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Saccharomyces cerevisiae Lacking Btn1p Modulate Vacuolar ATPase Activity to Regulate pH Imbalance in the Vacuole*

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The vacuolar H^+-ATPase (V-ATPase) along with ion channels and transporters maintains vacuolar pH. V-ATPase ATP hydrolysis is coupled with proton transport and establishes an electrochemical gradient between the cytosol and vacuolar lumen for coupled transport of metabolites. Btn1p, the yeast homolog to human CLN3 that is defective in Batten disease, localizes to the vacuole. We previously reported that Btn1p is required for vacuolar pH maintenance and ATP-dependent vacuolar arginine transport. We report that extracellular pH alters both V-ATPase activity and proton transport into the vacuole of wild-type *Saccharomyces cerevisiae*. V-ATPase activity is modulated through the assembly and disassembly of the V_0 and V_1 V-ATPase subunits located in the vacuolar membrane and on the cytosolic side of the vacuolar membrane, respectively. V-ATPase assembly is increased in yeast cells grown in high extracellular pH. In addition, at elevated extracellular pH, *S. cerevisiae* lacking *BTN1* (*btn1*-Δ), have decreased V-ATPase activity while proton transport into the vacuole remains similar to that for wild type. Thus, coupling of V-ATPase activity and proton transport in *btn1*-Δ is altered. We show that down-regulation of V-ATPase activity compensates the vacuolar pH imbalance for *btn1*-Δ at early growth phases. We therefore propose that Btn1p is required for tight regulation of vacuolar pH to maintain the vacuolar luminal content and optimal activity of this organelle and that disruption in Btn1p function leads to a modulation of V-ATPase activity to maintain cellular pH homeostasis and vacuolar luminal content.

The neuronal ceroid-lipofuscinoses (NCLs)[2] are the most common group of progressive neurodegenerative diseases in children, with an incidence as high as 1 in 12,500 live births (1, 2). The NCL disorders are inherited in an autosomal recessive manner, with mutations in seven distinct genes resulting in pathologically similar disease with a different age of onset (3, 4). The NCLs are characterized by the accumulation of autofluorescent hydrophobic material in the lysosomes of neurons, and to lesser extent, other cell types (5, 6); however, the molecular basis behind this storage and the disease remains unknown. The juvenile form of NCL results from mutations in the *CLN3* gene, which codes for a lysosomal transmembrane protein (7–9).

We have previously reported that the *Saccharomyces cerevisiae BTN1* gene product has high sequence similarity with the human *CLN3* gene product. *BTN1* encodes a non-essential protein that is 39% identical and 59% similar to human *CLN3* (10). Studies have revealed that Btn1p is located in the vacuolar membrane (11, 12) and, although the primary function of this protein remains unclear, it has been implicated in several cellular pathways. Lack of *BTN1* resulted in resistance to D-(−)-threo-2-amino-1-[*p*-nitrophenyl]-1,3-propanediol (ANP), and this phenotype is complemented by expression of human Cln3p, indicating that yeast Btn1p and human Cln3p likely have a conserved function (13). Resistance of *btn1*-Δ yeast strain to ANP was caused by an apparent decrease in the pH of the growth media brought about by an elevated ability to acidify growth medium through an increased activity of the plasma membrane H^+-ATPase (12). Elevated plasma membrane H^+-ATPase activity results from an imbalance in pH homeostasis within the cell, manifest as an abnormally acidic vacuolar pH in *btn1*-Δ (12). This disturbance in pH homeostasis becomes normalized through growth, most likely through up-regulation of *HSP30* and *BTN2*, the only two genes to have altered expression through growth in *btn1*-Δ as compared with normal (12). Although deletion of *HSP30* or *BTN2* did not alter vacuolar pH, vacuolar H^+-ATPase (V-ATPase) activity was increased in both *hsp30*-Δ and *btn2*-Δ at late growth phase (14), suggesting a direct relationship between these gene products and V-ATPase activity. In addition, by raising the acidic pH of the vacuole in *btn1*-Δ with chloroquine, elevated activity of the plasma membrane H^+-ATPase, vacuolar pH, and increased expression of *BTN2* and *HSP30* were normalized (15).

More recently, we have shown an involvement of Btn1p in regulating intracellular levels of L-arginine within the cell. Specifically, *btn1*-Δ has a deficiency in the intracellular and in particular vacuolar levels of the basic amino acids arginine and lysine. Furthermore, vacuoles isolated from *btn1*-Δ have a decreased ability to transport L-arginine *in vitro* (16). However, a direct role for Btn1p in L-arginine transport into the vacuole remains unclear.

The V-ATPase along with ion channels and transporters maintains vacuolar pH (17–19). Moreover, the V-ATPase-driven proton transport is coupled with ATP hydrolysis and establishes an electrochemical gradient between the cytosol and vacuolar lumen. This gradient is used for coupled transport of metabolites such as amino acids (20–22), ions such as calcium (23) or sodium (24), and another metabolites (25, 26). The yeast V-ATPase consists of a complex of peripheral subunits containing the ATP binding sites, termed the V_1 sector, attached to a complex of membrane proteins containing the proton pore, termed the V_0 sector. Interaction between the V_1 and V_0 sectors is essential for ATP-driven proton transport, and this interaction is manipulated *in vivo* as a means of regulating V-ATPase activity (18). For example, when yeast are deprived of glucose the V-ATPase complexes are disassembled into cytoplasmic V_1 sectors and membrane-bound V_0 sectors (27).

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[2] The abbreviations used are: NCL, neuronal ceroid-lipofuscinosis; V-ATPase, vacuolar-type H^+-ATPase; MES, 3-(*N*-morpholino)ethanesulfonic acid; ACMA, 9-amino-6-chloro-2-methoxy-acridine; $C_{12}E_9$, nonaethyleneglycolmonodecyl ether (9-lauryl ether); BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; ANP, D-(−)-threo-2-amino-1-[*p*-nitrophenyl]-1,3-propanediol.

