**Batten disease: evaluation of CLN3 mutations on protein localization and function**

Ronald E. Haskell¹, Carrie J. Carr³, David A. Pearce³, Michael J. Bennett⁴ and Beverly L. Davidson¹,²⁺

Program in Gene Therapy, Departments of ¹Internal Medicine and ²Neurology, University of Iowa, Iowa City, IA 52242, USA, ³Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA and ⁴Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX 75235, USA

Received 25 October 1999; Revised and Accepted 18 January 2000

Juvenile neuronal ceroid lipofuscinosis (JNCL), Batten disease, is an autosomal recessive lysosomal storage disease associated with mutations in CLN3. CLN3 has no known homology to other proteins and a function has not yet been described. The predominant mutation in CLN3 is a 1.02 kb genomic deletion that accounts for nearly 85% of the disease alleles. In this mutation, truncation of the protein by a premature stop codon results in the classical phenotype. Additional missense and nonsense mutations have been described. Some missense substitutions result in a protracted phenotype, with delays in the onset of classical clinical features, whereas others lead to classical JNCL. In this study, we examined the effect of naturally occurring point mutations on the intracellular localization of CLN3 and their ability to complement the CLN3-deficient yeast, *btn1-Δ*. We also examined a putative farnesylation motif thought to be involved in CLN3 trafficking. All of the point mutations, like wild-type CLN3, were highly associated with lysosome-associated membrane protein II in non-neuronal cells and with synaptophysin in neuronal cell lines. In the yeast functional assay, point mutations correlating with a mild phenotype also demonstrated CLN3 activity, whereas the mutations associated with severe disease failed to restore CLN3 function completely. CLN3 with a mutation in the farnesylation motif trafficked normally but was functionally impaired. These data suggest that these clinically relevant point mutations, causative of Batten disease, do not affect protein trafficking but rather exert their effects by impairing protein function.

**INTRODUCTION**

Juvenile neuronal ceroid lipofuscinosis (JNCL), or Batten disease, is caused by mutations in CLN3 (1). CLN3 is a hydrophobic protein of 438 amino acids containing 5–7 transmembrane domains. The amino acid sequence of CLN3 is highly conserved between vertebrates, *Caenorhabditis elegans* and yeast (2). Analysis of the primary structure did not show targeting signals or motifs that indicate function or intracellular location. The biochemical role of CLN3 currently is not known, although investigators have localized the protein to lysosomal compartments in mammalian cells (3) and the vacuole in yeast (4). Further studies indicated that in the CLN3-deficient yeast strain (*btn1-Δ*), vacuolar pH was decreased during the early phases of growth (5). Interestingly, human CLN3 can complement the yeast phenotype (6).

Approximately 85% of Batten disease cases result from a 1.02 kb deletion (exons 7–8) in which 217 bp of the mRNA are deleted. This results in a frameshift and premature termination of the remaining protein (1,7). Patients homozygous for this mutation have an onset of disease at ages 5–8, with blindness followed by seizures, progressive mental deterioration and death, generally by the third decade. Pathological findings in JNCL patients revealed accumulation of autofluorescent lysosomal storage material with a characteristic fingerprint profile when viewed by electron microscopy (8). The predominant storage material is ATP synthase subunit C (9).

Other mutations in CLN3 have been described (1,7,10) and include missense and nonsense mutations. Patients heterozygous for the predominant 1.02 kb deletion mutation and the described nonsense mutations follow a classical clinical course (7). Patients heterozygous for the 1.02 kb deletion and a mutation that causes loss of the last 15 amino acids also have classical JNCL (7). In contrast, compound heterozygous patients with the predominant deletion and a missense mutation may have a protracted clinical course characterized by visual loss, less severe seizures and/or modest cognitive and motor impairment (7,10). Earlier studies showed that the missense mutations L101P, L170P and E295K caused protracted JNCL (7). However, compound heterozygous patients that had the 1.02 kb deletion and mutations V330F or R334H presented with classical JNCL (7).

