Intracellular trafficking of CLN3, the protein underlying the childhood neurodegenerative disease, Batten disease

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Abstract Juvenile neuronal ceroid lipofuscinoses (Batten disease) is a progressive neurodegenerative disorder resulting from mutations in the *CLN3* gene, which encodes a hydrophobic 438 amino acid protein of unknown function. Prior studies have shown that CLN3 is expressed in multiple tissues, with highest levels in brain and testis. Experiments using cells overexpressing CLN3 indicate that CLN3 is a lysosomal resident protein. However, studies to date have not addressed trafficking of endogenous CLN3. As such, the purpose of the present study was twofold. First, to develop a culture model to allow evaluation of native CLN3 transport. Second, to utilize available epitope-specific antibodies to determine if CLN3 reaches the plasma membrane en route to the lysosome. Our data using a NCCIT (embryonic testicular carcinoma) cell model coupled with surface biotinylation and antibody trapping demonstrated that at least a proportion of CLN3 trafficks to the lysosome via the cell membrane. Moreover, inhibition of the μ3A subunit of the AP-3 adapter protein complex increased levels of CLN3 at the cell surface.

Key words: Batten disease; Protein trafficking; CLN3; Neuronal ceroid lipofuscinose

1. Introduction

The neuronal ceroid lipofuscinoses (NCLs) are inherited neurodegenerative disorders characterized by the accumulation of autofluorescent lipopigments in many cell types and notably neurons [1]. In the past, the NCLs were classified exclusively on the basis of the age of onset and clinical pathological features, for example the electron microscopy profile of the storage deposits in biopsy tissue. More recently, the identification of genes causative of these disorders has assisted with appropriate diagnoses [2–5].

Batten disease, the juvenile onset form of NCL (ceroid lipofuscinosis type III), is the most common of this class of lysosomal storage diseases. Clinical onset typically initiates with visual failure at approximately 4–6 years followed by seizures and progressive mental decline. Magnetic resonance imaging studies show progressive cerebral atrophy, and patients generally succumb by the second or third decade of life [6].

The Batten disease gene (*CLN3*) contains 15 exons and encodes a 1689 bp product [2]. Biochemical studies to assess the topology revealed an extremely hydrophobic 438 amino acid protein having five transmembrane-spanning domains [7]. The CLN3 transcript has no notable homology with other proteins [2] and is conserved amongst *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila*, mouse and dog, and other species.

Recent work on transfected or viral vector-infected cells showed that CLN3 is a resident lysosomal enzyme, co-localizing with lysosomal markers LAMP-1, LIMP II and cathepsin D in non-neuronal cells [8–10] and synaptophysin in neuronal cell lines [10]. Most patients with CLN3 deficiency harbor a 1.02 kb deletion on both alleles [2], with overexpressed mutant protein retained in the endoplasmic reticulum (ER) [9]. Interestingly, lesions representative of the most common point mutations did not grossly impair lysosomal targeting using yeast as a model system [10]. Although well-established that the loss of functional CLN3 leads to a lysosomal storage disease characterized by the accumulation of autofluorescent inclusions containing in part adenosine triphosphate (ATP) synthase subunit c [11], the mechanisms underlying neuronal degeneration as a consequence of the deficiency remain equivocal.

Following their transit through the Golgi complex, newly synthesized lysosomal membrane proteins reach late endosomes and/or lysosomes through direct transport from the *trans*-Golgi network via clathrin-coated vesicles, or indirect transport via the secretory pathway to the plasma membrane and endocytosis [12–16]. The cytoplasmic domains of transmembrane proteins contain tyrosine- and dileucine-based sorting motifs that allow adaptor protein complex (AP) binding, and appropriate segregation [17–19]. Both motifs are present in the carboxy-terminus of CLN3. In mammalian cells, AP-1 and AP-3 can mediate vesicle membrane protein (e.g. LAMP-1 and LIMP II) transport to endosomal and subsequently lysosomal compartments [1,12,16,20]. Additionally, lysosomal membrane proteins (e.g. LAMP-1) may traffic to late endosomes and lysosomes via AP-2-dependent endocytosis after first being transported to the plasma membrane [21].

The development of hypotheses regarding how mutations in CLN3 lead to disease would be aided substantially by understanding how native CLN3 reaches the lysosome. Initial studies on CLN3 trafficking have utilized overexpression systems because CLN3 is undetectable using available antibodies in most cell types. Here, we capitalized on earlier work by Lerner and colleagues demonstrating that CLN3 expression is

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highest in brain and testis [2] and found relatively robust levels of CLN3 protein in NCCIT cells, an embryonic testicular carcinoma cell line, relative to other cell lines tested. Our data reveal that CLN3 reaches the lysosome via the plasma membrane, and that plasma membrane localization can be enhanced by inhibition of AP-3.

2. Materials and methods

2.1. Materials

Only analytical grade reagents were used. Retinoic acid (RA) was purchased from Sigma (St. Louis, MO, USA). 30% (w/v) acrylamide, 1% (w/v) bisacrylamide solution was supplied from Bio-Rad Laboratories (Hercules, CA, USA). [^{32}P]deoxycytidine triphosphate (dCTP) (3000 Ci/mmol) was acquired from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

2.2. Cell culture

NCCIT (American Type Tissue Culture collection, Rockville, MD, USA) cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. RA (10^{-5} M) was added to the medium to induce differentiation [22].