We report that V-ATPase activity is modulated by extracellular pH, showing a down-regulation of its activity at low extracellular pH. Our results show that assembly of V_0/V_1 subunits is increased with the pH of the medium. Importantly, we report that *btn1-Δ* down-regulates ATP hydrolysis by V-ATPase, most likely to maintain normal H^+ transport across the vacuolar membrane and to compensate a pH imbalance caused by the lack of Btn1p. We propose that Btn1p has active involvement in regulation of vacuolar pH and that a disruption in the function of this protein results in altered vacuolar luminal content through alterations in transport of metabolites that are usually sequestered in this organelle.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The B11718 wild-type strain (*MATα his3Δ1 lys2Δ0 ura3Δ0*) was purchased from RESGEN. The KANMX system was used to delete *BTN1* using homologous recombination and Cre-recombinase driven excision of the KANr cassette as described by Guldener and colleagues (28). Yeast strains were grown as indicated in YNB medium (6.7 g/liter yeast nitrogen base without amino acids/2% glucose) plus the amino acids supplement. Buffered medium was made as above but by adding 50 mM MES. The pH of the medium was adjusted after autoclaving.

Measurement of Vacuolar pH—Vacuolar pH measurements were performed using methods previously described (29, 30). Briefly, yeast were harvested by centrifugation after the incubation in medium at low and high pH, then 40×10^6 cells were resuspended in 200 μl of the same medium with 50 μM BCECF-AM, and incubated for 30 min at 30 °C. Cells were collected again by centrifugation, washed three times, and resuspended in 1 ml of the same medium. 100 μl of the cell suspension were used by determination of the vacuolar pH by fluorescence measurement.

Fluorescence intensity and absorbance values were acquired using a Spectra Max M5 multimode microplate reader (Molecular Devices). Emission fluorescence at 490 nm excitation wavelength was acquired, and background fluorescence was measured using BCECF-AM-free cultures. The values were normalized to cell density (NI_{490}) to calculate the vacuolar pH. At the end of each experiment, a calibration curve of fluorescence intensity *versus* pH was obtained for each yeast strain tested and growth medium used by incubating yeast cultures in experimental medium containing 50 mM MES, 50 mM HEPES, 50 mM KCl, 50 mM NaCl, 0.2 M ammonium acetate, 10 mM NaN_3, 10 mM 2-deoxyglucose, 50 μM carbonyl cyanide *m*-chlorophenylhydrazone, titrated to five different pH values within the range of 4.0–8.0 using 1 M NaOH. To estimate vacuolar pH, experimental NI_{490} values from a given strain were compared with a calibration curve generated from the same yeast strain, as growth and the amount of dye loaded varied between strains.

Yeast Cell Fractionation and Vacuole Isolation—Yeast vacuoles were obtained by using the method of Ohsumi and Anraku (22) with some modifications. Yeast cells were grown in YNB (yeast nitrogen base) medium as indicated and then washed once with distilled water. Cells were converted to spheroplasts by suspending the cell pellet in 2 ml/g of pellet wet weight of buffer B (20 mM potassium phosphate, pH 7.6, 1.2 M sorbitol) containing zymolyase 20T (2.5 mg/g of pellet wet weight). Cultures were incubated for 60 min at 30 °C with gentle shaking. Spheroplasts were collected by centrifugation (500 × *g* for 5 min) and then washed once with buffer B. The pellet was suspended in 10 ml of buffer C (10 mM Tris-MES, pH 6.9, 0.1 mM $MgCl_2$, 12% Ficoll 400) and homogenized by ten strokes in a Dounce homogenizer. The lysate was cleared by centrifugation at 100,000 × *g* for 1 h. The top layer was collected in a Dounce homogenizer containing 8 ml of buffer C, and clumps were broken up by six or seven strokes. The homogenate was transferred to an ultracentrifuge tube and layered with buffer D (10 mM Tris-MES, pH 6.9, 0.1 mM $MgCl_2$, 8% Ficoll 400). The mixture was centrifuged at 100,000 × *g* for 1 h. The top layer was collected and dispersed in a tube containing 2× buffer E (1× buffer E is 10 mM Tris-MES, pH 6.9, 5 mM $MgCl_2$, 25 mM KCl). Vacuoles were converted to vesicles by adding 2 volumes of 1× buffer E, and a pellet was obtained by centrifugation at 34,400 × *g* for 1 h. Purity of the vacuoles was verified by SDS-PAGE and Western blot analysis with vacuolar markers. The antibodies used were: Anti-Vma2p (vacuolar membrane), anti-Vph1p (vacuolar membrane), anti-Alkaline phosphatase (vacuolar membrane), anti-Porin (mitochondrial outer membrane), anti-Dol-P-Man synthase (endoplasmic reticulum membrane), and anti-Vps10p (Golgi membrane). All the antibodies were obtained from Molecular Probes and used following the manufacturer's instructions.

Measurement of the Vacuolar ATPase Complex Activities—Vacuolar H^+-ATPase activity (10 μg of protein of isolated vacuolar membranes in 250 μl of assay mixture) essentially as described previously (31). The activity was assessed in the presence of sodium azide (5 mM, to inhibit mitochondrial ATPase), ammonium molybdate (0.2 mM, to inhibit acid phosphatases), and sodium orthovanadate (100 μM, to inhibit plasma membrane H^+-ATPase). In addition, the activity measured in the presence of 1 μM concanamycin A (a specific inhibitor of the vacuolar H^+-ATPase) was subtracted from the activity measured without the inhibitor to calculate the V-ATPase activity. Under the assay conditions used, the ATPase activity determined could predominantly be attributed to V-ATPase.

The proton transport activity into the lumen of isolated vacuoles was measured by monitoring the formation of pH gradient across the vacuolar membrane by fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) in the presence or absence of 1 μM concanamycin A essentially as described by Camarasa *et al.* (32). Thus, 25 μg of vacuolar membrane vesicles was added to a cuvette containing 2 ml of reaction buffer (10 mM BisTris Propene (BTP)-MES, pH 7.0, 25 mM KCl, 2 mM $MgSO_4$, 10% (v/v) glycerol, and 2 μM ACMA). The reaction was started by the addition of 1 mM BTP-ATP (pH 7.5), and fluorescence emission signal was recorded at 480 nm after excitation at 412 nm.

In Vivo Interaction between V_0 and V_1 Subunits of the Vacuolar ATPase Assays—Yeast cells were grown in supplemented minimal medium and harvested by centrifugation (1000 × *g* for 5 min). Spheroplasts were generated by treatment with zymolyase 20T for 30 min in YNB medium and 1.2 M sorbitol in the presence or absence of 2% glucose. Then, spheroplasts were pelleted and lysed in phosphate-buffered saline containing 1% $C_{12}E_9$, protease inhibitors (2 μg/ml aprotinin, 0.7 μg/ml pepstatin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and 0.67 mM dithiobis(succinimidyl propionate). The V_1 domain of the vacuolar ATPase was immunoprecipitated using the monoclonal antibody 13D11 against Vma2p and protein A-Agarose beads. Samples were then separated by electrophoresis on 10% acrylamide gel and transferred to a nitrocellulose membrane by standard techniques. Western blotting was performed using the monoclonal antibodies 8B1 (Vma2p) and 13D11 (Vph1p) from Molecular Probes, followed by a horseradish peroxidase-conjugated secondary antibody. The obtained blots were analyzed by densitometry using the software GelPro analyzer version 3.1.

