BTN1, the *Saccharomyces cerevisiae* homolog to the human Batten disease gene, is involved in phospholipid distribution

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**SUMMARY**

BTN1, the yeast homolog to human CLN3 (which is defective in Batten disease), has been implicated in the regulation of vacuolar pH, potentially by modulating vacuolar-type H⁺-ATPase (V-ATPase) activity. However, we report that Btn1p and the V-ATPase complex do not physically interact, suggesting that any influence that Btn1p has on V-ATPase is indirect. Because membrane lipid environment plays a crucial role in the activity and function of membrane proteins, we investigated whether cells lacking BTN1 have altered membrane phospholipid content. Deletion of BTN1 (*btn1⁻¹*) led to a decreased level of phosphatidylethanolamine (PtdEtn) in both mitochondrial and vacuolar membranes. In yeast there are two phosphatidylserine (PtdSer) decarboxylases, Psd1p and Psd2p, and these proteins are responsible for the synthesis of PtdEtn in mitochondria and Golgi-endosome, respectively. Deletion of both BTN1 and PSD1 (*btn1⁻¹ psd1⁻¹*) led to a further decrease in levels of PtdEtn in ER membranes associated to mitochondria (MAMs), with a parallel increase in PtdSer. Fluorescent-labeled PtdSer (NBD-PtdSer) transport assays demonstrated that transport of NBD-PtdSer from the ER to both mitochondria and endosomes and/or vacuole is affected in *btn1⁻¹Δ* cells. Moreover, *btn1⁻¹Δ* affects the synthesis of PtdEtn by the Kennedy pathway and impairs the ability of *psd1⁻¹* cells to restore PtdEtn to normal levels in mitochondria and vacuoles by ethanolamine addition. In summary, lack of Btn1p alters phospholipid levels and might play a role in regulating their subcellular distribution.

**INTRODUCTION**

We have previously reported that the *Saccharomyces cerevisiae* BTN1 gene encodes a nonessential protein that is 39% identical and 59% similar to human CLN3 (Pearce and Sherman, 1997). Mutations in human CLN3, which codes for a lysosomal transmembrane protein, causes Batten disease, the juvenile form of neuronal ceroid lipofuscinosis (NCL) (The International Batten Disease Consortium, 1995; Jarvela et al., 1998; Kytälä et al., 2004; Storch et al., 2004; Phillips et al., 2005). Btn1p is located in the vacuole, the yeast membrane compartment that is equivalent to the mammalian lysosomal organelle (Croopnick et al., 1998; Pearce et al., 1999), although several recent studies in a *S. cerevisiae* model found Btn1p associated with punctate membrane structures (Vitiello et al., 2010; Wolfe et al., 2011). Furthermore, localization studies of overexpressed Btn1p in *Schizosaccharomyces pombe* associated this protein to Golgi membranes (Codlin and Mole, 2009). The primary function of this protein remains unknown; however, Btn1p has been implicated in several cellular pathways. In a *S. cerevisiae* yeast model, lack of Btn1p (*btn1⁻¹*) led to a decreased vacuolar pH during the logarithmic phase of growth (Pearce et al., 1999). Furthermore, *btn1⁻¹* cells downregulate ATP hydrolysis by vacuolar-type H⁺-ATPase (V-ATPase) most likely as a cell response to control H⁺ transport across the vacuolar membrane and compensate for pH imbalance caused by the lack of Btn1p (Padilla-Lopez and Pearce, 2006). This alteration at the vacuole has been implicated in deficient regulation of intracellular L-arginine levels, with *btn1⁻¹* cells showing a lower ability to transport this amino acid into isolated vacuoles in vitro (Kim et al., 2003). However, this latter defect could be a secondary consequence from the primary vacuolar pH alteration observed in *btn1⁻¹* cells.

Little is known about the mechanisms regarding lipid transport and distribution within eukaryotic cells. PtdSer is synthesized by PtdSer synthase in a specialized region of the endoplasmic reticulum (ER) membrane that is associated to mitochondria [mitochondria-associated membrane (MAM)] (Gaigg et al., 1995). PtdSer is transported to the locus of PtdSer decarboxylases Psd1p or Psd2p, where it is decarboxylated to form phosphatidylethanolamine (PtdEtn). PtdEtn is a well-known inner mitochondrial protein (Kuchler et al., 1986). However, whereas previous studies associated Psd2p to Golgi-vacuole membranes (Trotter et al., 1993; Trotter and Voelker, 1995), a recent study refined Psd2p localization to the endosome, arguing that this enzyme controls vacuolar membrane phospholipid content (Gulsahan et al., 2010). Following its formation, PtdEtn is transported back to the ER for the synthesis of phosphatidylinositol (PtdCho) (Atkinson et al., 1980; Trotter et al., 1993). Alternatively, PtdEtn can be synthesized through the cytidyldiphosphate (CDP)-ethanolamine branch of the Kennedy pathway (Kennedy and Weiss, 1956). PtdEtn is essential for growth of the yeast *S. cerevisiae* and is required for the function and integrity of mitochondrial

