Phenotypic reversal of the *btn1* defects in yeast by chloroquine: A yeast model for Batten disease

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ABSTRACT *BTN1* of *Saccharomyces cerevisiae* encodes an ortholog of *CLN3*, the human Batten disease gene. We have reported previously that deletion of *BTN1*, *btn1-Δ*, resulted in a pH-dependent resistance to D-(–)-threo-2-amino-1-[p-nitrophenyl]-1,3-propanediol (ANP). This phenotype was caused by *btn1-Δ* strains having an elevated ability to acidify growth medium through an elevated activity of the plasma membrane H⁺-ATPase, resulting from a decreased vacuolar pH during early growth. We have determined that growing *btn1-Δ* strains in the presence of chloroquine reverses the resistance to ANP, decreases the rate of medium acidification, decreases the activity of plasma membrane H⁺-ATPase, and elevates vacuolar pH. However, an additional effect of this phenotypic reversal is that activity of plasma membrane H⁺-ATPase is decreased further and vacuolar pH is increased further as *btn1-Δ* strains continue to grow. This phenotypic reversal of *btn1-Δ* can be considered for developing a therapy for Batten disease.

Neuronal ceroid-lipofuscinoses (NCL) are the most common group of progressive neurodegenerative diseases in children, with an incidence as high as 1 in 12,500 live births and with about 440,000 carriers in the United States (1, 2). These disorders are autosomal recessive, with similar early symptoms and disease progression. Diagnosis is often based on visual problems, behavioral changes, and seizures. Progression is characterized by a decline in mental abilities, increased severity of untreatable seizures, blindness, loss of motor skills, and premature death. Traditionally, several NCL disorders have been divided into subtypes based on the age of onset and pathology and are denoted by the following *CLN* genes responsible for each disease: infantile-NCL, *CLN1* (Santavuori-Haltia disease); late infantile-NCL, *CLN2* (Jansky-Bielschowsky disease); juvenile-NCL, *CLN3* (Batten disease); adult-NCL, *CLN4* (Kufs' disease); and two variant late infantile forms, *CLN5* and *CLN6*. The gene products of *CLN1* and *CLN2* have been identified as a lysosomal protein thiolesterase and a lysosomal pepstatin-insensitive protease, respectively (3, 4). Recently, *CLN5* was identified as a protein of unknown function (5). Although the *CLN3* gene responsible for Batten disease was positionally cloned in 1995 (6), with most individuals with the disease harboring a 1.02-kilobase deletion of the gene, the function of this protein and the molecular basis for this disease still remain elusive. The NCL are characterized by the accumulation of autofluorescent hydrophobic material in the lysosomes of neurons and, to a lesser extent, other cell types (7, 8). Furthermore, protein sequencing and immunological studies have revealed that subunit *c* of mitochondrial ATP synthase is the major component of the lysosomal storage material in *CLN2*, *CLN3*, and *CLN4* but not *CLN1* (9, 10). This accumulation of mitochondrial ATP synthase subunit *c* is not a result of increased expression of the P1 and P2 nuclear genes

that encode the protein, nor does the stored protein have a different encoded sequence from that for normal individuals (11, 12). Furthermore, slower degradation of mitochondrial ATP synthase subunit *c* was found to occur in NCL fibroblasts compared with normal cells. Although initially located in the mitochondria, mitochondrial ATP synthase subunit *c* accumulated in lysosomes of NCL cells, whereas the degradation of another mitochondrial inner membrane protein, cytochrome oxidase subunit IV, was unaffected, with no lysosomal accumulation (13, 14).