The 1.02 kb deletion mutation resulted in a protein that does not traffic out of the endoplasmic reticulum (ER) (11). Alterations to the wild-type protein by the addition of artificial epitopes to either the N- or C-terminus also affected appropriate trafficking of CLN3 (12). Tags on the N-terminus appeared to inhibit the ability

*To whom correspondence should be addressed. Tel: +1 319 353 5511; Fax: +1 319 353 5572; Email: beverly-davidson@uiowa.edu*
of CLN3 to fold properly and caused retention in the Golgi. Epitopes cloned onto the C-terminus were also shown to affect trafficking of the protein, possibly by masking sites important for post-translational modification (12). One site that could be sensitive to such a modification is the putative farnesylation motif, CQLS, located at the C-terminus. In support of this speculation, studies using synthetic peptides representative of the C-terminal region of CLN3, or GST–CLN3 fusion proteins, showed evidence for farnesylation in vitro (13,14).

In this study, we tested how a mutation in the CQLS sequence, in the context of the full-length protein, affected protein localization and function. In the absence of any known biological role for CLN3, we chose to take advantage of the yeast model to address the latter question. Similarly, we tested whether the identified missense mutations influenced either or both of these properties. cDNA sequences representing a mutated CQLS motif and many of the characterized human mutations were generated and cloned into mammalian and yeast expression vectors, and their localization and function tested. Our results show that the missense mutations studied do not inhibit correct localization within the cell, but they variably affect CLN3 function.

RESULTS

Evaluation of the CQLS motif

We first sought to confirm that our method of confocal analysis could clearly identify fluorescent signals arising from discrete compartments. Second, we confirmed that the CLN3 antibody, Q438, is specific for CLN3. Representative photomicrographs in Figure 1A show a positive control for overlapping compartments by analyzing A549 cells for lysosomal-associated membrane protein (LAMP) II localization. Cells were labeled with LAMP II antibody H4B4 and visualized using two secondary antibodies labeled with a fluorescein isothiocyanate (FITC) derivative (Alexa488) or L-rhodamine. Because both of these antibodies detect the same primary antibody, we expected a complete overlap of fluorescence (Fig. 1A). Cells were evaluated by using confocal microscopy and plotting the fluorescent profile along one pixel width of the Z-series. The synchronous profile plot along the merged Z-series clearly demonstrates the overlap of the two fluorophores. As a positive control for non-overlapping compartments, A549 cells were labeled with MitoTracker ROS, which labels mitochondria with rhodamine, followed by immunohistochemistry for LAMP II- and Alexa488-conjugated secondary antibodies. The photomicrographs (Fig. 1B) show distinctive staining patterns, with little overlap observed on merged images or the intensity plot through the Z-series.

To test the specificity of Q438 for CLN3, A549 cells were transfected with epitope-tagged CLN3. Although we previously showed that N- and C-terminal tagging disrupts CLN3 trafficking (12), the epitope tag allowed for testing of CLN3 antibody specificity because dual antibody staining on the same protein can be performed. After transfection with C-terminal, FLAG-tagged CLN3, A549 cells were incubated with antibodies to FLAG and CLN3 (Q438). The panels in Figure 1C

![Figure 1](image_url)

Figure 1. Confocal analysis for overlapping and non-overlapping fluorescent signals, and anti-CLN3 immunohistochemistry. (A) A549 cells were stained with anti-LAMP II and both rhodamine-conjugated goat anti-mouse (rhM) and Alexa488-conjugated goat anti-mouse (488αM). The relative intensity of both fluorescent signals was similar and the distribution of fluorescent peaks identical. (B) A549 cells were metabolically labeled with MitoTracker Ros (MTr) and then immunohistochemically stained for LAMP II using Alexa488-conjugated goat anti-mouse (488αM). (C) A549 cells were transfected with FLAG-tagged CLN3 and stained with anti-CLN3 (left) or anti-FLAG (right) antibodies. The merged middle panel shows complete overlap that is also evident on the representative Z-series (bottom). For (A), (B) and (C), the top row (1) shows individual fluorescence patterns of a single optical section acquired from the rhodamine (left) and Alexa488 (right) channels, obtained from the same field. The middle row (2) shows the merged image. The bottom panel (3) shows a stacked Z-series for rhodamine, Alexa488 and the merged image. The Z-series varied in thickness depending on the thickness of the individual cell being imaged. The plot shows the pixel intensity along the thin red line (arrow) taken through the merged Z-series. The x-axis is the distance in µm from the left side of the photomicrograph. Photomicrographs in (A)–(C) are representative images of cells from each transfection.
Figure 2. Human CLN3 missense mutants. Vectors expressing representative
missense mutations in CLN3 are shown in bold. New restriction sites are indicated and underlined. The deduced primer sets shown. The alterations from the wild-type CLN3 sequence are localized to vesicles containing LAMP I (Fig. 3A), as was shown in prior studies (3). We hypothesized that a signal in or near the C-terminus may be important in CLN3 trafficking beyond the ER, leading eventually to localization within lysosomal compartments. To test whether the putative farnesylation signal at the C-terminus was required for appropriate intracellular targeting, we used PCR site-directed mutagenesis to change the CQLS motif to SQLS (C435S, Fig. 2).