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membranes; a lack of the major PtdSer decarboxylase, Psd1p, leads to a substantial decrease of PtdEtn in total cellular and mitochondrial membranes, conferring a petite phenotype (Birner et al., 2001). Moreover, depletion of PtdEtn causes defects in the assembly of mitochondrial protein complexes and loss of mitochondrial DNA (Birner et al., 2001; Storey et al., 2001; Birner et al., 2003). PtdEtn also plays a crucial role in GPI anchor biosynthesis (Menon and Stevens, 1992) and seems to be involved in the autophagy and cytosol-to-vacuole-targeting delivery pathways (Huang and Klionsky, 2002; Wang and Klionsky, 2003; Yoshimori, 2004; Nebauer et al., 2007).

We report a potential newly identified role for Btn1p: being necessary for the distribution of phospholipids in cell membranes. We report that yeast cells lacking BTN1 show decreased transport of PtdSer from ER membranes to either mitochondria or endosome and/or vacuole membranes, resulting in a significant decrease of PtdEtn synthesis in cells lacking Psd1p (psd1-Δ). Furthermore, a lack of Btn1p (btn1-Δ) also partially impairs the synthesis of PtdEtn by the Kennedy pathway. Thus, the multiple cellular phenotypes associated to a lack of Btn1p, or indeed CLN3, could be a consequence of altered membrane phospholipid content.

**RESULTS**

**Btn1p is not a component of the V-ATPase complex**

Our previous study (Padilla-Lopez and Pearce, 2006) indicated that Btn1p has the ability to modulate the activity of the V-ATPase complex. However, evidence was lacking in support of a direct role for Btn1p in the regulation of V-ATPase. An attractive explanation for the role of Btn1p in V-ATPase regulation would be to demonstrate either that Btn1p physically interacts with one or more subunits of the V-ATPase complex, or that Btn1p could be part of the macrocomplex formed by the V-ATPase-complex subunits and other proteins that associate with them; for example, those involved in the glycolytic pathway (Lu et al., 2001; Su et al., 2003; Lu et al., 2004; Sautin et al., 2005; Lu et al., 2007).

Because specific antibodies against all 13 of the V-ATPase subunits are not available to test Btn1p interaction with them all, we decided to test whether Btn1p is a component of the V-ATPase complex by analyzing the vacuolar proteome using two-dimensional (2D) blue-native/SDS-PAGE electrophoresis and western blotting. Vacular fractions isolated from wild-type strains harboring the empty plasmid pRS426 or pmBTN1-V5 were used to perform a blue-native electrophoresis. This technique allowed us to resolve the different protein complexes of the vacuolar proteome. The individual components of these complexes were separated in the second dimension by SDS-PAGE, transferred to a nitrocellulose membrane and probed with antibodies against the V5 epitope and three V-ATPase subunits (Vma1p and Vma2p) and one component of the membrane V0 sector (Vph1p). Fig. 1A shows the normal profile for V-ATPase complexes in a wild-type strain. Three kinds of V-ATPase complexes (Vph1p). Fig. 1A shows the normal profile for V-ATPase complexes and Vma2p) and one component of the membrane V0 sector (Vph1p). Fig. 1A shows the normal profile for V-ATPase complexes by analyzing the vacuolar proteome using two dimensional (2D) blue-native/SDS-PAGE electrophoresis and western blotting.

**Changes in Btn1p levels alter the membrane phospholipid profile in yeast cells**

Different organelles in the cell have a specific composition of membrane phospholipids that is tightly regulated in order to maintain an optimal membrane protein function. Alterations of the phospholipid profile could easily change the way membrane proteins interact with each other and with the phospholipids themselves, leading to the loss of protein activity and causing a number of pleiotropic effects on the cell. We investigated whether the observed effect on V-ATPase activity by Btn1p could be a consequence of alterations in the lipid environment of the vacuolar membrane.