2.3. Antibodies and absorption tests

Dr. M. Bennett (Southwestern Medical Center, University of Texas, Dallas, TX, USA) kindly provided the antibodies Q438 and Q516. The rabbit polyclonal antibodies Q516 and Q438 were generated against synthetic peptides of human CLN3 protein sequence corresponding to amino acids 2–18 (GGCAGSRRRFSDSEGEE; CLN3.1) and 251–265 (QPLIRTEAPESKPGS; CLN3.3), respectively.

Q438 and Q516 specificities were confirmed by absorption of sera with peptide-conjugated Sepharose beads. Peptides, 5 mg/ml, were brought up in 50 μl phosphate-buffered saline (PBS). 5 μl of normal horse serum (NHS)-activated Sepharose 4 fast flow (Pharmacia Biotech, Uppsala, Sweden) were washed with 50 μl ice-cold 1 mM HCl and diluted in 100 μl PBS. The washed beads and the peptide solution were mixed and allowed to bind for 4 h at room temperature while rotating end over end. Non-reacted groups were subsequently blocked by incubation in 1 ml 0.05 M Tris buffer pH 7.4 overnight. Beads were washed in 0.05 M Tris buffer pH 8 and 0.2 M acetate buffer pH 4, 150 μl each, for a total of six repetitions.

2.4. Immunohistochemistry

For ABC immunostaining, NCCIT cells were fixed with 2% paraformaldehyde in 0.1 M PBS pH 7.4 for 15 min at 4°C, and rinsed three times with ice-cold 0.01 M PBS pH 7.4. Cells were then incubated with antibodies diluted with PBS containing 3% bovine serum albumin (BSA) and 0.3% Triton X-100 overnight at 4°C. The antibody dilutions are as follows: Q438, 1:1000; Q516, 1:500; monoclonal antibody to human LAMP-1 (H4A3, from University of Iowa Tissue Culture Hybridoma Facility), 1:4; polyclonal antibody from rabbit to human mannose-6-phosphate receptor (M6PR) (kindly provided by Peter Lobel, Rutgers, NJ, USA), 1:1000. After rinsing three times with PBS, cells were incubated in 1:200 biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA) followed by incubation with ABC complex (Vector Laboratories, Inc., Burlingame, CA, USA). The complex was then reacted with diaminobenzidine (DAB) and H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) in the presence of nickel sulfate to give a dark blue precipitate according to the kit manual. Sections were then mounted with GelMount medium (Biomeda Corp, Foster City, CA, USA) and analyzed using a Leica DM RBE. Images were acquired with a SPOT RT camera and associated software (Diagnostic Instruments, Sterling Heights, MI, USA).

For immunofluorescence assay and confocal microscopy, NCCIT cells were fixed and incubated with Q438 and H4A3 as above. The secondary antibodies lissamine–rhodamine-conjugated goat anti-mouse and fluorescein-conjugated goat anti-rabbit (Jackson Immunoresearch Laboratories, West Grove, PA, USA) were used at 1:200 in diluent. Confocal microscopy was performed using a 40× objective on a Zeiss LSM 510, with images captured using associated software.

2.5. Northern blots

Total RNA from NCCIT cells was prepared by using Trizol® reagent (Gibco-BRL, Grand Island, NY, USA). A 534 bp probe specific for the 495–1029 region of human CLN3 was cloned into pMCG-16 by polymerase chain reaction (PCR) using a primer pair, 5′-AAAGAATTCATGGGGGAGTCACCTTCCTC-3′ and 5′-AAAGAATTCTCAACGGATGCGACAGCAGCG-3′. The probe was generated using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN, USA) according to the kit manual. Hybridizations were performed in Northern Max-Plus following the manufacturer's instructions (Ambion, Dallas, TX, USA).

2.6. Antibody uptake experiments

NCCIT cells were grown on plastic Petri dishes (60 mm) and treated with RA for 48 h. Q438 (1:1000) and Q516 (1:500) were then added to the culture medium. The cells were incubated at 37°C for 1, 2, or 6 h, then washed and processed for immunostaining, immunoprecipitation, or biotinylation. For immunostaining, the cells were fixed with 2% paraformaldehyde in 0.1 M PBS, pH 7.4 for 15 min at 4°C. Biotinylated goat anti-rabbit IgG diluted in PBS, 0.3% Triton X-100, was added for 1 h at 4°C, followed by incubation with ABC complex and reaction with DAB in the presence of nickel sulfate.

For immunoprecipitation, cells were washed and proteins extracted without phase separation. Briefly, extracts were prepared by incubating cells in ice-cold Tris-buffered saline (TBS) (10 mM Tris, pH. 7.4, 0.15 M NaCl) containing 1 mM ethylenediamine tetraacetic acid (EDTA,) 1% (vol/vol) Triton X-114, and protease inhibitors (see below) for 45 min on ice. Extracts were transferred to Eppendorf tubes and clarified by centrifugation at 14 000×*g* for 10 min at 4°C. Protein A-Sepharose was then added to the cell lysates for 2 h at 4°C. The immune complexes were pelleted, washed, eluted with lithium dodecyl sulfate (LDS) sample buffer, and subjected to LDS–polyacrylamide gel electrophoresis followed by Western blotting (see below).