Genes encoding predicted proteins with high sequence similarity to Cln3p have been identified in mouse, dog, rabbit, *Caenorhabditis elegans*, and the yeast *Saccharomyces cerevisiae* (refs. 15 and 16; see also Swiss-Prot accession no. O 29611 and GenBank accession nos. U 92812 and Z 49335). We previously reported that the corresponding yeast gene, *BTN1*, encodes a nonessential protein that is 39% identical and 59% similar to human Cln3p (17). Deletion of *BTN1* had no effect on the degradation of mitochondrial ATP synthase subunit *c*. We further showed that yeast strains lacking Btn1p, *btn1-Δ*, were resistant to D-(–)-threo-2-amino-1-[p-nitrophenyl]-1,3-propanediol (ANP) and that this phenotype was complemented by expression of human Cln3p, indicating that yeast Btn1p and human Cln3p share the same function (18) and are therefore orthologs. This resistance to ANP depended on the ability of *btn1-Δ* yeast strains to decrease the pH of growth medium because of an enhanced ability to acidify growth medium through an initial increase in the activity of plasma membrane H⁺-ATPase (19, 20). This elevated activity of the plasma membrane H⁺-ATPase is most likely a response to an imbalance in pH homeostasis within the cell, resulting from an abnormally acidic vacuolar pH in *btn1-Δ* strains (20). As *btn1-Δ* strains grow, activity of the plasma membrane H⁺-ATPase and vacuolar pH are returned to normal. Examination of the expression of all yeast genes in *btn1-Δ* strains revealed that expression of *HSP30* and *BTN2* was increased. We speculated that altered gene expression is involved in normalizing the activity of plasma membrane H⁺-ATPase and vacuolar pH (20). Therefore, through coordinate gene expression, pH homeostasis in *btn1-Δ* strains is maintained.

Chloroquine, a lysosomotropic agent, is widely used as an antimalarial agent because of its toxicity to *Plasmodium falciparum* trophozoites (21). Chloroquine accumulates in the acidic food vacuole causing an increase in pH, which is believed to inhibit the mobilization of food reserves during this stage of the parasite's development (22–28). It is the ability of chloroquine to raise the pH of the acidic vacuolar compartment that prompted us to investigate whether the pH of the vacuole in *btn1-Δ* yeast strains could be raised. We report that growing *btn1-Δ* strains in the presence of chloroquine results in the loss

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Abbreviations: NCL, neuronal ceroid-lipofuscinosis; ANP, D-(–)-threo-2-amino-1-[p-nitrophenyl]-1,3-propanediol; YPD, bacto-yeast extract/bacto-peptone/glucose.

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of resistance to ANP. During early growth of *btn1*-Δ strains in the presence of chloroquine, plasma membrane H⁺-ATPase activity is decreased, and vacuolar pH is increased—changes that result in similarity to *BTN1*⁺ strains.

Although it remains to be shown that a defective lysosomal pH occurs in individuals with Batten disease and that altered lysosomal pH or possibly the altered gene expression acting to correct this defect is responsible for this devastating neurological disease, the use of drugs that can modulate this lysosomal pH may lead the way to a potential therapy in humans.

MATERIALS AND METHODS

Yeast Strains and Growth. The isogenic *btn1*-Δ yeast strain B-10195 (*MATa btn1*-Δ::HIS3 *CYC1*⁺ *cyc7*-Δ::CYH2 *leu2*,3-112 *ura3*-52 *his3*-Δ1 *trp1*-289; denoted *btn1*-Δ) was derived from B-7553 (*MATa BTN1*⁺ *CYC1*⁺ *cyc7*-Δ::CYH2 *leu2*,3-112 *ura3*-52 *his3*-Δ1 *trp1*-289; denoted *BTN1*⁺) by gene disruption (17). Yeast strains were grown as indicated in YPD medium [1% wt/wt bacto-yeast extract/2% wt/wt (vol/vol) bacto-peptone/2% wt/wt glucose]. ANP and chloroquine were added at the indicated concentrations after autoclaving. Growth in liquid medium was measured with a Klett Summerson Photoelectric Colorimeter (Klett Manufacturing, New York).

Acidification of External Medium. Extracellular acidification was measured as change in pH according to the method of Hemenway *et al.* (29), except that the starting pH for each experiment was not adjusted. Briefly, cells harvested at the times indicated were washed twice and resuspended in sterile distilled water to a concentration of 150 mg cells (wet weight) per ml. A 4-ml suspension of cells was stirred and diluted with 14 ml of sterile distilled water, and a glucose pulse was added as 2 ml of a 20% (vol/vol) glucose solution. The pH of the medium was measured every 60 s for the indicated time.