In *S. cerevisiae*, the different phospholipids are synthesized de novo from CDP-diacylglycerol (CDP-DAG). Addition of serine to CDP-DAG synthesizes PtdSer in the MAMs (Gaigg et al., 1995) that is subsequently transported to other organelles. In the mitochondria, PtdSer is decarboxylated to form PtdEtn by Psd1p.
In the endosomes and vacuole compartments, PtdEtn is formed by the action of Psd2p (Gulshan et al., 2010). PtdEtn in the mitochondrial and vacuolar compartments can be subsequently exported from those organelles back to the ER to be transformed to PtdCho by the action of PtdEtn methyltransferases. Alternatively, PtdEtn can be synthesized from ethanolamine (Etn) through the Kennedy pathway. In this pathway, ethanolamine needs to be activated to CDP-ethanolamine (CDP-Etn) and fused to diacylglycerol (DAG) to synthesize PtdEtn.

In the endosomes and vacuole compartments, PtdEtn is formed by the action of Psd2p (Gulshan et al., 2010). PtdEtn in the mitochondrial and vacuolar compartments can be subsequently exported from those organelles back to the ER to be transformed to PtdCho by the action of PtdEtn methyltransferases. Phosphatidylinositol (PtdIns) is also synthesized from CDP-DAG by the addition of inositol molecule (Fig. 2). Therefore, lipid extracts of whole cell extracts, vacuolar membrane, mitochondria and MAMs were obtained from wild-type and btn1Δ cells, and phospholipid content was analyzed by thin layer chromatography (TLC; see Methods). Mitochondrial and vacuolar extracts obtained from btn1Δ cells showed a slight but significant decrease of PtdEtn levels compared with the wild-type strain (Fig. 3). To further understand the observed changes, potential genetic interaction between BTN1 and either PSD1 or PSD2 was investigated by analyzing the phospholipid profile in psd1Δ and psd2Δ cells carrying an additional BTN1 deletion (btn1Δ psd1Δ and btn1Δ psd2Δ).

The most dramatic alterations were observed in the psd1Δ genetic background. The PtdSer decarboxylase Psd1p is a component of the inner mitochondrial membrane, which is responsible for the major part of PtdEtn synthesis in yeast cells. It has been described that lack of Psd1p leads to a substantial decrease of PtdEtn in total cellular and mitochondrial membranes, thereby conferring a petite phenotype, which is linked to the loss of respiratory capacity. Thus, this mutant strain requires supplementation with ethanolamine when cultured in nonfermentable carbon sources (Birner et al., 2001). Results obtained in this work confirmed decreased PtdEtn in whole cell, mitochondrial and vacuolar fractions of this strain (Fig. 3). Interestingly, psd1Δ cells did not show a significant decrease of PtdEtn levels in the MAM fraction. Additionally, deletion of psd1 led to an accumulation of PtdIns that could be observed in all the analyzed fractions, which suggests that blocking the main route for synthesizing PtdEtn is compensated by the overproduction of PtdIns in yeast cells. Surprisingly, combining btn1Δ with psd1Δ (btn1Δ psd1Δ) led to both PtdSer accumulation in mitochondrial and MAM fractions and a further decrease of PtdEtn levels that was mainly focused in the MAM fraction. Moreover, btn1Δ psd1Δ also led to a normalization of PtdIns level.

Analysis of psd2Δ strains also showed a decrease of PtdEtn in all the studied fractions and, although the mitochondrial fraction was slightly less affected than in psd1Δ cells, the MAM and vacuole fractions showed lower PtdEtn levels than cells lacking psd1Δ. In addition, psd2Δ cells did not show accumulation of PtdIns, but levels of PtdSer were increased in the MAM and vacuole fractions.
This suggests that \( \text{psd}2^- \) does not trigger the same compensatory response as the one observed in \( \text{psd}1^- \) cells that leads to the accumulation of PtdIns. In fact, \( \text{psd}2^- \) cells showed a significant decrease of PtdIns in mitochondrial and MAM fractions. \( \text{btn}1^- \) \( \text{psd}2^- \) did not lead to any significant changes on the phospholipid profile as compared with \( \text{psd}2^- \) cells (Fig. 3).