2.7. Western blot

For Western analysis, all samples were diluted to 1 mg/ml protein in 1% LDS (Sigma-Aldrich, St Louis, MO, USA), 5% β-mercaptoethanol, 5% glycerol, 0.025% bromophenol blue, and 0.035 M Tris–HCl (pH 6.8) and incubated for 30 min at room temperature. Samples were subjected to LDS–PAGE on 12% gels and transferred to nitrocellulose. Nitrocellulose sheets were blocked with 5% non-fat dry milk for 1 h in PBS containing 0.1% (vol/vol) Tween 20. Primary antibodies Q438 (1:2000) or Q516 (1:1000) were added and incubated for 1 h at room temperature. Detection was enhanced by chemiluminescence using horseradish peroxidase conjugated to anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA).

2.8. Biotinylation and streptavidin precipitations

NCCIT cells were washed four times with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-C/M). Sulfo-NHS-biotin (Pierce, Rockford, IL, USA) 0.5 mg/ml in ice-cold PBS-C/M was added to the dishes for 30 min at 4°C. Cells were then washed once with serum-free media and three times with PBS-C/M [23] and extracted with phase separation as described below. Approximately 50 μl of immobilized streptavidin (6% cross-linked agarose, Pierce, Rockford, IL, USA) was added to 1 ml of cell lysate and allowed to bind overnight at 4°C while rotating end over end. After binding, immobilized streptavidin was recovered by centrifugation (14 000×*g* for 30 s) and washed (five times) with ice-cold TBS, pH 8.0, containing 1% (vol/vol) Triton X-100. The addition of 100 μl Laemmli sample buffer and three rounds of agitation and boiling (5 min each) eluted bound proteins. Fresh 2-mercaptoethanol was added after each boiling step to a final concentration of 5% (vol/vol) [23].

2.9. Phase separation

Washed NCCIT cells were extracted with ice-cold TBS (10 mM Tris, pH 7.4, 0.15 M NaCl) containing 1 mM EDTA, 1% (vol/vol) Triton X-114, protease inhibitors (20 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml papain, 176 μg/ml phenylmethylsulfonyl fluoride) at 2–5×10^7 cells/ml for 45 min on ice. Cell extracts were transferred to Eppendorf tubes and clarified by centrifugation (14 000×*g* for 10 min) at 4°C. To separate phases, the lysate was warmed to 32°C for 3 min and centrifuged at 10 000×*g* for 20 s at room temperature. The aqueous phase (upper) was removed and residual amphipathic proteins re-

extracted by the addition of 100 μl of 11% Triton X-114 followed by an additional phase separation. The detergent phase was also re-extracted by the addition of a 10-fold volume of TBS with 1 mM EDTA, protease inhibitors (see above) and 0.06% Triton X-114 [6,24].

2.10. Antisense experiments

Selective inhibition of the μ3A subunit of AP-3 was used essentially as described by Le Borgne et al. [12]. Briefly, phosphorothioate-modified oligodeoxynucleotides were synthesized (IDT, Santa Clara, CA, USA). The sequence sites selected were centered on the initiation ATG (ATG antisense) and on a non-overlapping site located immediately downstream (inner antisense), deduced from the human μ3A. A reversed ATG antisense was synthesized as the control oligonucleotide (reversed antisense). Cells were incubated for 48 h with 5 μM ATG antisense together with 5 μM inner antisense, 10 μM reversed antisense only, or left untreated. Fresh oligonucleotides were added every 24 h. Oligonucleotide sequences are as described in Le Borgne et al. [12].

3. Results

3.1. Native CLN3 expression in NCCIT cells

The study of CLN3 trafficking would be greatly aided by the availability of cell lines expressing immunologically detectable levels of CLN3. We screened numerous cell lines and found that the testicular cancer cell line, NCCIT, expressed significant levels of human CLN3 (Fig. 1A, top left panel). As was found previously with other integral lysosomal membrane proteins [25], the induction of differentiation by the addition of RA substantially increased CLN3 immunoreactivity (Fig. 1A). The most notable rise in expression was at 1 day post addition of RA with intense intracellular CLN3 immunoreactivity (Fig. 1A, top right panel). At later time points (studies to 14 days), CLN3 immunoreactivity was reduced and punctate, reflective of its known lysosomal localization in non-neuronal cells (Fig. 1B). The increase in CLN3 expression could be attributed in part to increased mRNA expression (Fig. 1C).