RESULTS

btn1-Δ Results in an Alteration of the Vacuolar pH through Growth—We have previously reported that *btn1-Δ* results in an alteration of the vacuolar pH at early growth phase compared with wild-type yeast (12).

A

Time (h)	pH 4		pH 7.5	
	wt	*btn1-Δ*	*wt*	*btn1-Δ*
6	5.57± 0.22	4.65± 0.3	6.48± 0.09	6.52± 0.05
12	4.99± 0.006	5.02± 0.08	5.76± 0.04	6.19± 0.13
24	5.03± 0.025	4.88± 0.123	5.87± 0.01	6.02± 0.02

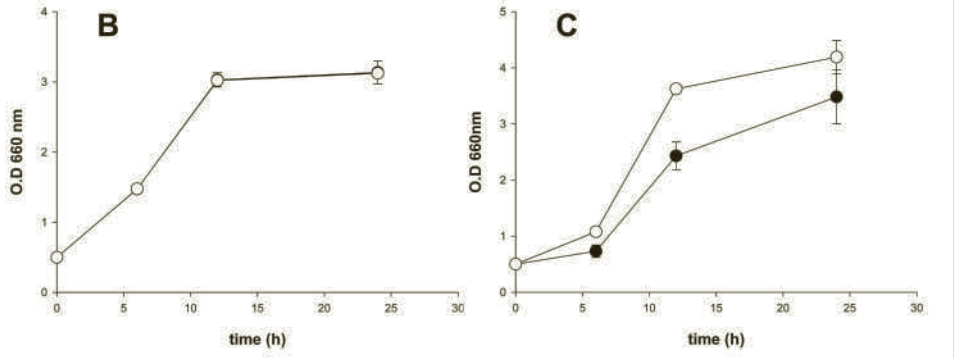

FIGURE 1. **Vacuolar pH and growth curves in wild type and *btn1*-Δ grown in YNB medium at pH 4 and pH 7.5.** Yeast cells were inoculated at initial A_{660} of 0.5. Samples were harvested at 6, 12, and 24 h of incubation in the medium, and vacuolar pH was measured as described under "Experimental Procedures." At the same time cell growth was determined by measurement of A_{660}. *A*, measured vacuolar pH at the indicated time points. *B*, growth curve of wt and *btn1*-Δ cells in YNB medium at pH 4. *C*, growth curve of wt and *btn1*-Δ cells in YNB medium at pH 7.5. *Filled symbols* represent the measured growth in wild type strain. *Open symbols* represent the results obtained with *btn1*-Δ cells. The plots represent the mean of the results obtained from three identical experiments.

These studies demonstrated that *btn1*-Δ had a decreased vacuolar pH at 6 h of growth in YPD (1% yeast extract, 2% peptone, 2% glucose) and that, as *btn1*-Δ grew, after 6 h the vacuolar pH became normalized. In addition, it is well known that yeast lacking subunits of the V-ATPase complex have impaired growth in culture medium buffered at pH 7.5 or higher (33). Therefore, extracellular pH could influence maintenance of an acidic vacuolar pH. To confirm this hypothesis, wild type and *btn1*-Δ were grown in YNB medium at starting pH values of 4 and 7.5, respectively, and vacuolar pH was measured at different time points following the method described by Ali *et a*l (29). The results, summarized in Fig. 1, show that vacuoles of *btn1*-Δ had decreased pH (4.65) at 6 h compared with wild type (5.57) grown in YNB medium with an initial pH of 4. However, no differences were observed at the same time point in cells grown in YNB medium at pH 7.5. Wild type and *btn1*-Δ grown at pH 4 show similar vacuolar pH at 12 h remaining the same at 24 h of growth. However, *btn1*-Δ shows a slightly higher vacuolar pH than wild type after 12 h of growth in medium at pH 7, reaching values of 6–6.2 compared with 5.75–5.9 for wild-type vacuoles. Therefore, a lack of Btn1p appears to result in an altered ability to acidify the vacuolar lumen. The vacuolar pH imbalance apparent for *btn1*-Δ vacuoles led us to postulate that Btn1p could have a function in the modulation of V-ATPase activity. We therefore investigated whether there is Btn1p involvement in modulating V-ATPase activity.

btn1-Δ *Results in Decreased V-ATPase Activity Resulting in Altered* H^+*-transport Coupling at Early Growth Phase at High pH*—To further understand the role of extracellular pH regulation of vacuolar acidification, vacuole fractions were isolated from *btn1*-Δ cultured in YNB medium at a starting pH of 7.5. Purity of the vacuolar fractions was tested using immunological markers (see under "Experimental Procedures"). Concanamycin A-sensitive V-ATPase activity and H^+-transport activity were measured at 6, 12, and 24 h of growth. The pH of the medium was monitored at the same time points. Supporting previous reports, *btn1*-Δ showed an increased ability to acidify the medium (12) (Fig. 2*A*). Thus, the rate of acidification of medium of *btn1*-Δ cultures until 12 h was 0.18 pH units/h compared with wild-type cultures (0.1 pH unit/h). Moreover, vacuoles isolated from both wild type and *btn1*-Δ showed an increase in the activity of ATP hydrolysis by V-ATPase with time. However, V-ATPase activity at 6 h was significantly lower for *btn1*-Δ, as compared with wild type, being only 20% of that for wild type at 6 h (Fig. 2*B*). No significant difference in the hydrolysis of ATP by the vacuolar ATPase was detected at 12 and 24 h between *btn1*-Δ and wild type.

Proton transport into isolated vacuoles was measured by ACMA fluorescence quenching, Fig. 2*C*. The activity measured in wild type was relatively constant through growth. Moreover, no significant difference in H^+-transport activity between wild type and *btn1*-Δ vacuoles was observed throughout growth in YNB medium at pH 7.5.