**Lack of \( \text{BTN1} \) alters phospholipid transport from the ER**

We investigated the potential involvement of Btn1p in phospholipid distribution among cell membranes through a transport assay using fluorescence labeled PtdSer analog, 1-palmitoyl-2-{N-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl}-PtdSer (NBD-PtdSer). To determine how NBD-PtdSer is incorporated by yeast cells, wild-type and \( \text{btn}1^- \) yeast strains were grown in synthetic complete (SC) media to logarithmic phase, incubated with 5 \( \mu \text{M} \) NBD-PtdSer for 5 minutes and analyzed by fluorescence microscopy. Fig. 4 shows that NBD-PtdSer was incorporated to the same degree in both wild-type and \( \text{btn}1^- \) yeast strains. Moreover, fluorescence was concentrated in similar punctate structures in both strains. It has been described that NBD-PtdEtn is incorporated by yeast cells by flip across the plasma membrane and is enriched in ER membranes (Hanson et al., 2002). NBD-PtdSer fluorescence (Fig. 4) accumulates in the same kind of structures as published for NBD-PtdEtn. Therefore, NBD-PtdSer probably accumulates in the ER of yeast cells.

Transport of NBD-PtdSer from ER to either mitochondria or endosomes and vacuole was determined by the rate of synthesis of NDB-PtdEtn in a \( \text{psd}2^- \) or \( \text{psd}1^- \) genetic background, respectively. As shown in Fig. 5A and 5B, the rate of NBD-PtdEtn synthesis in \( \text{psd}1^- \) cells was significantly lower than in \( \text{psd}2^- \) cells, confirming Psd1p as the main PtdSer decarboxylase in yeast cells. Moreover, combining \( \text{btn}1^- \) in either the \( \text{psd}1^- \) or \( \text{psd}2^- \) genetic background led to a slower rate of NBD-PtdEtn synthesis, suggesting that transport of NBD-PtdSer from the ER to both mitochondria and vacuolar membranes is partially impaired in those strains.

It has been described that lack of Psd1p leads to a substantial decrease of PtdEtn in total cellular and mitochondrial membranes, thereby conferring a \textit{petite} phenotype and inability to grow on nonfermentable carbon sources. However, exogenous ethanolamine addition allows \( \text{psd}1^- \) cells to synthesize PtdEtn by the Kennedy pathway (Kennedy and Weiss, 1956) in which ethanolamine is activated in the cytosol to CDP-ethanolamine and further bound to diacylglycerol in the ER membrane to synthesize PtdEtn. Thus, PtdEtn synthesized by the Kennedy pathway can be delivered to either the mitochondria or the vacuolar membrane and restore normal levels in those organelles. Because Btn1p seems to be necessary for normal delivery of phospholipids to the mitochondria, we tested whether a lack of Btn1p affected restoration of PtdEtn in the mitochondria and vacuole of \( \text{psd}1^- \) cells when grown in...
btn1-Δ and phospholipids

SC-glucose with 5 mM ethanolamine supplementation (Fig. 6). Although ethanolamine supplementation did not significantly raise the level of PtdEtn in total cell fractions of any of the analyzed strains, mitochondrial and vacuolar fractions of psd1-Δ cells grown with ethanolamine supplementation elevated and returned PtdEtn to wild-type levels. No changes in the psd1-Δ MAM fraction of PtdEtn was observed after supplementation with ethanolamine. Interestingly, ethanolamine supplementation of btn1-Δ psd1-Δ did not raise PtdEtn in either mitochondria or vacuoles, whereas a significant increase was observed in MAMs, compared with nonsupplemented media, suggesting that PtdEtn synthesized at the ER by the Kennedy pathway does not reach the other two cellular organelles. However, PtdEtn accumulation in MAMs of btn1-Δ psd1-Δ cells did not reach the levels observed in a single psd1-Δ strain, raising the issue of whether the observed phenotype is caused by an impairment of PtdEtn synthesis through the Kennedy pathway rather than a failure to transport the phospholipid to either mitochondria or vacuoles.

Impairment of the Kennedy pathway could lead to accumulation of free ethanolamine in the cytosol, which potentially could become toxic to cells. Therefore, we wondered whether yeast strains lacking some steps of the salvage pathway for PtdEtn synthesis are sensitive to high concentrations of ethanolamine in the media. Fig. 7 shows that yeast mutant strains lacking either the EKI1 or ECT1 genes, coding for the first and second enzymes of the Kennedy pathway for PtdEtn synthesis, respectively, were unable to grow in the presence of 10 mM ethanolamine. Moreover, ethanolamine was shown to be toxic for btn1-Δ strains, suggesting that lack of BTN1 also impairs the synthesis of PtdEtn through the Kennedy pathway.

DISCUSSION

We report a newly identified genetic interaction for BTN1 and the major PtdSer decarboxylase, Psd1p, suggesting a potential role for Btn1p in phospholipid transport and distribution among yeast membranes.