3.2. Antibody-induced capping of CLN3

The lumen of vesicular compartments along the biosynthetic secretory and endocytic pathways is topologically equivalent to the exterior of the cell. Recent experiments have shown that CLN3 is a type IIIB transmembrane protein containing five transmembrane domains [7]. As such, Q516, which recognizes amino acids 2–18, is directed to an extracellular or

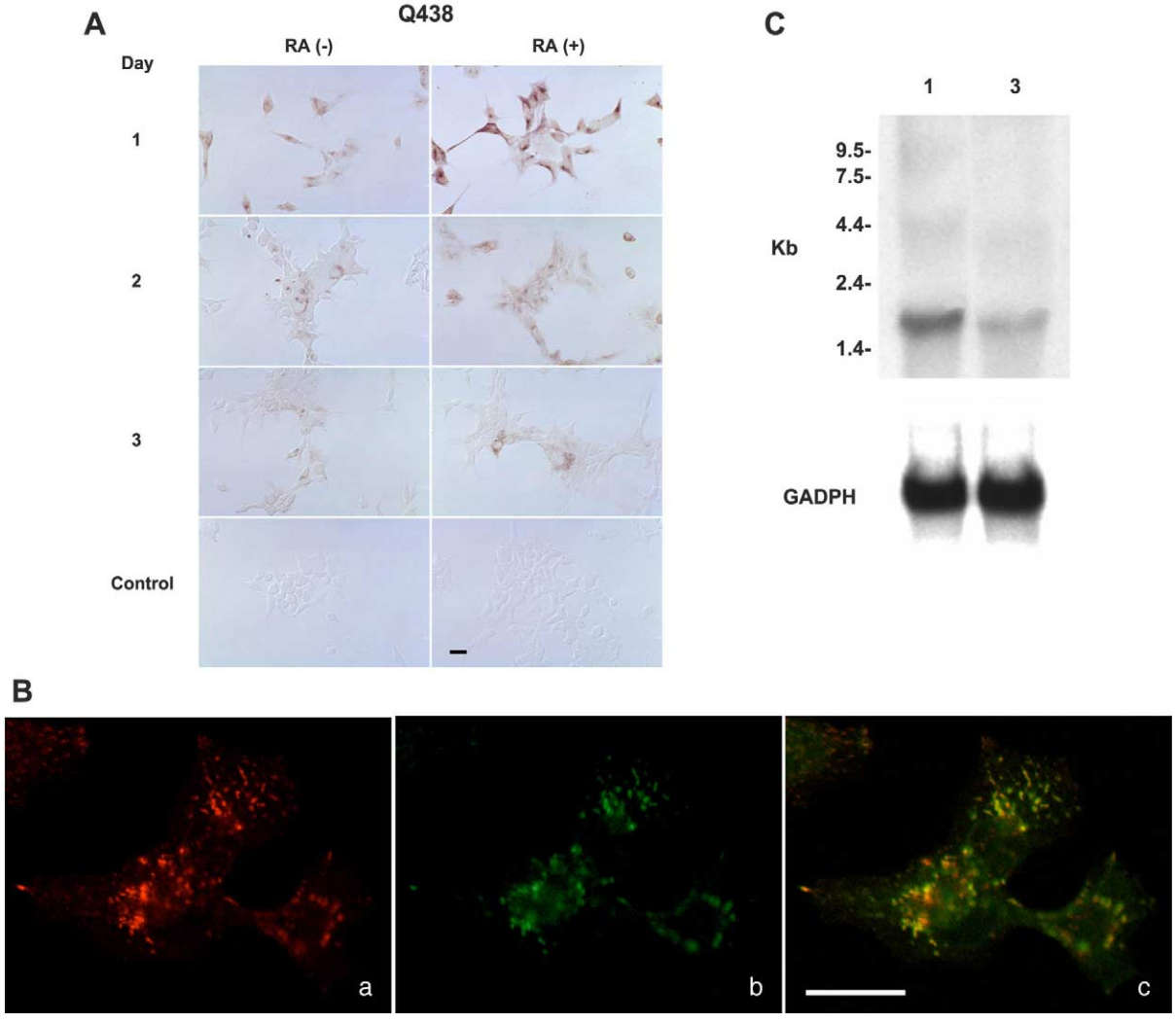

Fig. 1. CLN3 expression in RA-induced NCCIT cells. NCCIT cells were stained with anti-CLN3 antibody Q438 (A), 1, 2 and 3 days later following mock treatment (left panels) or induction with RA (right panels). Lower panels, anti-CLN3 antibody pre-absorbed to immunizing peptides. B: CLN3 co-localizes with LAMP-1. NCCIT cells were differentiated with RA for 3 days, and immunofluorescence with LAMP-1 (red, a) and Q438 (green, b) evaluated by confocal microscopy. c is the merged image. Bar, 25 μm. C: Northern blot for CLN3-specific or GAPDH-specific RNA in RA-induced NCCIT cells. RNA was prepared 1 or 3 days after RA treatment.