Subcellular Fractionation and Assay of Plasma Membrane H⁺-ATPase Activity. Plasma membranes were isolated and purified as described (30). In brief, cell extracts were prepared, and plasma membranes were collected at the interface of a discontinuous sucrose density gradient. Plasma membrane H⁺-ATPase activities were assessed by inclusion of the pertinent inhibitors of mitochondrial, plasma membrane, or vacuolar H⁺-ATPases, and Pi release was quantitated as described (31, 32).

Measurement of Vacuolar pH. Vacuolar pH was measured by using the fluorescent dye 6-carboxyfluorescein as described by Preston *et al.* (33), except that fluorescence was measured with a Fluorolog 2 spectrofluorometer (Instruments SA, Edison, NJ). Labeling of cells with 5 μM 6-carboxyfluorescein was performed in YPD, which contained 50 mM citric acid adjusted to pH 3.0, and the cells were washed with YPD.

Vacuolar pH was calculated from an *in vivo* calibration prepared by pretreating cells with ionophores to equilibrate the *in vivo* pH.

Northern Analysis. Expression of *HSP30* and *BTN2* was measured at the time point indicated by Northern blot analysis. mRNA and probes for *HSP30* and *BTN2* were prepared, and measurement of yeast gene expression was performed as described (34).

RESULTS

ANP Resistance of *btn1*-Δ Strains Is Reversed by Chloroquine. Chloroquine is a lysosomotropic agent best known for use as an antimalarial agent. Chloroquine is a weak-base amine that, in its neutral form, enters acidic compartments, such as the vacuole or lysosome, and becomes protonated. The pH becomes elevated as chloroquine accumulates in the compartment. This well characterized ability to elevate pH in an acidic compartment prompted us to test whether chloroquine would affect the decreased vacuolar pH in *btn1*-Δ strains during early growth, which results in resistance to ANP. We had already established that the resistance to ANP of *btn1*-Δ strains depended on pH (18). We examined the effect of chloroquine on growth of *BTN1*⁺ and *btn1*-Δ strains over a range of pHs in either the presence or absence of ANP. Chloroquine at a concentration above 0.5 mM in growth medium slows the growth of both *BTN1*⁺ and *btn1*-Δ strains (data not shown). Specifically, we show that, over a pH range of 6.0–7.0, 0.1 mM chloroquine has no effect on yeast growth (Fig. 1). As previously shown, both *BTN1*⁺ and *btn1*-Δ strains can grow in the presence of 2.25 mM ANP at pH 6.5 and below. However, *btn1*-Δ strains are resistant to 2.25 mM ANP at pH 6.8–7.0, whereas *BTN1*⁺ strains are not (Fig. 1). Both *BTN1*⁺ and *btn1*-Δ strains are able to grow in the presence of 0.1 mM chloroquine and 2.25 mM ANP at pH 6.5 and below. However, at pH 6.8–7.0, *BTN1*⁺ and *btn1*-Δ are unable to grow in a medium containing 0.1 mM chloroquine and 2.25 mM ANP, indicating that *btn1*-Δ strains are no longer resistant to ANP. This result may indicate that a combination of ANP and chloroquine is toxic to the *btn1*-Δ strains at pH 6.8–7.0. However, it is the reason that ANP becomes toxic to *btn1*-Δ strains in the presence of chloroquine that is of interest. Similar effects on the ANP resistance of *btn1*-Δ strains were found for concentrations of chloroquine between 0.05–0.5 mM (data not shown). Growth is diminished above 0.5 mM chloroquine, and *btn1*-Δ strains retain resistance to ANP below 0.05 mM chloroquine.