The idea that the lipid environment modifies the activity of membrane proteins is not new. Crider and Xie demonstrated that phospholipids play a crucial role in the functional coupling of V-ATPases in bovine brain (Crider and Xie, 2003). It also known that Btn1p could have a role in modulating V-ATPase activity (Padilla-Lopez and Pearce, 2006) and importing basic amino acids to the vacuolar lumen (Kim et al., 2003), and that overexpression of the plasma membrane L-arginine transporter Can1p is toxic in btn1-Δ cells (Vitiello et al., 2007). In addition, Opekarová et al. demonstrated that PtdEtn is an essential factor for targeting Can1p to the plasma membrane of yeast (Opekarova et al., 2002). Therefore, taken together, it can be suggested the observed alterations in btn1-Δ cells in V-ATPase activity and L-arginine metabolism might be a secondary consequence of a defect in phospholipid metabolism that we now describe.

The two major alterations in the phospholipid profile in btn1-Δ psd1-Δ yeast include: (1) accumulation of PtdSer in the mitochondrial and MAM fractions, and (2) a further decreased PtdEtn in the MAM fraction. Both alterations suggest an impairment of the transport of PtdSer from ER membranes and/or MAMs to the Psd2p locus at endosomes and/or the vacuole, which would lead to a decreased synthesis of PtdEtn, as observed. However, it is intriguing that only PtdEtn levels of MAMs are diminished in btn1-Δ psd1-Δ cells, whereas mitochondrial and vacuolar fractions did not show any significant differences compared with the psd1-Δ strain. The latter observation might be explained if we assume that PtdEtn transport from vacuolar membranes back to ER is also impaired in the double mutant, whereas its delivery to mitochondria remains unaffected. NBD-PtdSer transport analysis presented in Fig. 5 supports a role for Btn1p in the transport of phospholipids among cells membrane. Because lack of Btn1p does not affect the uptake of the fluorescent analog of PtdSer by yeast cells (Fig. 4), decreased synthesis of NBD-PtdEtn in btn1-Δ cells is probably a result of an impaired delivery of NBD-PtdSer from ER and/or MAMs to the cell organelles in which the two PtdSer decarboxylases are located. Finally, it is also interesting how additional BTN1 deletion normalizes PtdIns accumulation in psd1-Δ cells, raising the issue of whether lack of Btn1p has deeper consequences in the regulation of the phospholipid biosynthetic pathway.

The distribution of PtdEtn synthesized in the ER through the Kennedy pathway further supports a phospholipid transport role.
for Btn1p (Fig. 6). However, if BTN1 deletion only impaired transport of phospholipids from ER to other cellular membranes, high amounts of PtdEtn accumulated in ER and MAM fraction would be expected. As this is not the case, it is possible that synthesis of PtdEtn through the Kennedy pathway is also impaired in btn1-Δ cells. Interestingly, we show that yeast strains lacking genes of the Kennedy pathway for the synthesis of PtdEtn are sensitive to ethanolamine, supporting that the Kennedy pathway is impaired in this strain.

It has been proposed that Btn1p in S. pombe affects the transport of membrane proteins through the Golgi. In this yeast model, deletion of BTN1 leads to secretion of the vacuolar protease Cpyp as a consequence of mistrafficking of its receptor, Vps10p (Codlin and Mole, 2009). Therefore, the findings presented in the current work could partially explain the effect described above as a result of an alteration of the phospholipid content of the cell membranes. However, deletion of BTN1 does not lead to a mistrafficking of Cpyp in S. cerevisiae (supplementary material Fig. S1), suggesting that membrane protein trafficking is not affected by Btn1p in this yeast model.

In summary, this work points to a role for Btn1p in the synthesis and distribution of phospholipids in yeast cells. Although Btn1p has been described mainly as a vacuolar protein in S. cerevisiae, a recent study shows that Btn1p changes its subcellular localization from the vacuole to unidentified punctae in a pH-dependent manner and that the glycosylation state of Btn1p probably regulates this process (Wolfe et al., 2011). It is possible that, although unconfirmed in this study, these unidentified punctae might belong to regions of the ER such as the MAM. Thus, in addition to being highly hydrophobic, Btn1p could be a mobile protein with the potential capacity of transporting different kinds of lipid species among cell membranes.

