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Lysosomal localization of the neuronal ceroid lipofuscinosis CLN5 protein

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The Finnish variant late infantile neuronal ceroid lipofuscinosis (vLINCL) belongs to the neuronal ceroid lipofuscinosis group of common recessively inherited neurodegenerative disorders. The *CLN5* gene responsible for this brain disorder codes for a novel protein with no homology to previously reported proteins. In this study, we have investigated the biosynthesis and intracellular localization of this protein in transiently transfected BHK-21 cells using a CLN5-specific peptide antibody. Confocal immunofluorescence microscopy showed that wild-type CLN5 is predominantly targeted to lysosomes and immunoprecipitation analysis recognized a 60 kDa polypeptide. The molecular weight of this protein was reduced to 40 kDa by deglycosylation with Endo H and to 38 kDa with PNGase F. The same-sized glycosylated polypeptides were also observed in the media, suggesting that the 60 kDa glycosylated CLN5 polypeptide represents a soluble lysosomal glycoprotein, not an integral transmembrane protein as predicted earlier. The most common human vLINCL mutation blocked the lysosomal targeting of expressed polypeptides. This would imply that the pathogenesis of vLINCL would be associated with the defective lysosomal trafficking, preventing the normal biological function of the corresponding polypeptide.

INTRODUCTION

Neuronal ceroid lipofuscinoses (NCLs) are a group of common recessively inherited neurodegenerative disorders of childhood (1). The term NCL was proposed by Zeman to define disorders characterized by the accumulation of autofluorescent lipopigment storage material in various tissues (2). All types of NCL diseases cause progressive visual and mental decline, motor disturbances, epilepsy and behavioral changes, and lead to premature death. Based on the clinical course of the disease, the childhood forms have traditionally been divided into three main subtypes: infantile NCL (INCL; locus definition CLN1; MIM256730), classical late infantile NCL (LINCL; CLN2; MIM204500) and juvenile NCL (JNCL; CLN3; MIM204200). One of the subtypes, Finnish variant late infantile NCL (vLINCL; CLN5; MIM256731) has its clinical onset at 2–7 years of age. The first symptom is motor clumsiness, followed by progressive visual failure, mental and motor deterioration, and later by myoclonus and seizures. The age at death varies from 14 to 36 years (3–5). Cerebellar atrophy is the most striking abnormality in brain imaging studies (6) and in autopsy specimens (7).

The defective genes behind six human NCL diseases are known. Two NCL genes encode the following soluble lysosomal enzymes: palmitoyl protein thioesterase 1 (PPT1), defective in CLN1 (8–10), and tripeptidyl peptidase (TPP1), defective in CLN2 (11). Recent evidence indicates that in neurons, PPT1 is localized in synaptosomes and synaptic vesicles (12,13). The CLN3 gene was identified in 1995 (14). The CLN3 protein is an integral transmembrane protein, which may play a role in the regulation of vacuolar pH (15). In addition to lysosomes (16), several other intracellular localizations have been proposed for CLN3 (17–19). In neurons, the protein has been shown to be transported along the neuronal extensions and to be targeted to neuronal synapses (20,21). The CLN8 gene encodes a membrane protein with unknown function (22). It has been shown to be transported between the endoplasmic reticulum (ER) and ER–Golgi intermediate compartment (23). The CLN6 gene was cloned very recently, and is predicted to encode a novel transmembrane protein with unknown function (24,25). The sixth known NCL gene is CLN5, and the predicted amino acid sequence shows no homology to previously reported proteins (26). In a recent study, CLN5 was shown to be expressed in embryonic human

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brain at the beginning of cortical neurogenesis, and its expression was increased as cortical development proceeded (27). To date, four disease mutations have been described (5,26), of which three result in premature termination of the polypeptide chain. The most common mutation among Finnish CLN5 patients is a 2 bp deletion, del(AT)2467-2468 (called FIN_M below), resulting in Tyr392Stop. Another disease mutation found among Finnish patients is G1517A, leading to a very truncated polypeptide (Trp75Stop). The SWE mutation, ins(C)1961, was found in one Swedish and one Finnish CLN5 patient, both being compound heterozygotes for the mutation. The fourth CLN5 mutation, G2127A, was found in a Dutch family, and results in an amino acid substitution of Asp279Asn.