Coupling between ATP hydrolysis and H^+-transport can be expressed as the ratio between the H^+ transport activity and V-ATPase activity. We plotted V-ATPase/H^+-transport coupling through growth (Fig. 2*D*), which indicates that the V-ATPase/H^+-transport coupling ratio for *btn1*-Δ is increased by 5 times compared with wild type at 6 h of growth. As *btn1*-Δ cells grow beyond 6 h, the coupling of V-ATPase/H^+-transport in *btn1*-Δ falls to similar values to that of wild type. It is important to note that the coupling in wild type only showed slight variations throughout growth in YNB medium. These results suggest that, during the early phase of growth, *btn1*-Δ can regulate V-ATPase activity to keep a normal transport of protons across the vacuolar membrane and normalize the pH of the vacuole at this time.

btn1-Δ *Results in Decreased V-ATPase Activity and Increased Vacuolar* H^+*-transport throughout Growth at Low pH*—To further understand the role of extracellular pH on regulating vacuolar proton pumping, we repeated the previous experiments in strains cultured in YNB with a starting pH of 4. Both the wild type and *btn1*-Δ show lower V-ATPase and vacuolar H^+-transport activities throughout growth as compared with cells grown at pH 7.5. Lower extracellular pH therefore appears to down regulate activity of the V-ATPase and vacuolar

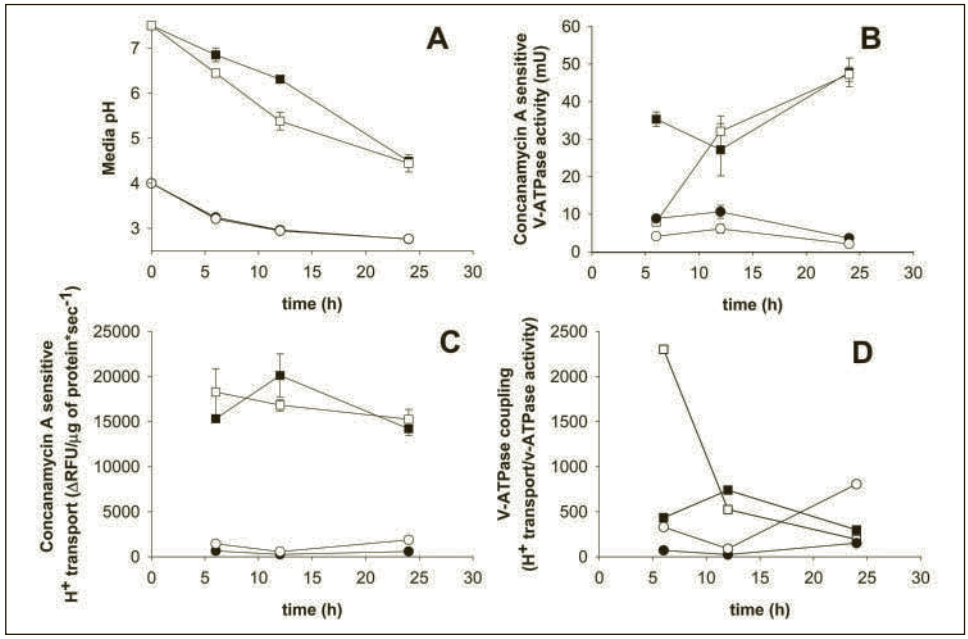

FIGURE 2. **Vacuolar H^+-ATPase activities in wild type and *btn1*-Δ grown at low and high pH.** Yeast cells were inoculated at initial A_{660} 0.5. Samples were harvested at 6, 12, and 24 h of incubation in the medium, then vacuoles were isolated as indicated under "Experimental Procedures." *A*, pH of the medium at the indicated times. *B*, ATP hydrolysis activity by vacuolar ATPase complex measured as described under "Experimental Procedures." *C*, H^+ translocation activity measured by fluorescence quenching of ACMA. *D*, vacuolar ATPase coupling calculated by the ratio between the mean of the H^+ translocation activity and the mean of the ATP hydrolysis activity. ■, wild-type strain, pH 7.5; □, *btn1*-Δ strain, pH 7.5; ●, wild-type strain, pH 4; ○, *btn1*-Δ strain, pH 4. The plots represent the mean and standard errors of the results obtained from three identical experiments.