As shown above, and in prior studies (12), placing an epitope tag on the C-terminus of CLN3 leads to retention of CLN3 within the ER. We hypothesized that a signal in or near the C-terminus may be important in CLN3 trafficking beyond the ER, leading eventually to localization within lysosomal compartments. To test whether the putative farnesylation signal at the C-terminus was required for appropriate intracellular targeting, we used PCR site-directed mutagenesis to change the CQLS motif to SQLS (C435S, Fig. 2).

Plasmids expressing wild-type CLN3 and the C435S mutant were transfected into A549 cells. Co-localization of CLN3 and the missense mutants in a yeast strain lacking the predicted farnesylation motif was not required for proper targeting of CLN3 to the lysosome.

As antibodies directed against rat LAMP I and LAMP II were unavailable, and the CLN3 staining pattern in neurons was consistent with synaptic vesicle transport, we tested whether CLN3 co-localized with synaptophysin, a component of the presynaptic vesicle membrane (19). Transfected PC6-3 cells were stained using anti-CLN3 (rhodamine-labeled secondary antibody) and anti-synaptophysin (Alexa488-labeled secondary antibody). Co-localization of synaptophysin with each of the individual mutations or the wild-type protein along neuronal extensions and terminal growth cones (data not shown). As antibodies directed against rat LAMP I and LAMP II were unavailable, and the CLN3 staining pattern in neurons was consistent with synaptic vesicle transport, we tested whether CLN3 co-localized with synaptophysin, a component of the presynaptic vesicle membrane (19).

Analysis of naturally occurring CLN3 missense mutations

We next tested how point mutations known to cause classical (V330F and R334H) or protracted (L101P, L170P and E295K) JNCL influence localization of CLN3. Clones containing these mutations were generated (Fig. 2) and tested in A549 transfection experiments as described above. Regardless of whether the mutation was associated with a classical or a protracted JNCL phenotype, the naturally occurring missense mutations co-localized with LAMP I (Fig. 4) and LAMP II (Fig. 5), similarly to wild-type CLN3. As noted with wild-type CLN3, the mutant proteins, in general, demonstrated a higher degree of overlap with LAMP II than with LAMP I.

CLN3 co-localization with synaptophysin in neuronal cell lines

JNCL is predominantly a neuronal disease. To test whether subcellular targeting of the identified point mutations were similarly unaffected in the context of neuronal cells, we transfected PC6-3 cells (18) with wild-type and mutant forms of CLN3. Preliminary staining of neuronal cells transfected with wild-type CLN3 showed staining patterns consistent with vesicular transport to terminal growth cones (data not shown). As antibodies directed against rat LAMP I and LAMP II were unavailable, and the CLN3 staining pattern in neurons was consistent with synaptic vesicle transport, we tested whether CLN3 co-localized with synaptophysin, a component of the presynaptic vesicle membrane (19).