Here we have studied the biosynthesis, post-translational processing and intracellular targeting of the CLN5 protein in transiently transfected BHK-21 cells. CLN5 proved to be a lysosomally targeted 60 kDa glycoprotein, and the lysosomal targeting of the FIN_M mutant fails to occur in vitro. The results link CLN5 to other lysosomal NCL proteins and imply that the defective intracellular transport of CLN5 could be responsible for the molecular basis of vLINCL.

RESULTS

Biosynthesis of wild-type (WT) and FIN_M CLN5 proteins in BHK-21 cells

To monitor the biosynthesis and intracellular processing of the CLN5 protein, BHK-21 cells were transiently transfected with wild-type (WT) and FIN_M CLN5 cDNA constructs. Cells were metabolically labeled for 2 h and the cells and media were harvested immediately after the labeling period. Samples were immunoprecipitated with the 5289 peptide antibody and analyzed by SDS–PAGE and autoradiography. Cell samples of WT CLN5 revealed a 60 kDa band and in the case of FIN_M CLN5, a 52 kDa band on the gel. Several background bands were also observed, but specific CLN5 signals were evident when the results were compared with non-transfected cells (Fig. 1A). It also became evident that our peptide antibody 5289 did not detect endogenous CLN5 polypeptide. WT and FIN_M polypeptides with similar molecular weights were also observed in media samples, which revealed no background bands (Fig. 1B). The mobility of either WT or FIN_M CLN5 polypeptide bands obtained from cells and media did not change after a chase period of 2 h (data not shown).

Based on the primary amino acid sequence, CLN5 has eight potential N-glycosylation sites (26). In order to examine whether CLN5 is glycosylated after synthesis, immunoprecipitated polypeptides from cells and media were subjected to treatment with Endo H, an enzyme that releases high mannose-type, but not complex-type, N-linked oligosaccharides from polypeptides. This experiment resulted in the collapse of the 60 kDa WT protein to a single 40 kDa polypeptide and the 52 kDa FIN_M protein to a single 37 kDa band (Fig. 1A). To further characterize the nature of the N-glycosylation in CLN5, the corresponding immunocomplexes were treated with PNGase F, an enzyme that removes all N-linked oligosaccharide side chains from glycoproteins. After PNGase F treatment of WT CLN5, a single 38 kDa protein became visible. The same treatment of FIN_M resulted in a 35 kDa protein (Fig. 1A). These observations suggest that in addition to high mannose-type sugars, complex-type sugars are also found in both WT and FIN_M CLN5 polypeptides. Deglycosylation of polypeptides in media with PNGase F resulted in the same-sized bands. However, after Endo H treatment of the WT media sample, an additional smear between 40 and 52 kDa was observed, and treatment of the FIN_M media sample resulted in at least five different protein bands, with molecular weights between 37 and 52 kDa (Fig. 1B). These findings suggest that some oligosaccharide chains of secreted forms of CLN5 are further processed to Endo H-resistant forms.