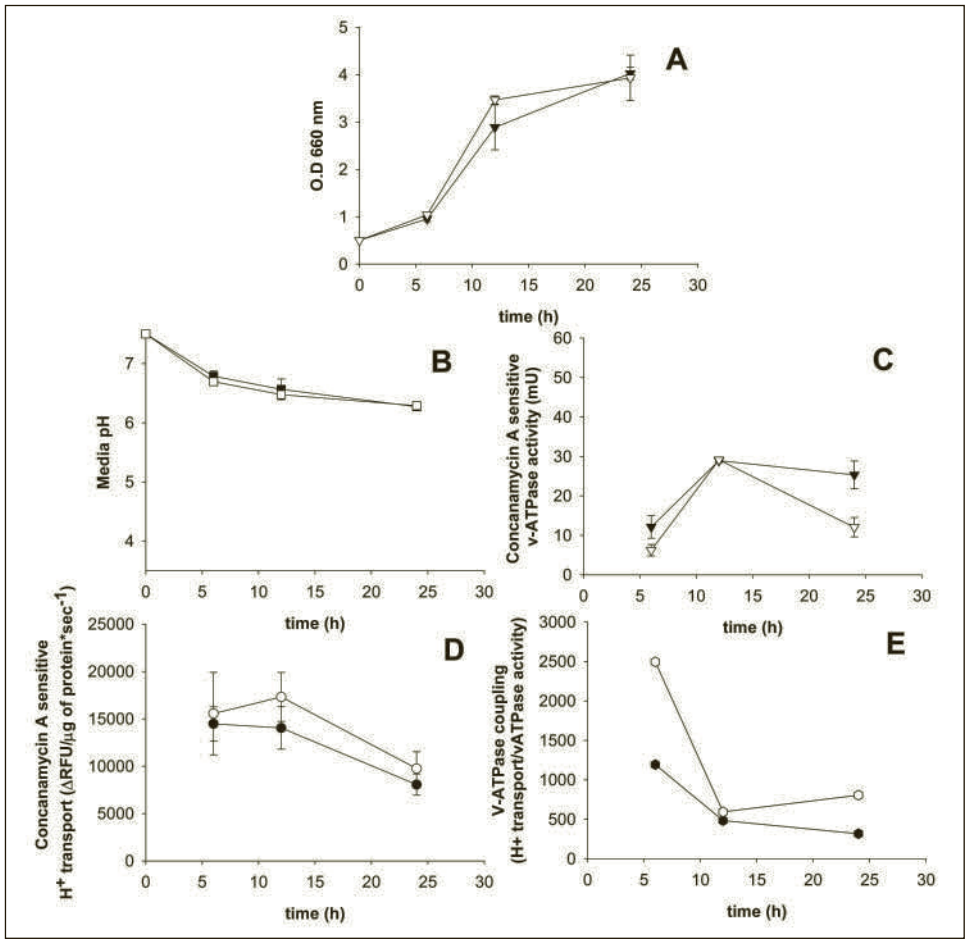

FIGURE 3. **Vacuolar H^+-ATPase activities in wild type and *btn1*-Δ grown in pH 7. 5 buffered YNB medium.** Yeast cells were inoculated at initial A_{660} of 0.5. Samples were harvested at 6, 12, and 24 h of incubation in the medium, then vacuoles were isolated as indicated under "Experimental Procedures." *A*, cell growth measured by optical density at 660 nm. *B*, pH of the medium at indicated times. *C*, ATP hydrolysis activity by vacuolar ATPase complex measured as described under "Experimental Procedures." *D*, H^+ translocation activity measured by fluorescence quenching of ACMA. *E*, vacuolar ATPase coupling calculated by the ratio between the mean of the H^+ translocation activity and the mean of the ATP hydrolysis activity. *Filled symbols* represent the activities measured in wild-type strain. *Open symbols* represent the results obtained with *btn1*-Δ cells. The plots represent the mean of the results obtained from three identical experiments.

H^+-transport (Fig. 2). This supports the notion that there is an extracellular pH-dependent regulation of V-ATPase activity, vacuolar H^+-transport, and likely vacuolar acidification. Interestingly, concanamycin A-sensitive V-ATPase activity was decreased throughout growth for *btn1*-Δ as compared with wild type, although, at the later time point of 24 h, activities were very similar. Concomitantly, *btn1*-Δ has a higher H^+-transport activity than wild type throughout growth. Thus, the V-ATPase/H^+ transport coupling stayed higher in *btn1*-Δ compared with wild type throughout growth and in particular at the later time point of 24 h. It could therefore be postulated that a lack of Btn1p partially impairs the ability of the cell to down regulate the transport of protons to the vacuolar lumen at low pH.

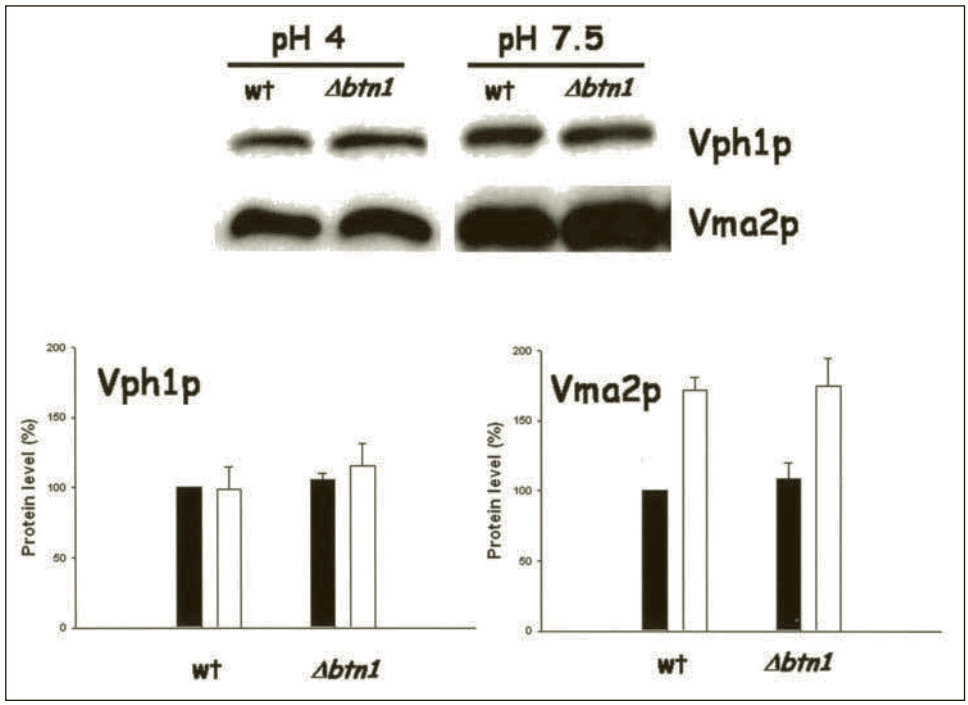

FIGURE 4. **Vph1p and Vma2p levels in vacuoles of wild type and *btn1*-Δ cells.** Yeast cells were inoculated at initial A_{660} 0.5. Samples were harvested at 6 h of incubation in the medium, then vacuoles were isolated as indicated at "Experimental Procedures," and 10-μg samples were analyzed by SDS-PAGE and Western blot using monoclonal antibodies 10D7 and 13D11 that recognize 100-kDa Vph1p (V_0) and 60-kDa Vma2p (V_1), respectively. The amount of protein was calculated by densitometry analysis using Gel-Pro analyzer version 3.1 software. The plots represent the mean and standard errors of the results obtained from three identical experiments.

Buffering Growth Media to a High pH Alters the Ability of Cells to Modulate V-ATPase Vacuolar H^+-transport Activities—We are studying how extracellular pH affects the maintenance of intracellular pH in both wild type and *btn1*-Δ. Our results demonstrate that *btn1*-Δ clearly alters extracellular pH at a different rate than wild type (Fig. 2*A*) most likely as a means to balance intracellular pH homeostasis (12). The decrease in V-ATPase activity we report for *btn1*-Δ occurs simultaneously with an increased rate of media acidification. Thus, it could be proposed that the down-regulation of V-ATPase during the early growth phase is released when the pH of the vacuolar lumen becomes "normal" or "optimal," such that the vacuolar compartment is at its steady state. Importantly, this "normalization" of vacuolar pH in *btn1*-Δ would coincide with a normalization of media acidification by the plasma membrane H^+-ATPase such that *btn1*-Δ would behave as if it is wild type in regards to overall pH homeostasis.