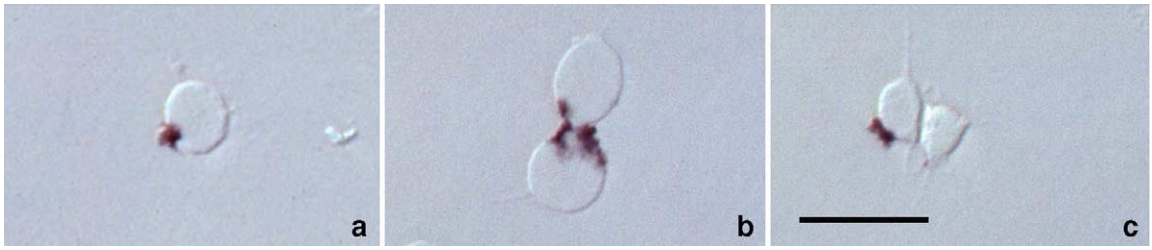

Fig. 2. Anti-CLN3 antibodies directed against an extracellular epitope of CLN3 induced capping on the surface of NCCIT cells. RA-induced NCCIT cells were incubated in media with the anti-CLN3 antibody Q516. After 6 h, cells were fixed incubated with biotinylated goat anti-rabbit IgG and developed using DAB substrate. a–c are representative. Bar, 25 μm. Similar results were seen in cells incubated with Q516 for 1 or 2 h (not shown).

intraluminal epitope. We took advantage of the Q516 epitope specificity to test if CLN3 is transported to the cell surface. RA-treated NCCIT cells were incubated with Q516 for 1, 2 or 6 h, after which the cells were fixed, permeabilized and stained with biotinylated goat anti-rabbit IgG. Capping of the antigen–antibody complex, as represented by the photomicrographs in Fig. 2, was evident in approximately 1–5% of cells at all time points (not shown and Fig. 2). Internalized antibody was also evident, although in a smaller percentage of the cells. When RA-treated NCCIT cells were incubated with the antibody Q438, which recognizes a cytoplasmic epitope, neither capping nor internalization was evident (data not shown).

Immunoprecipitation and surface biotinylation experiments confirmed that CLN3 trafficked to the cell surface after induction of differentiation with RA. RA-treated NCCIT cells were incubated with Q516 or Q438 for 1, 2 (not shown) or 6 h at 37°C, harvested, and cell extracts (see Section 2) reacted with protein G coupled to Sepharose beads. The immunocomplex was dissociated and electrophoresed through 12% LDS gels, blotted onto nitrocellulose, and incubated with Q438 to detect CLN3. Fig. 3A is representative of all time points, and shows that CLN3 could be immunoprecipitated when NCCIT cells were incubated with Q516 (lane 2) but not when incubated with buffer alone or Q438 (lanes 1 and 3, respectively). When NCCIT cells were surface biotinylated after Q516 incubation, the amount of streptavidin–biotin-linked CLN3 precipitate was greatly increased (Fig. 3B, compare lanes 1 and 2). Together, the data suggest that CLN3 is transported to the cell surface in RA-induced NCCIT, and that incubation with antibodies targeted to extracellular epitopes enhanced surface expression further. Our results also confirm the topology of CLN3 predicted from in vitro transcription/translation experiments [7].

3.3. The role of AP-3 in CLN3 trafficking

CLN3 is predominantly lysosomal in differentiated NCCIT cells, with very little surface staining evident (Fig. 1). To test if CLN3 trafficking to the lysosome occurs via an AP-3-dependent mechanism, NCCIT cells were cultured in antisense oligonucleotides targeted against the μ3A subunit of AP-3 using methods previously described by Le Borgne and colleagues [12]. In Fig. 4, representative photomicrographs demonstrate that incubation of NCCIT cells in AP-3 antisense oligonucleotides caused increased surface expression of CLN3 and LAMP-1 (b and e), but not the M6PR (h), a receptor known to use AP-1-dependent pathways [13,14]. Western blotting and immunostaining confirmed inhibition in μ3A subunit expression (data not shown). Incubation of cells in control oligonucleotides (reverse antisense) did not change the extent of surface expression (Fig. 4c, f, and i). When cells treated with AP-3 antisense oligonucleotides were subsequently incubated with antibodies directed against extracellular epitopes, internalization of the antibody was evident when directed against CLN3 (Fig. 5b) or LAMP-I (e). Incubation with control oligonucleotides (Fig. 5c and f, for CLN3 and LAMP-1, respectively) was indistinguishable from untreated cells (Fig. 5a and d). As expected, the presence or absence of AP-3 antisense oligonucleotides did not affect M6PR immunoreactivity.

To confirm the increased surface expression of CLN3 in the presence of antisense AP-3 oligonucleotides, surface biotinylation studies were done. NCCIT cells were cultured in either AP-3 antisense or control oligonucleotides for 48 h followed by biotinylation and extraction of the detergent soluble fraction (see Section 2). Biotinylated proteins were immunoprecipitated with immobilized streptavidin followed by fractionation by LDS–PAGE gels and blotting to nitrocellulose. Blots were probed with the anti-CLN3 antibody Q438. As shown in Fig. 6, treatment of NCCIT cells with AP-3 antisense oligonucleotides led to substantial increases in surface biotinylated