As antibodies directed against rat LAMP I and LAMP II were unavailable, and the CLN3 staining pattern in neurons was consistent with synaptic vesicle transport, we tested whether CLN3 co-localized with synaptophysin, a component of the presynaptic vesicle membrane (19). Transfected PC6-3 cells were stained using anti-CLN3 (rhodamine-labeled secondary antibody) and anti-synaptophysin (Alexa488-labeled secondary antibody). Co-localization of synaptophysin with each of the individual mutations or the wild-type protein along neuronal extensions and terminal growth cones (Fig. 6) was evaluated by confocal microscopy as in Figure 1. Synchronous red and green profiles indicated that the distribution of CLN3 and of the integral membrane protein synaptophysin was contained within the same vesicular compartment. Mutant forms of CLN3 showed co-localization with synaptophysin in all cases. In additional experiments examining co-staining for CLN3 and MitoTracker ROS, no co-localization was noted (data not shown). Together, our data show that CLN3 is an endosomal/lysosomal protein in non-neuronal cells and resides within synaptic vesicles in neuronal cells. The results also show that the missense mutations in CLN3 do not inhibit the ability of the encoded proteins to reach their appropriate compartment.

CLN3 rescue of the yeast strain btm1Δ

The lack of any overt mistargeting of mutant proteins raised the possibility that the resultant phenotype, classical or protracted, was mediated through a loss of function. We tested the function of CLN3 and the missense mutants in a yeast strain lacking the previously described (3), and also to vesicles containing LAMP II (Fig. 3A). We observed consistent localization with LAMP II vesicles, whereas some LAMP I vesicles did not contain detectable levels of CLN3, especially in areas near the Golgi. The distribution of the C435S mutant was indistinguishable from that of wild-type CLN3 (Fig. 3B). These data suggest that the predicted farnesylation motif was not required for proper targeting of CLN3 to the lysosome.

Materials and Methods

In this study, we performed immunocytochemistry to detect the localization of CLN3 in neuronal cells. We used primary antibodies directed against LAMP I or LAMP II to detect the lysosomal localization of CLN3. Additionally, we used secondary antibodies conjugated to Alexa488 to visualize the localization of the primary antibodies. Our data indicate that wild-type CLN3 localized to vesicles containing LAMP I (Fig. 3A), as was
yeast ortholog to human CLN3, btn1 (5,6). Prior work showed that the btn1-Δ strain could grow in medium containing D-(−)-threo-2-amino-1-(p-nitrophenyl)-1,3-propanediol (ANP). The ability of btn1-Δ to grow on ANP-containing medium was due to a decreased vacuolar pH (5). The alteration in vacuolar pH caused an up-regulation in plasma membrane H⁺-ATPase activity, which in turn led to acidification of the medium. The acidification detoxified ANP and allowed the yeast to grow (5).

We previously demonstrated that the ANP-resistant phenotype could be reversed completely by btnlp or wild-type human CLN3 (6). Here, we tested the various human CLN3 mutants for their ability to restore ANP sensitivity to the btn1-Δ strain.
Strains containing vector alone, or vectors expressing wild-type \textit{CLN3} or the \textit{CLN3} point mutants, were plated onto medium containing ANP, and a comparison of growth made (Table 1). \textit{Btn1-Δ} cells transfected with control vector (no insert) grew in the presence of ANP, whereas expression of wild-type \textit{CLN3} completely restored ANP sensitivity. As shown in Table 1, the mutants L170P and E295K did not grow in medium containing ANP, suggesting that these mutants functionally complement the yeast \textit{btn1p} deficiency. Intermediate growth of mutants L101P, V330F, R334H and C435S was noted. Therefore, in the case of the naturally occurring human mutations, the data strongly suggest that these proteins were localized properly but were functionally impaired.