Phospholipid synthesis is regulated by the transcription repressor Opi1p. Opi1p binds precursor phosphatidic acid in ER membranes. Glucose starvation causes a rapid decrease in intracellular pH that protonates phosphatidic acid and breaks its interaction with Opi1p. Once free in the cytosol, Opi1p moves into the nucleus and represses the expression of several genes involved in phospholipid synthesis (Young et al., 2010). Interestingly, Btn1p changes localization from vacuole to punctate structures at acidic pH (Wolfe et al., 2011). Therefore, it could be that low pH mimics glucose starvation conditions and triggers both repression of phospholipid synthesis through Opi1p action, and Btn1p retention in the MAM fraction, inhibiting any further transport of phospholipids to the vacuole. However, in order to confirm this hypothesis, further study will be required.

Fig. 5. Cells lacking BTN1 show impaired transport of NBD-PtdSer from ER membranes. Decarboxylation of NBD-labeled PtdSer to NBD-PtdEtn by either Psd1p or Psd2p was used to monitor PtdSer transport from ER to either mitochondria or endosomes and/or vacuole, respectively, as described in the Methods. (A) Transport to endosomes and/or vacuole was determined measuring NBD-PtdEtn synthesis in psd1-Δ yeast protoplasts resuspended in SC medium with 1.2 M sorbitol and 5 μM NBD-PtdSer and incubated for the indicated time at 30°C. (B) Transport to mitochondria was determined by performing the same procedure with psd2-Δ yeast protoplasts. Lipid extracts were obtained, analyzed by TLC and the fluorescence associated to NBD-PtdEtn was plotted. The plots represent the mean ± s.e.m. of the results obtained from three identical experiments. Differences between strains are statistically significant with *P<0.005 as determined by standard t-test.

Fig. 6. Lack of BTN1 leads to alterations in the phospholipids profile of yeast cells. Yeast cells were inoculated at initial A660 0.1 in SC medium with or without 5 mM ethanolamine and grown for 24 hours, then the indicated cell fractions were isolated and lipid extracts were obtained as described in the Methods. The plots represent the mean and standard errors of the results obtained from five identical experiments. Differences in lipid content are statistically significant with *P<0.005 as determined by standard t-test. TF, total fraction; Mitto, mitochondrial fraction; MAM, mitochondria-associated membrane fraction; VAC, vacuolar fraction. Differences in lipid content are statistically significant with *P<0.005 as determined by standard t-test. Symbols denote statistically significant differences to the same membrane fraction of wild-type (+), psd1-Δ (+) or btn1-Δ psd2-Δ (Δ) strains grown in the absence of ethanolamine.
Finally, several studies describing CLN3, the human homolog for BTN1, involvement in lipid metabolism has been reported. Thus, it has been proposed that CLN3 is involved in the synthesis of bis(monoacylglycerol) phosphate (BMP), a phospholipid component of lipid rafts (Hobert and Dawson, 2007). Alternatively, an in vitro palmitoyl-protein Δ-9 desaturase activity, which could convert membrane-associated palmitoylated proteins to their respective palmitoylated derivatives, has been associated to this component of lipid rafts (Hobert and Dawson, 2007). Alternatively, an in vitro palmitoyl-protein Δ-9 desaturase activity, which could convert membrane-associated palmitoylated proteins to their respective palmitoylated derivatives, has been associated to this protein (Narayan et al., 2006). Although neither study shows direct involvement of CLN3 in either BMP synthesis or palmitoyl-protein Δ-9 desaturation, respectively, these results might reflect downstream alterations in underlying membrane dynamics owing to the alterations in the phospholipids we report.

In conclusion, we propose a new role for Btn1p in the regulation of phospholipid distribution among the membranes of S. cerevisiae. Because BTN1 and human CLN3 are homologs, the Btn1p-related biochemical alterations presented in the current work might underlie altered lysosomal function in Batten disease and result in the pathological consequences of this disease.

METHODS

Yeast strains and media

The B4742 wild-type (MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), btn1-Δ (MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 btn1::KAN), psd1-Δ (MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1::KAN), psd2-Δ (MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd2::KAN), ect1-Δ (MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ect1::KAN) and eki1-Δ (MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 eki1::KAN) yeast strains were purchased from RESGEN. The NATMX4 system was used to delete BTN1 in the BTN1 background using homologous recombination (Goldstein and McCusker, 1999). The NATMX4 cassette was obtained by PCR amplification from plasmid p4339 using primers containing 50-55 bp of the sequence upstream and downstream of the BTN1 open reading frame (ORF). Deletions were tested for resistance to Nourseothricin (clonNAT) and confirmed by PCR.

Yeast strains were grown as indicated in SC medium (6.7 g/l yeast nitrogen base without amino acids, 2% glucose and complete amino acid supplement). When denoted, 5 and 10 mM ethanolamine was added to the media.