Chloroquine Decreases Rate of Extracellular Acidification and Activity of Plasma Membrane H⁺-ATPase and Increases Vacuolar pH. Previously, we showed that ANP resistance in *btn1*-Δ strains was caused by an elevated ability to acidify

FIG. 1. Chloroquine reverses resistance to ANP of *btn1*-Δ strains. Identical serial dilutions of *BTN1*⁺ and *btn1*-Δ strains were plated on the following media at the indicated pH: YPD with no addition; YPD + 0.1 mM chloroquine; YPD + 2.25 mM ANP; and YPD + 0.1 mM chloroquine + 2.25 mM ANP.

growth medium through an increased activity of plasma membrane H^+ -ATPase in the early phase of growth. To confirm that chloroquine was specifically countering the absence of Btn1p, we measured the rates of extracellular acidification and activities of plasma membrane H^+ -ATPase in *BTN1*⁺ and *btn1*-Δ strains grown in the presence and absence of chloroquine. Plasma membrane H^+ -ATPase activity clearly is decreased in both *BTN1*⁺ and *btn1*-Δ strains (Fig. 2A). In the absence of chloroquine in the early phase of growth, at 6.5 h, plasma membrane H^+ -ATPase activity is elevated in *btn1*-Δ compared with *BTN1*⁺ strains. Later in the growth curve, at 25 h, plasma membrane H^+ -ATPase activity in *btn1*-Δ strains becomes normalized at a level similar to that of *BTN1*⁺ strains. In the early phase of growth in the presence of 0.1 mM chloroquine, a concentration that reverses ANP resistance in *btn1*-Δ strains, plasma membrane H^+ -ATPase activity is decreased to an activity close to that of *BTN1*⁺ strains grown in either the presence or absence of 0.1 mM chloroquine. An examination of the rate of extracellular acidification early in growth reveals a decreased rate of acidification for *btn1*-Δ strains in the presence of chloroquine (Fig. 2C), most likely caused by the decrease in plasma membrane H^+ -ATPase activity. The most likely explanation for the reversal of the ANP resistance of *btn1*-Δ strains by chloroquine is that plasma membrane H^+ -ATPase activity is now close to that of normal *BTN1*⁺ strains. Therefore, *btn1*-Δ strains no longer have an increased rate of acidification, which would normally result in decreasing the pH of the ANP containing medium, enabling growth. In summary, chloroquine has rendered *btn1*-Δ strains like *BTN1*⁺ strains with respect to ANP resistance through normalization of the plasma membrane H^+ -ATPase activity.

FIG. 2. Plasma membrane H^+ -ATPase activities and acidification of external media by actively growing *BTN1*⁺ and *btn1*-Δ yeast strains. (A) Plasma membrane H^+ -ATPase activity determined with isolated plasma membranes at the indicated time of the growth of the *BTN1*⁺ and *btn1*-Δ strains. (B) Growth curves of *BTN1*⁺ and *btn1*-Δ strains in YPD medium were identical. (C) Acidification profiles at 6.5 and 25 h, as measured by the change in external pH of the medium for (a) *BTN1*⁺, YPD; (b) *btn1*-Δ, YPD; (c) *BTN1*⁺, YPD + 0.1 mM chloroquine; (d) *btn1*-Δ, YPD + 0.1 mM chloroquine. These plots are representative of the results obtained from three identical experiments.

Table 1. Vacuolar pH of *BTN1*⁺ and *btn1*-Δ strains grown in the presence or absence of chloroquine

Time of growth, h	Optical density, Klett units	pH			
				Chloroquine	
		<i>BTN1</i> ⁺	<i>btn1</i> -Δ	<i>BTN1</i> ⁺	<i>btn1</i> -Δ
6.5	25	6.15	5.80	6.20	6.20
25	300	6.10	6.10	6.30	6.30

It is important to note that, when we examine the effect of chloroquine on the plasma membrane H^+ -ATPase in both *BTN1*⁺ and *btn1*-Δ strains later in growth, there is a statistically relevant decrease in activity. The objective of this study was to examine the possibility of reversing the effects of deleting *BTN1*. Clearly, in the early phase of growth, chloroquine achieves this phenotypic reversal. However, it is clear that, later through the growth curve, both *BTN1*⁺ and *btn1*-Δ strains treated with chloroquine have a less than normal activity of plasma membrane H^+ -ATPase.

