanamycin A. The specific activity of the ATPase in control vacuoles in the absence of concanamycin A was 1.2 μmol of ATP/min/mg of protein.

| Relative ATPase activity | + concanamycin | - concanamycin |
|--------------------------|---------------|---------------|
| Control                  | 100           | 100           |
| Nocodazole-treated       | 98 ± 4        | 96 ± 5        |

| Relative proton transport | + concanamycin | - concanamycin |
|---------------------------|---------------|---------------|
| Control                   | 3 ± 1         | 3 ± 1         |
| Nocodazole-treated        | 4 ± 1         | 4 ± 1         |

![Fig. 8. The yeast V-ATPase does not directly associate with β-tubulin.](image_url)

Because disruption of microtubules leads to fragmentation of the central vacuole (42), whereas glucose depletion leads to formation of a single large vacuole (38), it is possible that nocodazole inhibits dissociation by preventing vacuolar coalescence. However, dissociation cannot be occurring as a direct result of vacuolar coalescence since other stress conditions that lead to formation of a single large vacuole (i.e. ethanol shock and nitrogen depletion (43, 44)) do not cause dissociation of the V-ATPase complex. Thus, formation of a single large vacuole in yeast may be a necessary, but not sufficient, condition for dissociation of the V-ATPase to occur.

We have also found that dissociation of the V-ATPase is not dependent upon a particular stage of the cell cycle. Thus, nocodazole treatment does not affect V-ATPase dissociation through disruption of the cell cycle. This suggests that the signaling pathways involved in activating dissociation are not specific to a particular stage of the cell cycle. Further work will be required to identify these signaling pathways and to determine whether an intact microtubular network is required for dissociation of the V-ATPase in other systems.

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