Plasmid pmBTN1 was constructed by ligating the BTN1-containing EcoRI fragment of pAB1793 (Pearce and Sherman, 1998) with an EcoRI-digested 2-μ URA3 multicopy plasmid pRS426. To create the V5-tagged versions of pAB1793 and pmBTN1, the complete BTN1 ORF except the stop codon was amplified by PCR using the oligonucleotide primers 5’-ATGAGTGACAAATCTCATCA-3’ and 5’-TTCCATCTTACACCAAAG-3’ and inserted in frame into pYES2.1/V5-His-TOPO to produce a C-terminal V5-His BTN1. This plasmid was used as a template to amplify BTN1-V5 and the downstream region that includes the transcription termination signal for CYC1. For the amplification of this fragment we used the sense primers 5’-TTTTGGCTCCTTTTGGTTTG-3’ and the two antisense primers 5’-AAAAACTCGAGGTCACTGAGGAGGAAAGC-3’ and 5’-AAAAAAGCTTGTACAGGTAGGAGGAAAGC-3’, which generated Xhol and HindIII restriction sites, respectively. To generate pAB1793-V5, the obtained PCR fragment was double-digested with Nhel and Xhol and inserted in pAB1793 digested with the same restriction enzymes. We obtained pmBTN1-V5 by digestion of the PCR fragment with Nhel and HindIII and insertion in pmBTN1 digested with the same restriction enzymes.

Preparation of subcellular fractions

Yeast spheroplasts and crude mitochondrial fraction were isolated by published procedures (Gaigg et al., 1995; Glick and Pon, 1995). Mitochondria and MAM were purified using a sucrose gradient. Crude mitochondria were suspended in buffer A [0.6 M sorbitol, 10 mM K-MES [potassium 3-(N-morpholino)ethanesulfonic acid], pH 6.0], layered on top of a discontinuous gradient consisting of 5 ml 45% (w/v) sucrose and 5 ml 30% (w/v) sucrose in buffer A. After ultracentrifugation for 1 hour at 100,000 g, the purified mitochondria were collected from the 30-45% sucrose interface, diluted in buffer A and collected by centrifugation at 12,000 g for 15 minutes. A fraction containing MAMs was collected from the 0-30% sucrose interface, diluted in buffer A and centrifuged for 1 hour at 100,000 g. For further purification, the membranes were resuspended in buffer A with 2 mM EDTA and 1 mM DTT, adjusted to 50% (w/v) sucrose, loaded on the bottom of a flotation sucrose step gradient, and overlaid with 8 ml 20% (w/v) sucrose in buffer A and 2 ml of buffer A. After ultracentrifugation for 2 hours at 100,000 g, the MAM fraction was collected from the 0-20% sucrose interface, diluted in EM buffer (10 mM MOPS, pH 7.2, 2.5 mM EDTA) and subjected to 1 hour incubation at 30°C.
of centrifugation at 100,000 g. The resulting pellet was resuspended in EM buffer.

Yeast vacuoles were obtained by the method of Ohsumi and Anraku (1981) as previously described (Padilla-Lopez and Pearce, 2006).

Analysis of the vacuolar proteome by 2D blue-native/SDS-PAGE

2D blue-native/SDS-PAGE was performed using the NativePAGE Novex Bis-Tris gel system (Invitrogen). Briefly, 100 µg of isolated yeast vacuoles was pelleted by centrifugation at 20,000 g for 30 minutes and resuspended in NativePAGE sample buffer containing 0.5% digitonin, 0.5% n-dodecyl-β-D-maltoside (DDM) and 0.125% Coomasie Blue G-250. For first-dimensional blue-native gel electrophoresis, the solubilized samples were loaded onto a 4-16% Bis-Tris gel and run at 4°C for 1 hour at 150 V followed by 1 hour at 250 V. Individual lanes were then excised from the gel and incubated in SDS buffer (125 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 100 mM DTT) for 30 minutes. The gel strips were then placed horizontally onto 8-16% SDS-PAGE gels, sealed into place with molten agarose and run for 1 hour at 200 V at 10°C until the blue dye front had migrated from the bottom. The separated proteins were then transferred to nitrocellulose membranes and subjected to western blotting. Western blotting was performed using monoclonal antibodies against Vma1p (8B1), Vma2p (13D11) and Vph1p (10D7) from Molecular Probes and V5 epitope antibody from Invitrogen, followed by a horse-radish-peroxidase-conjugated secondary antibody. The obtained blots were analyzed by densitometry using the software Gel-Pro analyzer v. 3.1.