The remaining question was whether the reversal in ANP resistance and decrease in plasma membrane H^+ -ATPase activity by chloroquine in *btn1*-Δ strains were caused by an increase in vacuolar pH. As is shown in Table 1, vacuolar pH of *btn1*-Δ strains in the early phase of growth is in fact elevated from pH 5.80 to pH 6.20. It would seem that the underlying cause of ANP resistance in *btn1*-Δ strains, namely a decreased vacuolar pH, which in turn leads to an increased ability to acidify growth medium through an elevated activity of plasma membrane H^+ -ATPase, is indeed reversed by chloroquine. As with the activity of plasma membrane H^+ -ATPase, chloroquine has a noticeable effect on vacuolar pH for both *BTN1*⁺ and *btn1*-Δ strains in the later stages of growth. Both *BTN1*⁺ and *btn1*-Δ strains have an increase in vacuolar pH from 6.10 to 6.30.

Effect of Chloroquine on the Expression of *HSP30* and *BTN2* in *BTN1*⁺ and *btn1*-Δ Strains. We previously reported that *btn1*-Δ strains had increased expression of *HSP30* and *BTN2*. Because Hsp30p is a stress-induced down-regulator of plasma membrane H^+ -ATPase (35), we proposed that the increased expression of this protein was responsible for the normalizing of increased activity of this enzyme, leading to a return to a balanced pH homeostasis (20). The reason for increased expression of *BTN2*, which has similarity to human HOOK1, is less clear. The *Drosophila* HOOK1 has been implicated in endocytosis (36).

Therefore, we determined whether chloroquine affected the expression of *HSP30* and *BTN2* in *BTN1*⁺ and *btn1*-Δ strains. We confirmed that, late in growth, at 25 h, there was a significant increase in expression of *HSP30* and especially *BTN2* in *btn1*-Δ compared with *BTN1*⁺ strains. The effect of chloroquine was to decrease expression of *HSP30* in *BTN1*⁺ and *btn1*-Δ strains, whereas expression of *BTN2* was unchanged in *BTN1*⁺ strains and decreased in *btn1*-Δ strains (Fig. 3). In one sense, these results suggest that chloroquine partially returns *btn1*-Δ strains to the same state as *BTN1*⁺ as the level of expression of *HSP30* and *BTN2* is going down. However, again, we must be cautious in interpreting these results, because chloroquine clearly decreases expression of *HSP30* in *BTN1*⁺ strains, and *BTN2* expression is still higher than normal in *btn1*-Δ strains.

DISCUSSION

This study shows that the yeast model for Batten disease can be used to determine whether a drug can reverse defined biochemical and physiological phenomena associated with the deletion of *BTN1*. The fact that human CLN3 complements the resistance to ANP observed in *btn1*-Δ strains suggests that human CLN3 has the same function as Btn1p in yeast (18). It

FIG. 3. Data obtained from comparative Northern blot analyses of *HSP30* (Left) and *BTN2* (Right) mRNAs after 25 h of growth (300 Klett units). (A) *BTN1*⁺, YPD (closed black bars); (B) *btn1*-Δ, YPD (hatched bars); (C) *BTN1*⁺, YPD + 0.1 mM chloroquine (gray bars); (D) *btn1*-Δ, YPD + 0.1 mM chloroquine (open bars). The mRNA levels were normalized to *ACT1* mRNA, and the values for each type of mRNA are presented relative to a value of 1.0 for the *BTN1*⁺ strain at 25 h.

obviously needs to be established whether any of the defects found in *btn1*-Δ strains truly are associated with individuals with Batten disease. Nevertheless, the yeast model is currently a valuable but unproven model for gathering information on the pathogenesis of Batten disease. In this study, we examined the biology of *btn1*-Δ strains and attempted to suppress their phenotypes by adding the drug chloroquine. To our knowledge, this study is unique in its use of yeast as a clinical model for a human inherited disease. The results show that inclusion of chloroquine in the growth medium partially reverses the primary phenotype of ANP resistance in *btn1*-Δ strains. The resistance to ANP in *btn1*-Δ strains results from an increased activity of plasma membrane H⁺-ATPase, which is precipitated by a decreased vacuolar pH in the early phase of growth. It is apparent that chloroquine does in fact return both plasma membrane H⁺-ATPase activity and vacuolar pH to near normal in early growth of *btn1*-Δ strains. However, as growth continues, plasma membrane H⁺-ATPase becomes abnormally low and vacuolar pH becomes abnormally high in both *btn1*-Δ and *BTN1*⁺ strains. These abnormal conditions at the later growth period can be viewed as a secondary effect, and even other cellular changes may also occur from an abnormally low activity of plasma membrane H⁺-ATPase and an abnormally high vacuolar pH. The clearest indication that chloroquine does not complement the absence of Btn1p fully is the incomplete phenotypic suppression of the *HSP30* and *BTN2* mRNAs (Fig. 3). In addition, *BTN1*⁺ strains are again affected by chloroquine, with *HSP30* expression considerably decreased. No doubt chloroquine affects more than just the *BTN1*-mediated pH homeostasis in yeast, and it would be interesting to examine the effect of chloroquine on the expression of all yeast genes.