We therefore repeated our previous experiments in media with a starting pH of 7.5 with buffered medium to isolate the influence of media acidification in the regulation of V-ATPase complex activity. The measured proton transport into the vacuolar lumen for both wild type and *btn1*-Δ was similar to what we previously observed at a starting pH of 7.5 in non-buffered media (Fig. 3). Interestingly, wild type had a 50% lower V-ATPase activity after 6 and 24 h of growth compared with that in non-buffered medium (Fig. 3*C*). For wild type, this suggested that a constant high media pH results in a down-regulation of the regulatory mechanisms for maintaining a high or normal V-ATPase activity. For *btn1*-Δ at 24 h, V-ATPase activity was only 20% of the activity measured in non-buffered medium (Fig. 3*C*). This decrease also manifested as a 4-fold increase in V-ATPase/H^+-proton coupling for *btn1*-Δ vacuoles (Fig. 3*E*).

High Extracellular pH Leads to Increased Levels of V-ATPase Subunit Vma2p in Vacuolar Membranes—To test if the change in V-ATPase activity at different pH, and between wild type and *btn1*-Δ, was due to alterations in the protein levels of V-ATPase, the levels of two subunits of the V-ATPase, Vph1p (100-kDa polypeptide of the membrane V_0 subunit) and Vma2p (60-kDa polypeptide from the cytosolic V_1 subunit of the complex) were measured by Western blotting (Fig. 4). The amount of Vma2p in isolated vacuoles was 2-fold higher in strains grown for 6 h in pH 7.5 medium as compared with pH 4. However, no concomitant increase in the levels of Vph1p peptide was observed. Interestingly, our initial measurement of V-ATPase activity showed a 3-fold increase in wild type grown at pH 7.5 compared with those grown at pH 4. However, the H^+-transport activity measured was more than 20-fold higher at pH 7.5. No differences in the levels of Vph1p and Vma2p were observed between wild type and *btn1*-Δ under any of the conditions used, including cells grown in pH 7.5 buffered medium (not shown) and cells grown in YNB medium for 12 and 24 h (not shown). Therefore, these results suggest that it is unlikely that differences in V-ATPase coupling between wild type and *btn1*-Δ are due to altered subunit levels of the V-ATPase.

Assembly of v-ATPase Complex Is Regulated by Extracellular pH—It is been previously described that V_1 and V_0 subunits of the vacuolar ATPase complex assemble to form one active V-ATPase complex or disassemble to become inactive under different conditions (18). For example, the V_1 subunit dissociates from V_0 when cells are incubated in medium lacking glucose (27). Moreover, the level of assembly of both subunits may change with the carbon source of growth medium (27) or V-ATPase complexes lacking one of the peptides that form the enzymatic complex (34). This assembly mechanism has been described as the main process that regulates V-ATPase activity (27).

The observation that only Vma2p (which is a part of the cytosolic V-ATPase subunit V_1) but not Vph1p (membrane subunit V_0) had increased levels in vacuolar membranes isolated from cells grown in high pH medium does suggest a possible increase in the assembly between V-ATPase V_0 and V_1 under these conditions. Furthermore, there was no difference in the total levels of Vma2p in whole cell extracts at these or any other conditions tested (not shown). Altered assembly could contribute to the imbalance between V-ATPase ATP hydrolysis and H^+-transport activity observed in *btn1*-Δ. To test this, assembly of V_1 and V_0 subunits was assessed by co-immunoprecipitation of V-ATPase subunits, Vma2p (V_1) and Vph1p (V_0), for wild type and *btn1*-Δ grown for 6 h at low and high pH.

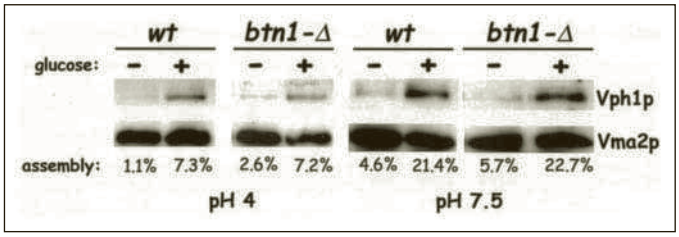

FIGURE 5. **Association between V_0 and V_1 subunits of the vacuolar ATPase in cells grown at low and high pH.** Yeast cells were grown in YNB medium at pH 4 and 7.5 and harvested by centrifugation at 6 h of incubation. Spheroplasts were generated by treatment with zymolyase 20T. After that, spheroplasts were incubated in YNB medium at pH 4 or 7.5 for 30 min in the presence or absence of 2% glucose and lysed in phosphate-buffered saline containing 1% $C_{12}E_9$ and protease inhibitors. The V_1 domain of the vacuolar ATPase was immunoprecipitated using the monoclonal antibody 13D11 against Vma2p and protein A-agarose beads. Samples were then separated by electrophoresis on 10% acrylamide gel and transferred to a nitrocellulose membrane by standard techniques. Western blotting was performed using the monoclonal antibodies 8B1 (*Vma2p*) and 13D11 (*Vph1p*) from Molecular Probes. The assembly grade was obtained by densitometry analysis using Gel-Pro analyzer version 3.1 software and calculating the relative intensity of Vph1p with respect Vma2p band (100%) in each lane.

The results of these assembly experiments show a 3-fold increase in the assembly of V_0 and V_1 when cells where incubated in high pH medium for both wild type and *btn1*-Δ (Fig. 5). However, there was no significant difference between wild type and *btn1*-Δ in assembly state of the V-ATPase in any of the conditions tested. Therefore, higher V-ATPase coupling in *btn1*-Δ compared with wild type could not be explained by an increase of the association between V_0 and V_1 subunits.

DISCUSSION

BTN1 Deficiency Results in Vacuolar pH Imbalance—The activity of the plasma membrane H^+-ATPase allows yeast cells to acidify the medium as they grow. The ability to pump protons out of the cell in combination with vacuolar-ATPase activity that provides the ability to acidify the vacuole is likely used by the cell to maintain a physiological pH in the cytosol. Thus, yeast cells show an incredible plasticity during a stress that challenges pH homeostasis, making it challenging to study any kind of disruption in cellular pH homeostasis. In this context, it has been described previously that *btn1*-Δ have an increased activity of the plasma membrane H^+-ATPase compared with wild type as a consequence of an imbalance in vacuolar pH at early growth phases (12). In the current study, we have analyzed how vacuolar pH changes during growth of *btn1*-Δ cultured in YNB medium with a starting low, 4, and high, 7.5, pH. Supporting previous results, we observed a decreased vacuolar pH in *btn1*-Δ at 6 h of growth in low pH medium, which became normalized after 12 h of growth. No difference in vacuolar pH was observed at 6 h in high pH medium between *btn1*-Δ and wild type. These results suggest that *btn1*-Δ results in reduced ability to maintain vacuolar pH homeostasis and that these cells could compensate the pH imbalance in a different manner, depending on extracellular pH.