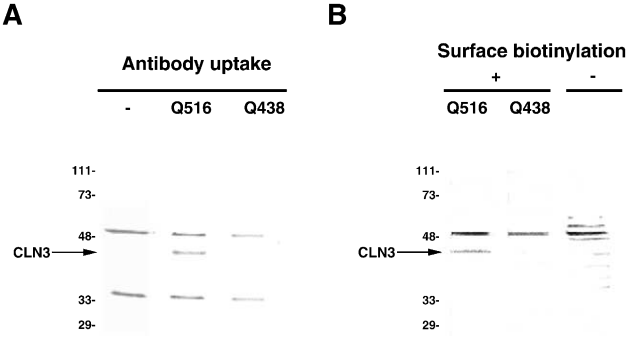

Fig. 3. CLN3 trafficks to the cell surface. A: Immunoprecipitation of internalized antibody. RA-induced NCCIT cells were left in media alone (lane 1) or incubated in Q516 (lane 2) or Q438 (lane 3). Cell extracts were prepared as described in Section 2, and CLN3 immunoprecipitated with protein A. The immune complexes were resolved by LDS–PAGE, blotted onto membranes, and CLN3 visualized by immunoblotting (arrow). B: Surface biotinylation reagents have access to CLN3 in NCCIT cells incubated with Q516, but not Q438. RA-induced NCCIT cells were incubated in Q516 (lane 1) or Q438 (lane 2), after which cell surface proteins were biotinylated (lanes 1 and 2) or left untreated (lane 3). Biotinylated proteins were isolated by incubation with streptavidin–agarose. The complex was disrupted and fractionated by LDS–PAGE, and immunoblotted with Q438.

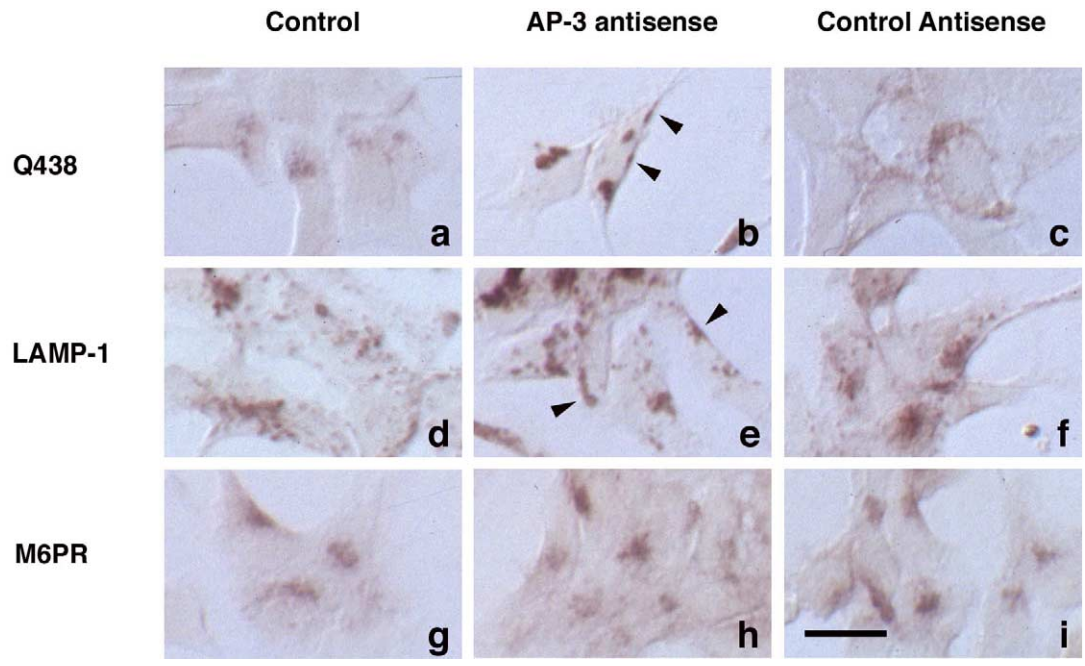

Fig. 4. Distribution of CLN3, LAMP-1 and MPR in μ3A antisense oligonucleotide-treated NCCIT cells. NCCIT cells were grown in media alone (a, d, and g), or in the presence of antisense (b, e, and h) or control (c, f, and i) oligonucleotides as previously described by Le Borgne and colleagues [12]. Representative photomicrographs of cells fixed, permeabilized and stained for CLN3 (Q516 Ab; a–c), LAMP-1 (d–f) or M6PR (g–i) are shown. Arrowheads depict apparent surface staining. Bar, 25 μm.

CLN3 (lane 2) while untreated (lane 1) or control oligonucleotide-treated cells (lane 3) did not. Together, the data support a role for AP-3 in trafficking of endogenous CLN3 in differentiated NCCIT cells.

4. Discussion

Three central findings emerged from these studies. First, NCCIT cells provide a valuable tool for evaluating endoge-