The questions of what biological processes are affected by chloroquine and whether a therapy for Batten disease can be derived by our phenotypic reversal studies in yeast clearly require further investigation. Chloroquine accumulates in acidic compartments and increases pH. One known effect of this elevation of pH in the lysosome is protease inhibition (37), which is most likely caused by displacing the pH from the enzymatic pH optimum. Curiously, chloroquine-treated neu-

rons were examined several years ago as a potential model for Batten disease because of the apparent induction of lysosome-associated granular aggregates (38), which was reminiscent of the pathology of Batten disease cells. Because leupeptin, a more potent protease inhibitor, was also used in this study and gave a more profound effect, it is assumed that defective degradation in the lysosome leads to the manifestation of these aggregates. In this case, if we assume that the chloroquine elevated the lysosomal pH above that of normal and that in Batten disease, in our model, the lysosome is more acidic than normal; thus, a deviation from optimal pH, either high or low, may well lead to a similar phenotype or accumulation of structures in the lysosome. Another parallel can be drawn when we consider reports that one of the side effects of chloroquine is retinopathy, the pathogenesis of which is yet to be elucidated (39). This side effect would indicate that retinal cells are particularly sensitive to the effects of chloroquine and perhaps an alteration in lysosomal pH. One of the first symptoms of Batten disease is deteriorating eyesight leading to blindness because of retinal degeneration. Other reported activities of chloroquine are inhibition of phospholipases, steroid synthesis, and protein phosphorylation, as well as binding or adsorbing to the plasma membrane inhibiting cell fusion (40).

Increased expression of *BTN2* in *btn1*-Δ strains, which is decreased to a degree by chloroquine, is significant. Btn2p shows 38% similarity over 104 amino acids to the human HOOK1 protein. The corresponding HOOK1 protein from *Drosophila* has been shown to be a protein involved in the endocytosis of transmembrane ligands (36), a process known to be driven by acidification of vesicles. It is still unclear as to why there is increased expression of Btn2p in *btn1*-Δ strains. It is tempting to speculate about a potential role of Btn2p in endocytosis because of the homology to HOOK1 and the fact that chloroquine, which no doubt decreases pH in all yeast-acidified compartments including those derived from endocytosis, decreases expression of *BTN2*. Btn1p has been localized to the vacuole (20, 41). CLN3 has been localized to the lysosome and late endosomes, and it was indicated that a small amount may be associated with the plasma membrane, which might suggest that CLN3 is recycled through the plasma membrane and potentially through endocytosis (42, 43). In murine telencephalic neurons, CLN3 was distributed toward synaptic processes, colocalizing with lysosomal and synaptic vesicle markers (43). Previously, we have speculated that any disturbance in pH homeostasis could affect the acidification of vesicles trafficked intracellularly and involved in the transmission of signals at axons of neural cells. The fact that chloroquine, which is known to elevate the pH of acidified compartments, has been shown to compensate at least partially for the defects associated with a lack of Btn1p and to reduce the increased expression of *BTN2* might suggest that *btn1*-Δ strains have a defect not only in vacuolar pH, but also in acidified vesicles involved in endocytosis. Such a presumption would make chloroquine an even more attractive mode of reversing the effects of Batten disease. However, because of known side effects such as retinopathy, more research is necessary before one can considering chloroquine as a potential therapeutic agent.

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