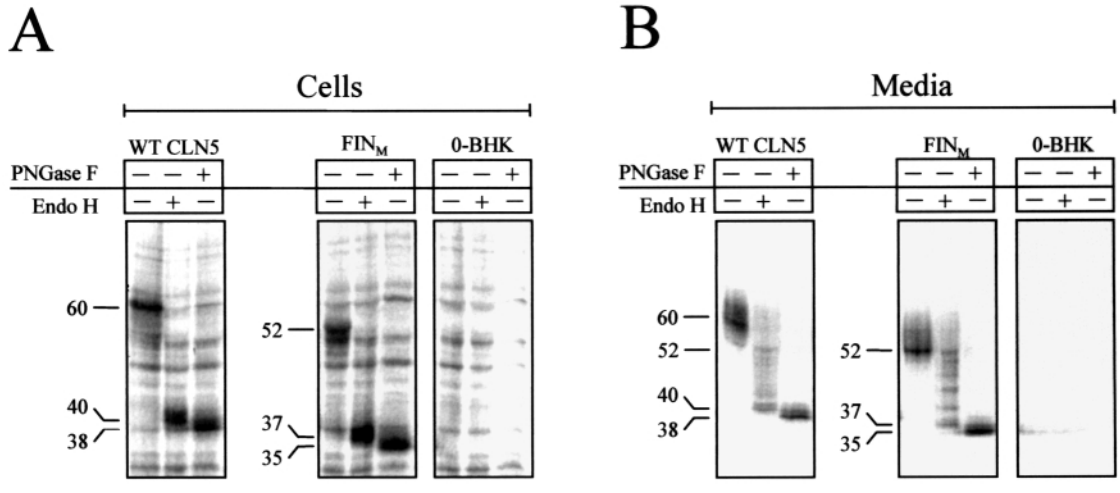

Figure 1. Biosynthesis of WT and FIN_M CLN5 in BHK-21 cells. Transiently transfected cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. Directly after the labeling period of 2 hours, cell (A) and media (B) samples were immunoprecipitated with CLN5-specific antibody (5289) and immunocomplexes were incubated in the absence or presence of glycosidases Endo H and PNGase F. Proteins were separated on SDS–PAGE and analyzed by fluorography. Constructs are shown above each panel and the molecular weights of observed bands are indicated on the left. 0-BHK, mock transfected.

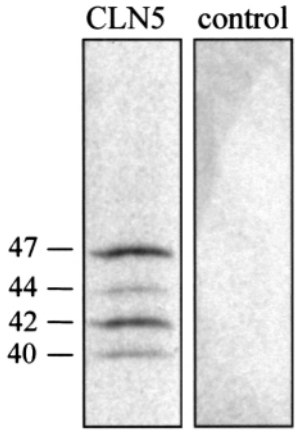

Figure 2. Cell-free synthesis of the WT CLN5 protein. CLN5 cDNA was subcloned to the pGEM3Z vector and translated in vitro using Promegas TNT T7 Quick Coupled Transcription/Translation System. Radiolabeled proteins were separated on SDS–PAGE and analyzed by autoradiography. Control reactions were performed without plasmid DNA. The molecular weights of produced polypeptides are shown on the left.

Cell-free synthesis of CLN5 protein

There are four in-frame AUG codons in the 5′ end of the CLN5 gene (26). The theoretical molecular weights of polypeptides translated from these alternative start codons are 46.3, 43.4, 41.5 and 40.3 kDa (measured using the Compute pI/Mw tool at http://www.expasy.org/tools/pi_tool.html). In transfected BHK-21 cells, WT CLN5 was expressed as a 60 kDa glycoprotein that after deglycosylation with PNGase F was shifted to 38 kDa (Fig. 1A and B). This would imply that the CLN5 polypeptide is not translated from the first AUG codon in BHK-21 cells, but rather the most 3′ initiation codon is mostly used. To further analyze this possibility, we expressed CLN5 cDNA in a cell-free system. This experiment produced protein bands of 47, 44, 42 and 40 kDa (Fig. 2). This result supports the idea that upstream AUG codons can potentially be used for the translation initiation of the CLN5 gene in some cells or tissues.