Down-regulation of V-ATPase Activity Maintains a Normal H^+-transport into btn1-Δ Vacuoles—We have studied the implications of altered V-ATPase activity in the context of altered vacuolar pH in *btn1*-Δ and report a 5-fold decrease of V-ATPase activity in *btn1*-Δ during early growth in high pH medium and a 2–3-fold decrease throughout growth for *btn1*-Δ cultured in low pH medium. Decreased V-ATPase activity could underlie a compensatory mechanism of maintaining normal vacuolar pH in cells lacking Btn1p function. This hypothesis is supported by the fact that there is similar vacuolar lumen acidification, in terms of H^+ transport activity and vacuolar pH at early growth phase in high pH medium for both *btn1*-Δ and wild type. The V-ATPase activity measured at later growth phases in high pH medium did not show any difference between wild type and *btn1*-Δ, which suggests that down-regulation of the vacuolar acidification through the ATPase complex is a regulatory mechanism no longer required at this stage. Therefore, lower vacuolar acidification observed in *btn1*-Δ following 12 h of growth in pH 7.5 medium could well mean alternative mechanisms exist to balance vacuolar pH in addition to altering V-ATPase activity. In this context, previous studies have described how increased expression of *HSP30* and *BTN2* in *btn1*-Δ contributes to the normalization of the plasma membrane H^+-ATPase activity at late growth phase in YPD (12). In addition, deletion of either *HSP30* or *BTN2* leads to an increase of V-ATPase activity at late growth phase (14). Thus, it is possible that the pH compensation observed in *btn1*-Δ vacuoles results from this same altered pattern of gene expression. However, there is no difference in vacuolar ATP hydrolysis at later growth phases between wild type and *btn1*-Δ. Therefore, it seems more possible that other factors, acting independently of the V-ATPase complex, result in the decreased acidification of the vacuole in *btn1*-Δ.

It has been described that transport proteins in the vacuolar membrane such as sodium exchanger Nhx1p (24, 35) or chloride channels (36) are involved in pH regulation of the vacuolar lumen (29, 37–39). We have previously shown that vacuoles isolated from *btn1*-Δ cultured to logarithmic phase in YPD have a decreased ability of transport the amino acid, L-arginine (16). Therefore, altered activity of arginine transport could contribute to pH normalization and the later over-compensation and elevated vacuolar pH observed in *btn1*-Δ at late growth phase. Again supporting this notion is the up-regulation of Btn2p in *btn1*-Δ (12). Btn2p is involved in the regulation of arginine and Na^+ uptake by the cell through the correct localization of Rhb1p, a down regulator of the activity of the plasma membrane arginine transporter Can1p, and Ist2p, a putative plasma membrane Na^+ channel (40–42). Thus, increased expression of Btn2p during late growth phase in *btn1*-Δ may represent a compensatory mechanism for balancing the disturbance in arginine and ion content entering the cell caused by the lack of Btn1p in the vacuolar membrane.

It is interesting that *btn1*-Δ has greater vacuolar pH imbalance during early growth in low pH medium. Under these conditions, the V-ATPase activity and vacuolar acidification measured in a wild type was 5 and 25 times lower, respectively, than those measured at pH 7.5. This inherent lower activity of the V-ATPase complex at low pH would make it more difficult to regulate the vacuolar pH in *btn1*-Δ. Previously, it has been described that yeast lacking subunits of the V-ATPase are able to acidify the vacuole in a V-ATPase-independent manner when they are grown in a low pH medium (30). Therefore, in low pH medium, there is a vacuolar acidification process that likely is not controlled through the regulation of the V-ATPase. This supports the idea that low extracellular pH would challenge the potential regulation of the proton content in vacuoles of *btn1*-Δ and could lead to the lower vacuolar pH we report. Our results show that down-regulation of the V-ATPase activity remains longer in *btn1*-Δ cultured at low pH compared with those cultured at high pH medium. This suggests that an additional alternate mechanism is needed to reach the observed normal pH in vacuoles for *btn1*-Δ at later growth phases in low pH medium.

We have shown that *btn1*-Δ results in a low V-ATPase activity at 24 h in pH 7.5 buffered medium. This suggests that maintaining high vacuolar pH through growth leads to a continuing down-regulation of the V-ATPase activity. One possible explanation for this phenomenon is that a high vacuolar pH would lead to a down-regulation of all the systems that could increase the pH of this organelle. This putative down-regulation would result in an increase of the proton concentration in the lumen of the vacuole, which could be controlled partially by Btn1p function. Therefore, cells lacking Btn1p would maintain down-