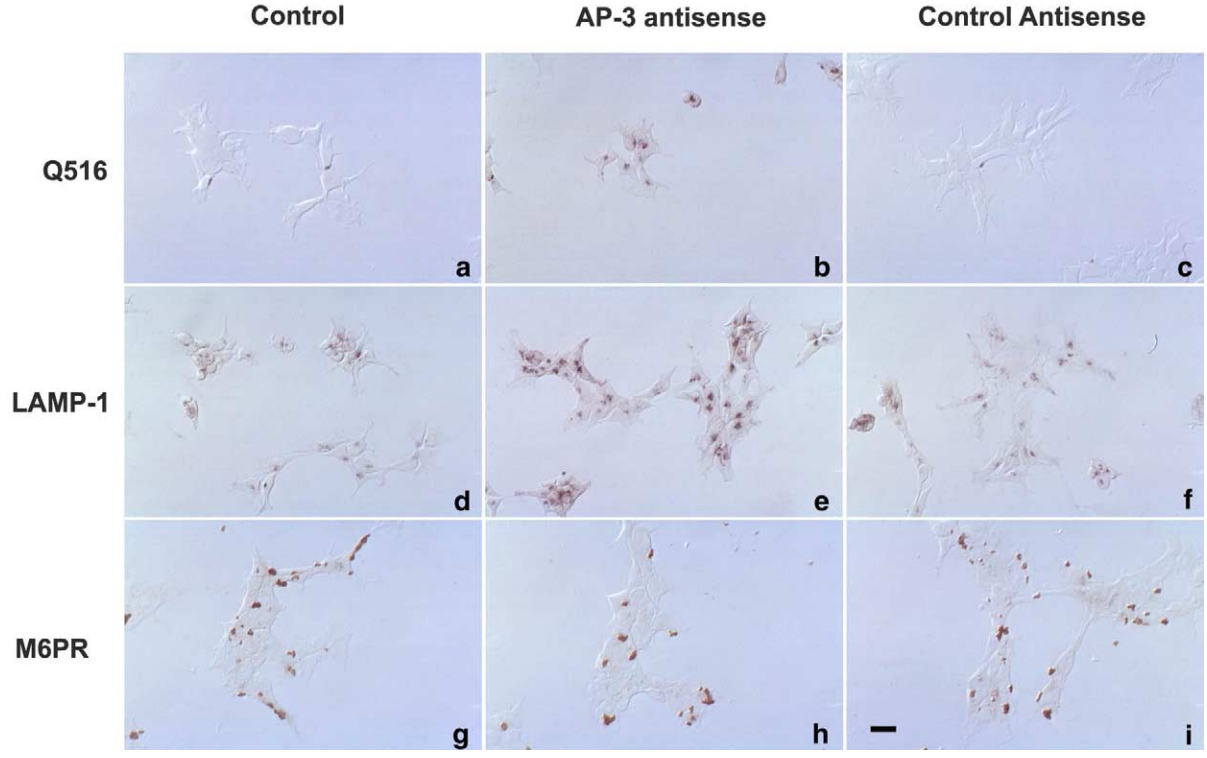

Fig. 5. Uptake of exogenous antibodies by NCCIT cells. NCCIT cells were grown in media alone (a, d, and g), or in the presence of μ3A antisense (b, e, and h) or control (c, f, and i) oligonucleotides. Antibodies to CLN3 (Q516), LAMP-1, or the M6PR were added to the culture medium 48 h later for an additional 6 h. The cells were washed, fixed and permeabilized, and the internalized antibodies subsequently detected with biotinylated goat anti-rabbit IgG as described (Section 2). Bar, 25 μm.

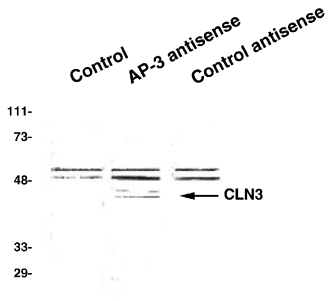

Fig. 6. Incubation in μ3A antisense oligonucleotides increased the accessibility of CLN3 to surface biotinylation. NCCIT cells were incubated in media alone (lane 1), μ3A antisense (lane 2) or control (lane 3) oligonucleotides for 48 h, after which cells were subjected to surface biotinylation. Biotinylated proteins were isolated from extracts (detergent soluble) by incubation with immobilized streptavidin, after which complexes were disassociated, fractionated by LDS–PAGE and immunoblotted. CLN3 was visualized by incubation of blots with Q438 antibodies.

nous CLN3 activity, expression, and trafficking. When NCCIT cells were induced to differentiate, CLN3-specific mRNA increased, as did the level of immunoreactive protein. Second, a proportion of CLN3 trafficked to intracellular compartments via the plasma membrane. Third, data using antisense oligonucleotides directed against the μ3A subunit of AP-3 indicate that trafficking of CLN3 in NCCIT cells occurs in part through AP-3-dependent mechanisms.

Studies using virally infected or transfected cells with CLN3 expression driven from strong viral promoters showed that CLN3 is lysosomal in non-neuronal cell lines [8–10]. However, no prior work has addressed how CLN3 is reaching the lysosome. NCCIT cells are developmentally pluripotent and can differentiate into derivatives of all three embryonic germ layers (i.e. ectoderm, mesoderm, and endoderm), and RA induction leads to vimentin-positive cells (5–20%), glial fibrillary acid protein (GFAP)-positive cells (10–30%) and neurofilament-positive cells (5%) [22]. The increase in CLN3 immunoreactivity in only 5% of this pluripotent line after RA induction reflects that CLN3 expression is higher in some differentiated cell types relative to others. Indeed Lerner and colleagues showed that CLN3 mRNA levels are not uniform amongst various tissues [2]. In studies in developing rodents, CLN3 immunoreactivity is highest in developing peripheral nervous system (PNS) and central nervous system (CNS) (Q. Mao and B.L. Davidson, unpublished results). It will be interesting to study further the phenotype of NCCIT cells induced to express higher levels of CLN3.

