Phenotypic reversal of the btm1 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, btm1-Δ, resulted in a pH-dependent resistance to D-(-)-threo-2-amino-1-[p-nitrophenyl]-1,3-propanediol (ANP). This phenotype was caused by btm1-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 btm1-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 btm1-Δ strains continue to grow. This phenotypic reversal of btm1-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 (Santa­ vuori–Haltia disease); late-infantile-NCL, CLN2 (Jansky–Bielchowsky 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, btm1-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 btm1-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 btm1-Δ strains (20). As btm1-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 btm1-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 btm1-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 btm1-Δ yeast strains could be raised. We report that growing btm1-Δ strains in the presence of chloroquine results in the loss of
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) times indicated were washed twice and resuspended in sterile distilled water to a concentration of 150 mg cells (wet weight)

**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 (Instrument 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.

Vacular 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.
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 Bnlp in yeast (18). It...
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 Bt1p 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 neurons 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. Bt2p 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 Bt2p in btn1-Δ strains. It is tempting to speculate about a potential role of Bt2p 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. Bt1p has been localized to the vacuole (40, 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 tenecephalic 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 Bt1p 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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