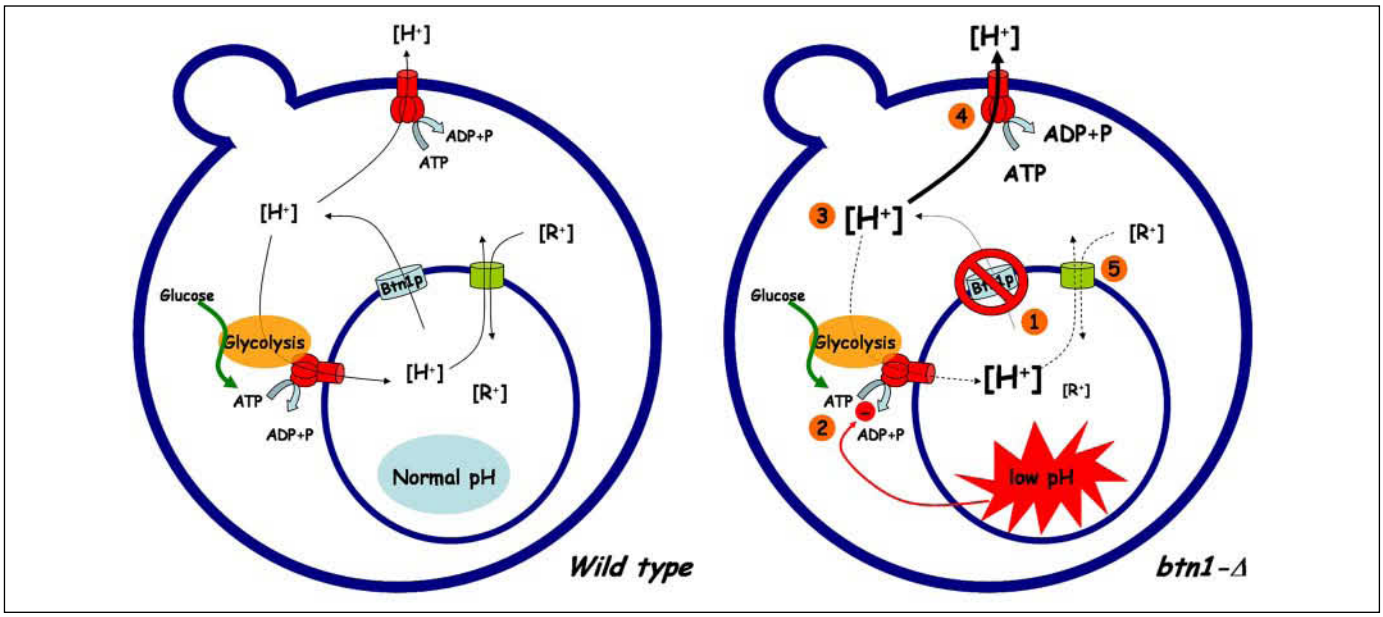

FIGURE 6. **Proposed Btn1p function in the vacuole of *S. cerevisiae*.** In a wild-type strain, Btn1p could control H^+ efflux from the vacuole during logarithmic phase of growth, when the glycolytic pathway is the main source of ATP, to maintain an optimal vacuolar pH for the different vacuolar process. Lack of Btn1p would lead potentially to an excess of protons into the vacuole and the decrease of the pH into this organelle if the cell is growing in low pH medium (*1*). The decrease of the vacuolar pH undergoes the regulation of the H^+ transport into the vacuole through the inhibition of the V-ATPase activity (*2*). As a consequence, the proton concentration in the cytosol would increase (*3*), but this is controlled by an increase of the proton pumping through the plasma membrane H^+-ATPase (*4*). Finally, the cell could regulate the transport of others metabolites (such as arginine) into the vacuole to maintain a normal proton content in the cytosol (*5*).

regulation of the ATP hydrolysis activity by the V-ATPase as the only way to maintain normal vacuolar pH.

Vacuolar Acidification through V-ATPase Activity Is Regulated by Extracellular pH Medium—We have shown that the ability to acidify the vacuole is down-regulated during growth in low pH medium. In addition, it has been shown that there is a decrease of the assembly of the V_0/V_1 subunits of the V-ATPase when cells were grown in low pH medium. However, decreased levels of the V-ATPase complex in the vacuole or a decrease in the assembly of the V_0/V_1 subunits cannot account for the 20-fold lower H^+ transport measured at low pH. Therefore, other unidentified regulatory mechanisms are likely to be involved in regulating the activity of the V-ATPase complex and maintaining the pH of the vacuole in a low pH environment.

Are We Closer to Knowing What Btn1p Does?—We do not show direct evidence that Btn1p is involved in the regulation of V-ATPase activity. However, the pH modulation by down-regulation of V-ATPase activity in *btn1*-Δ is not as tight as it is when Btn1p is present in the vacuolar membrane. We propose that Btn1p may have a role in balancing or regulating vacuolar pH particularly during the early phases of growth, when high energetic content leads to high transport of protons into the vacuolar lumen through V-ATPase activity (Fig. 6). During the logarithmic phase of growth, high glucose levels of the medium lead to the synthesis of high amounts of ATP by the glycolytic pathway. Several studies have demonstrated a direct interaction between glycolytic enzymes, such as aldolase (43–45) or phosphofructokinase-1 (46), and several subunits of the V-ATPase. Moreover, it has been postulated that the V-ATPase and glycolytic enzymes form a complex in the vacuolar membrane to maximize the efficiency of energy provision to acidify the vacuole. These studies imply that the energy source for V-ATPase proton pumping is glycolysis. It could therefore be hypothesized that *in vivo* proton pumping into the vacuole would occur primarily during logarithmic growth, when there is accessible ATP from glycolysis. Under these conditions Btn1p may be involved in the uncoupling of ATP hydrolysis and H^+ transport by the V-ATPase complex allowing H^+ leakage from the vacuolar lumen to the cytosol. This could potentially regulate an excess of protons in the vacuole. During later growth phases as oxidative phosphorylation takes over as the primary source of cellular ATP, less ATP is available to the V-ATPase, resulting in a decrease of the *in vivo* V-ATPase activity, and in *btn1*-Δ releasing down-regulation V-ATPase activity. Therefore, Btn1p could be a protein involved in H^+-transport from the vacuole to the cytosol when the H^+ concentration in the vacuolar lumen reaches high values in much the same way as uncoupling proteins do in mitochondria of mammalian cells (47–50). These uncoupling proteins transport protons across the mitochondrial inner membrane and divert energy from ATP synthesis to thermogenesis in the mitochondria of brown adipose tissue (51, 52) and are involved in decreasing the concentration of reactive oxygen species inside the mitochondria (53, 54). Although the role of Btn1p in the vacuole would be somewhat different to these uncoupling proteins in mitochondria, it is possible that these proteins could share a similar mechanism to transport protons across the membrane. However, further experimentation is required to confirm this hypothesis, because sequence analysis of Btn1p/CLN3 and mitochondrial uncoupling proteins does not reveal similarities.

Vacuolar membrane transport proteins are usually H^+-antiporters and allow vacuolar proton efflux in exchange for transport of different metabolites into the vacuole (22, 55). Therefore, an alternative role for Btn1p could be that as an exchange-protein transporting a specific metabolite into the vacuole. Although vacuoles isolated from *btn1*-Δ show decreased arginine uptake (16) suggesting that Btn1p could be a transporter of this amino acid, others studies have described that there are several proteins involved in the transport of amino acids into the vacuolar lumen (21). It is unlikely that the lack of one amino acid transporter would precipitate the vacuolar pH imbalance observed in *btn1*-Δ. Furthermore, we have measured the vacuolar pH of cells lacking *VBA2*, which has been described as a putative vacuolar arginine antiporter, and observed no difference with wild type (not shown). Therefore, it is more likely that a defect in arginine transport in *btn1*-Δ is in fact a secondary

consequence of a lack of the primary function of Btn1p. In conclusion, we propose that Btn1p has a direct role in maintaining optimal pH, and proton and ionic content of the vacuolar lumen. Regulation of vacuolar content is essential for optimal functional conditions for the lumen of the vacuole.

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