NCCIT cells show properties similar to F-9 embryonic carcinoma cells [25] as regards to the effects of RA on lysosomal membrane protein levels. In both cell lines, RA induction leads to increased expression of LAMP-1 and LAMP-2 [25] in only a proportion of cells, and detectable levels of LAMP-1 at the cell surface [25]. We also noted detectable levels of CLN3 on the cell surface of a proportion of NCCIT cells after RA induction by immunohistochemistry (Fig. 1), while only punctate intracellular staining was evident in non-induced cells.

The addition of antibody specific for an extracellular epitope of CLN3 induced very large aggregates of CLN3 at the cell surface (capping). Capping was not noted upon addition of antibody directed against residues 251–265. This result corroborated other work in our laboratory that showed the 251–256 region of CLN3 is cytoplasmic, making it inaccessible to extracellularly applied antibodies specific for this epitope [7]. Together with the surface biotinylation studies, these data support the idea that CLN3 in RA-induced NCCIT cells is transported to the cell surface.

An unusual consequence of deficiency of CLN3, a protein present in many cell types within multiple organ systems, is neurodegeneration characterized by loss of vision, epilepsy and progressive dementia. This highly hydrophobic protein is present in greatest amount in brain and testis [2]. In cell lines CLN3 has been shown to co-localize with LIMP II and cathepsin D in late endosomes/lysosomes at the electron microscopy level, and with LAMP-1 and LAMP-2 using confocal microscopy [9,10]. How is CLN3 transported to these compartments? Could it be dileucine, leucine/isoleucine pairs, or tyrosine-based motifs? Tyrosine-based sequences generally are YXXΦ (tyrosine, any amino acids, bulky hydrophobic amino acids), and are important for interaction with the adaptor protein subunits μ1, μ2, and μ3, of AP-1 through -3, respectively, for appropriate intracellular trafficking of vesicle membrane proteins [2,13,26]. Evaluation of the carboxy-terminus of CLN3 reveals dileucine and leucine/isoleucine pairs, and three tyrosine-based motifs, namely IYLVF, AYVNT, and LYEGL. The IYLVF motif in CLN3 lies in a highly hydrophobic region and is likely embedded within the membrane [7]. The remaining two motifs are in the predicted cytoplasmic COOH-terminus. The AYVNT motif may not be a true tyrosine-based motif, since there is considerable preference for highly hydrophobic amino acids such as phenylalanine, leucine, methionine, valine, or isoleucine at the Y+3 position; threonine is only weakly hydrophobic. Interestingly, the LYEGL motif is 100% homologous to the internalization motif in hIGAα [17]. Further studies to test the role of the LYEGL motif in CLN3 trafficking are warranted.

The LL or LI pairs in CLN3 are unlikely candidates for interactions with adaptor complexes. LL or LI pairs alone are not sufficient for internalization [27]. Earlier work by Höning showed that AP-3-dependent internalization by LL or LI was dependent on a similar pair of acidic residues DE or EE in the −4 and −5 position [27]. In contrast, the amino acids in the −4 and −5 positions for the LL or LI motifs in CLN3 are isoleucine, leucine, or serine.

Together, our data are consistent with AP-3's role in recruitment of lysosomal membrane proteins from endosomal compartments [28]. CLN3's localization on both late endosomes and lysosomes, coupled with the surface biotinylation and antibody capping experiments, suggests that a proportion of CLN3 trafficks to the plasma membrane, and that some AP-3-dependent sorting to the lysosome occurs. Moreover, we showed that disruption of the ubiquitous μ3A subunit of the AP-3 complex using previously described antisense approaches [12] caused increased routing of CLN3 to the plasma membrane. LAMP-1 was similarly misrouted while M6PR was not. Prior work by Dell'Angelica et al. demonstrated the requirement of the human β3A subunit of the AP-3 complex in trafficking of lysosomal membrane proteins, and showed that mutations in human β3A cause Hermansky–Pudlack syndrome [28].

Recent studies in yeast have shown that a deletion of *btn1* (yeast homolog of CLN3) leads to increased expression of btn*2*, a distant homolog of the cytoplasmic protein hook1

[29]. In *Drosophila*, hook1 is important in the internalization of the transmembrane protein boss (bride of sevenless), a ligand for the receptor tyrosine kinase sevenless on adjacent cells [30,31]. Although we may speculate that CLN3 expression on the plasma membrane may lead to its specific interaction with adjacent cells, there is little evidence to support this idea. A second possibility for the increased expression of btn2 in btn1Δ yeast is that vesicular transport in general is disrupted. Over time, impaired vesicular transport could lead to alterations in transport proteins secondarily as well as accumulation of storage material. The phenotype of CLN3 deficiency in humans supports this hypothesis, as the onset of disease is delayed until well after the PNS and CNS have formed. Thus, how an absence of CLN3, in the case of the 1.02 kb deletion, or reduced function, in the case of other identified point mutations [10] causes the recessive neurodegenerative disease with juvenile onset remains paradoxical. Our results, along with other studies on CLN3 biology, may help resolve this problem.

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