Phospholipid analysis

Total cellular (1 mg of protein) and purified membrane (250 µg protein) fractions were extracted by vigorous mixing with 2 ml chloroform:methanol (2:1, v/v). After 5 minutes centrifugation at 3000 g, the aqueous upper phase was discarded and the lower organic phase was washed three times with 3 ml of 10% methanol solution. The final lipid-containing chloroform phase was evaporated and the dried lipids were resuspended in 20 µl chloroform:methanol and then loaded on silica gel H TLC plates (Analtech). The plates were developed using a solvent system containing chloroform, methanol, 2-propanol, 0.25% aqueous KCl, triethylamine (30:9:5:6:18, v/v/v/v/v). Individual phospholipids were identified under ultraviolet light with authentic standards, by spraying the thin layer plates with 0.1% aqueous 8-anilino-1-naphthalenesulfonic acid. The lipid spots were scraped and quantified using the method of Rouser et al. (Rouser et al., 1966).

NBD-PtdSer transport assays

For the analysis of PtdSer transport to either mitochondria or vacuole, yeast cells lacking Psd1p were cultured in SC medium to logarithmic phase. Yeast protoplasts were generated by Zymoliase 20T incubation in SC media with 1.2 M sorbitol. Protoplasts were harvested by centrifugation, washed and resuspended in SC-1.2M sorbitol medium at 1 unit OD600/ml. Then, 5 µM NBD-PtdSer was added and cells were incubated at 30°C. 0.5 ml aliquots were taken at different time points, mixed with 0.5 ml of 5% SDS and disrupted by vortexing for 30 seconds. Lipids were extracted and resolved by TLC. Transport of PtdSer to the vacuole was determined by measuring the individual fluorescence associated to NBD-PtdEtn (λem=460 nm; λem=534 nm).

Yeast cell imaging

Cells were visualized under the 100× objective using an epifluorescent microscope (Olympus BX61, Melville, NY), a CoolSNAP HQ CCD camera (Photometrics, Tucson, AR) and IPLab 4.0 acquisition software (BD Biosciences, Rockville, MD). Image deconvolution was performed using the AutoDeblur software package (Media Cybernetics, Bethesda, MD), and overlays of fluorescent images were performed using Image J (NIH) and Photoshop software.

TRANSLATIONAL IMPACT

Clinical issue

Batten disease, the juvenile form of a group of disorders called neuronal ceroid lipofuscinoses (NCLs), is the most common neurodegenerative disease of childhood, with an incidence of one in 40,000 live births. Children with Batten disease are typically blind by age 7-8 years and suffer a decline in cognitive and motor function, as well as an increasing frequency of seizures, as the disease progresses. Other neurological complications can include schizophrenia, parkinsonism and behavioral problems. There is no cure or treatment, and sufferers commonly die blind, demented and bedridden in their late teens or early twenties.

A hallmark of Batten disease (and of all NCLs) is a lysosomal storage defect involving build up of lipofuscin (autofluorescent pigment granules composed of lipid-containing residues from lysosomal digestion) in the body’s tissues. In line with this phenotype, patients with the disease generally have a defect the CLN3 protein, which localizes to the lysosomal membrane, although other subcellular locations have also been reported. The exact function of CLN3, and how its dysfunction causes Batten disease, is unclear.

Results

In this paper, the authors investigate the potential involvement of BTN1, the yeast homolog of CLN3, in the distribution of membrane phospholipids among different organelles. They show that lack of btn1 (btn1Δ) leads to changes in the phospholipid profile that are even more pronounced in psd1Δ cells [which have reduced levels of the essential lipid phosphatidylethanolamine (PtdEtn)]. Specifically, btn1Δ cells have defects in the transport of phosphatidylinerine (a precursor of PtdEtn) from endoplasmic reticulum membranes to mitochondria, as well as to endosomal and vacuolar membranes. Moreover, the Kennedy pathway, which synthesizes PtdEtn, is also impaired when Btn1p is lacking.

Implications and future directions

These findings suggest an important role for BTN1 – and by extension its human homolog CNL3 – in the transport of lipids among cellular membranes. Altered lipid distribution among cell membranes could lead to their accumulation in lysosomes, impairing the normal function of these organelles and thereby triggering pleiotropic cellular defects that might collectively contribute to Batten disease. These findings will help to guide further work on the function of Btn1p/CLN3, and suggest that additional studies of intracellular lipid homeostasis in Batten disease are warranted.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.008490/-/DC1

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