\textbf{DISCUSSION}

Although \textit{CLN3} is ubiquitously expressed, the retina and neurons are severely affected in JNCL. The function of \textit{CLN3} is unclear at present and even the intracellular localization has been the subject of some debate. It has been localized variously to the mitochondria (20,21), lysosome (3,11), Golgi (22) and nucleus (15,23). Here we report that plasmids expressing wild-type \textit{CLN3} and clinically relevant \textit{CLN3} mutations transfected into non-neuronal cell lines produce \textit{CLN3} that co-localizes to LAMP I- and LAMP II-containing vesicles. LAMP I and LAMP II vesicles normally are associated with the endosomal–lysosomal pathway. LAMP II trafficks to the lysosome via the early endosome, and can accumulate at the cell surface (16,17).
In the neuronal cell line PC6-3, CLN3 co-localizes with synaptophysin, an integral membrane protein of the presynaptic vesicle. Interestingly, synaptophysin is also thought to traffick to the synaptic vesicle through an indirect route via the plasma membrane (24). Together, the data suggest that CLN3 could also traffick to early endosomes via the cell surface, to reside finally within the endosomal–lysosomal compartments.

Our previous experiments using green fluorescent protein (GFP) or FLAG C-terminal tagged CLN3 demonstrated that these fusion proteins did not traffick normally, leading to CLN3 accumulation within the ER (12). We hypothesized that the mistrafficking was due to a masking of the putative farnesylation motif in CLN3. The C435S mutation, which disrupts this motif, did not result in the retention of CLN3 within the ER or Golgi, suggesting that farnesylation of CLN3 probably is not required for traffickling to the endosomes, lysosomes or synaptic vesicles. However, yeast expressing the CLN3 C435S mutant grew in the presence of ANP, indicating that this mutation impaired function. Prior studies determined that a frameshift mutation after S423 (7) caused classical JNCL in humans, again illustrating the functional importance of the C-terminal region. A putative di-leucine lysosomal sorting motif and the putative farnesylation motif, CQLS, lie within this region. Although our results and the human data suggest that the C-terminus is critical for proper function, the direct effect of the 15 amino acid deletion is not known. Experiments to examine the effects of an analogous mutation in btn1p could confirm whether this motif is similarly important for btn1p function.

The mutations L101P and E295K occur within, or are very close to, two of the predicted transmembrane domains of CLN3.
Figure 6. Co-localization of wild-type and mutant CLN3 with synaptophysin in a neuronal cell line. PC6-3 cells were transfected with plasmids expressing wild-type CLN3 or one of the CLN3 missense mutations (Figure 2). After transfection, cells were differentiated with NGF. CLN3 immunoreactivity was examined after differentiation. The cells were stained with antibodies to CLN3 (red, top left panel for each mutant) and synaptophysin (green, top right panel for each mutant). In L101P, V330F and C435S, cells not expressing CLN3 are in the field and noted as positive for synaptophysin only. These non-CLN3-expressing cells are seen as green peaks only on the intensity profile (L101P, 30–35 µm; V330F, 10–12 µm; C435S, 40 µm). Representative photomicrographs are shown, with descriptions of the middle (2), bottom (3) and profile plots within each grouping as described in the legend to Figure 1.
Table 1. Wild-type or mutant CLN3: complementation of the btn1-∆ phenotype in yeast by CLN3-expressing plasmids, and the corresponding human phenotype

<table>
<thead>
<tr>
<th>Human CLN3 amino acid sequence</th>
<th>btn1-∆ growth in ANP</th>
<th>JNCL patientsa (no. of classical, protracted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>++ + + + +</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>L101P</td>
<td>+</td>
<td>0 1</td>
</tr>
<tr>
<td>L170P</td>
<td>0</td>
<td>1 1</td>
</tr>
<tr>
<td>E295K</td>
<td>0</td>
<td>0 1</td>
</tr>
<tr>
<td>V330F</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>R334H</td>
<td>+</td>
<td>7 4 1</td>
</tr>
<tr>
<td>C435S</td>
<td>+</td>
<td>Not known</td>
</tr>
</tbody>
</table>

aData adapted from Munroe et al. (7).
bBtn1p-deficient yeast (btn1-∆) were transfected with vector alone, allowing growth in the presence of ANP.
cHuman CLN3 can complement the btn1p deficiency, blocking growth in ANP. A value of 0 represents no growth and hence wild-type CLN3 function.
dCombined for mutations V330F, R334H and R334C.

(21). Transmembrane domains in synaptophysin and connexin32 are required for correct targeting to the presynaptic vesicle or gap junction, respectively (25). However, L101P and E295K mutations failed to alter localization. A recent study by Jarvela et al. (11) also demonstrated that the missense mutation E295K was virtually indistinguishable from wild-type CLN3 in both neuronal and non-neuronal cells. In our yeast assay, L101P showed functional impairment, whereas the E295K mutant remained indistinguishable from wild-type CLN3 or btn1p (data not shown). Prior studies of the yeast btn1p mutants L44P and E243K, which are analogous to L101P and E295K, respectively, support our findings. The data also corroborate the disease severity of JNCL cases (6,7). Both mutations cause protracted JNCL, with the E295K mutation inducing a less severe phenotype than L101P (7,10).

The mutation L170P affects an amino acid located within the vesicle lumen (B.J. Foster and B.L. Davidson, unpublished data), thus removed from the cytosolic machinery involved in transport of vesicles. Our data show that the human CLN3 mutant L170P (this report) and the corresponding btn1p mutant L112P (6) can fully complement the btn1-∆ phenotype, indicating that these mutations do not grossly impair CLN3 function. The difference in phenotype tagging the N-terminus of CLN3 disrupted trafficking (12). Tags on the N-terminus also lead to the exposure of an immunoreactive epitope that was masked in the native protein (12). Thus, the protracted form of JNCL caused by the L170P mutation may be due to the inability of the N-terminus to interact correctly with other regions of CLN3 or with other proteins. Alternatively, modest differences in protein sorting, not detectable using these assays, could result in the delayed-onset, mildly progressive form of JNCL reported for the L170P mutation.

It has been reported previously that patients harboring the V330F or R334H mutation follow a classical clinical course, with only one exception (7). Our data show that CLN3 harboring these mutations localizes to the same intracellular compartment as does the wild-type protein. Plasmids expressing V330F or R334H CLN3 did not similarly restore function to btn1-∆ yeast. The ANP sensitivity of the human V330F mutation closely mimics the previously reported V289F mutation in btn1p (6). However, the btn1p variant R293H was less sensitive to ANP-containing medium than the human R334 variant (6). How analogous mutations in the yeast and human proteins differentially affect function is unclear at this time.

In classical JNCL, non-neuronal tissues remain relatively unaffected except for the accumulation of storage bodies. However, PET scans revealed that cells within the visual cortex had decreased metabolic activity (26). Clinical progression paralleled a spreading diminution in metabolic activity from the visual to the entire cortex. Interestingly, regions of the ganglia and brainstem were spared. The co-localization of CLN3 and each of the CLN3 mutations with the synaptic proteins synaptophysin (this report), and of E295K with synaptic vesicle protein 2 (11) indicates that CLN3 could be important for synaptic vesicle physiology within these regions. Although the btn1-∆ yeast provide an excellent model for examining the general effect of mutations within CLN3, they may be less useful for addressing how a lack of CLN3 affects regulated secretion in neurons.

In summary, each of the mutant CLN3 proteins localizes to the same intracellular compartment as wild-type CLN3. All of the mutations tested rescued the yeast phenotype to some degree, suggesting that they also traffic normally in yeast and complement btn1p function. These data demonstrate the utility of the btn1-∆ yeast model as an aid in assessing the impact of human CLN3 mutations. Finally, knowledge about the role of CLN3 or btn1p in synaptic vesicle or yeast vacuole physiology could be facilitated through the use of the mutant proteins described.

MATERIALS AND METHODS

Mutations and cloning

Production of mutations in the CLN3 cDNA, shown in Figure 2, was carried out using the Quick Change Mutagenesis kit (Stratagene, La Jolla, CA) essentially according to the manufacturer’s directions. All primers were designed to cause a single mutation at the primary amino acid level along with silent mutations to facilitate restriction analysis. Each clone was sequenced by the University of Iowa DNA Sequencing Facility.
CLN3 expression vectors are under the control of the Rous sarcoma virus promoter.

Transfections and cell culture

PC6-3 or A549 cells were transfected by electroporation (Bio-Rad, Hercules, CA) using CsCl-purified plasmids. PC6-3 cells, a derivative of the PC-12 cell line (18), were maintained in Dulbecco’s modified Eagle’s medium with 15% horse serum and 5% fetal calf serum, and A549 cells were cultured in 10% fetal calf serum in minimal essential medium. All media were supplemented with antibiotics. Cells were trypsinized, washed with ice-cold medium to remove and inactivate residual trypsin, and pelleted by centrifugation. The cell pellet was resuspended in cytomix (10 mM K2HPO4, 10 mM KH2PO4, 25 mM HEPES, 2 mM EGTA, 120 mM KC1, 0.15 mM CaCl2, 5 mM MgCl2, 2 mM ATP, 5 mM glutathione, pH 7.6, with KOH) and 300 µl of the cell suspension mixed with each plasmid. The cell DNA solution was electroporated at 360 mA and 960 µF in 4 mm gap cuvettes. A549 cells were cultured overnight. PC6-3 cells were cultured for 7 days in the presence of 50 µg/ml nerve growth factor (NGF; Sigma, St Louis, MO) on collagen-coated plates (Becton Dickson, Bedford, MA) to induce arborization and expression of synaptophysin.

Yeast expression studies

The cultivation, manipulation and transformation of yeast strains followed standard procedures (27,28). The bt11Δ deletion yeast strains are deficient in bt1p, the yeast ortholog of CLN3. The yeast expression plasmids used in this study were made by subcloning the CLN3 sequences from the mammalian expression vectors into a single-copy plasmid pAB1793 (CEN6 UR.A3), under control of the Gal4 promoter. Yeast media (YPD glucose medium) and YPG (glycerol medium) were prepared with and without ANP added after autoclaving. Growth was compared for bt1pΔ, bt11Δ and bt11Δ yeast strains containing pAB1793 only, wild-type CLN3 or the various plasmids encoding mutant CLN3.

Immunohistochemistry and microscopy

A549 cells were cultured as above and processed for immunofluorescence assays. Cells receiving 500 nM MitoTracker Ros (Molecular Probes, Eugene, OR) for 1 h at 37°C were washed twice with Hank’s balanced salt solution with Ca2+ and Mg2+ and then fixed with 3.7% paraformaldehyde (PFA) at 37°C for 15 min. Cells were then treated with 1:1 acetonemethanol for 5 min. Cells not labeled with MitoTracker Ros were washed once with phosphate-buffered saline (PBS) and then fixed with ice-cold 3.7% PFA for 5 min. All cells were blocked and permeabilized with 3% bovine serum albumin (BSA) and 0.1% saponin for 20 min. Primary antibody against CLN3, Q438, was generated by immunization of rabbits with the peptide RQFLRTEAPSFKPG (residues 250–264) (15). Primary antibodies Q438 (1:1000), LAMP I (H4A3, 1:2), LAMP II (H4B4, 1:2) (University of Iowa Tissue Culture Hybridoma Facility) or synaptophysin (1:150) (Sigma) were diluted in 0.1% BSA, 0.1% saponin in PBS (diluent). The secondary antibodies lissamine-rhodamine-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa488-conjugated goat anti-mouse (Molecular Probes) were used 1:200 in diluent.

CLN3 and CLN3 mutations were always stained with rhodamine secondary antibodies. Confocal microscopy was performed using a 100× oil-immersion objective on a Zeiss LSM 510, with images captured using associated software. A single en face optical section is shown in Figures 1 and 3–6 with a corresponding 0.6 µm wide Z-series. The merged Z-series was then evaluated for the distribution of fluorescent structures as an indicator of co-localization. The photomicrographs are representative of images seen in three or four independent experiments, with >25 cells examined per transfection.

ACKNOWLEDGEMENTS

The authors thank Dr Fred Sherman for support and Chad M. Stocker for excellent technical assistance. We are also indebted to the University of Iowa Hybridoma Facility, DNA Sequencing Core and Microscopy Core personnel. The Hybridoma and DNA sequencing facilities are supported in part by the Diabetes and Endocrinology Research Center. This work was funded in part by a grant from the Batten Disease Support and Research Association (B.L.D.), the NIH (R01 NS36610 to D.A.P.) and from support provided by the Roy J. Carver Trust (B.L.D).

REFERENCES