Intracellular targeting of WT and FIN_M CLN5 proteins in BHK-21 cells

The subcellular location of the CLN5 protein was studied in transiently transfected BHK-21 cells using the 5289 antibody, organelle-specific antibodies and confocal microscopy. To determine the target organelle of the WT polypeptides, protein synthesis was stopped by cycloheximide treatment. When cells were fixed with 4% PFA followed by permeabilization with − 20°C methanol, the 5289 antibody showed a vesicular staining pattern in more than 90% of the transfected cells. These vesicles overlapped almost completely with the lysosomal/endosomal marker lgp120 (Fig. 3A–C). In a small fraction of the cells, the 5289 antibody showed co-localization with Golgi- and ER-specific antibodies (data not shown). However,

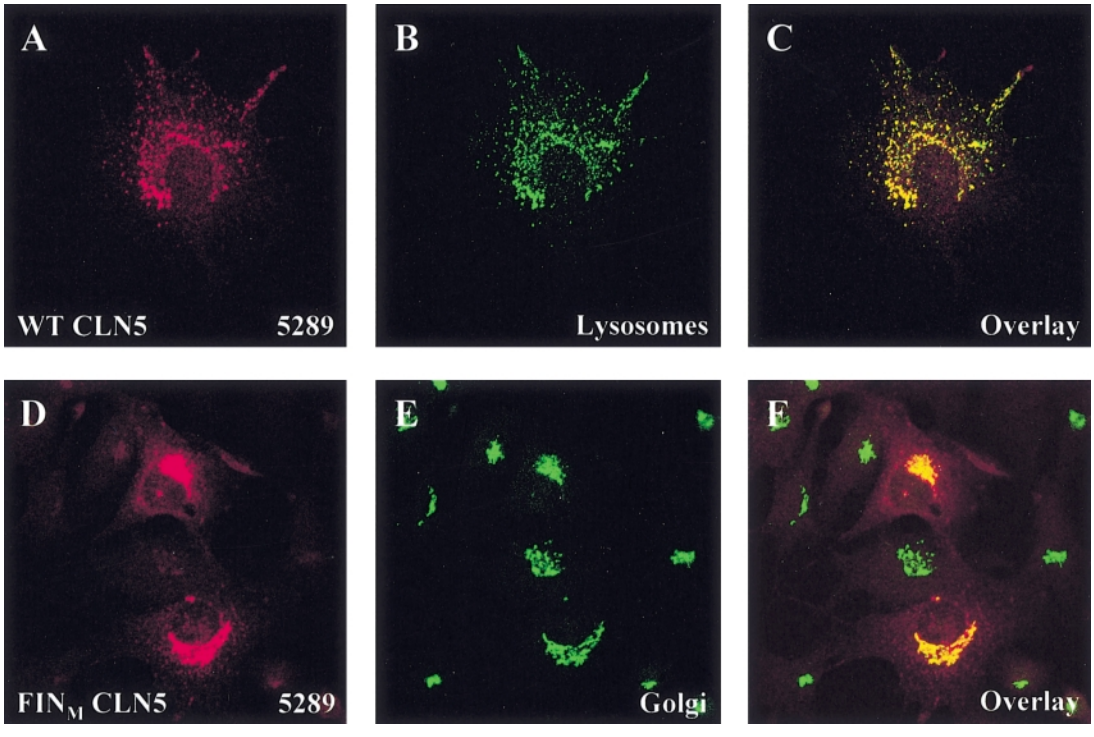

Figure 3. Subcellular localization of WT (A–C) and FIN_M (D–F) CLN5 in BHK-21 cells. Transiently transfected cells were fixed, permeabilized, blocked and stained with CLN5-specific antibody (5289), and either a lysosome-specific (lgp120) or medial Golgi-specific (CTR433) antibody. The secondary antibodies were conjugated with either FITC or TRITC. Cells were viewed with a confocal immunofluorescence microscope. The antibody used is shown in the lower right corner and the transfected construct in the lower left corner of each frame. The right-most figures show the overlay of both CLN5 and the organelle-specific staining. Co-localization is indicated by yellow.

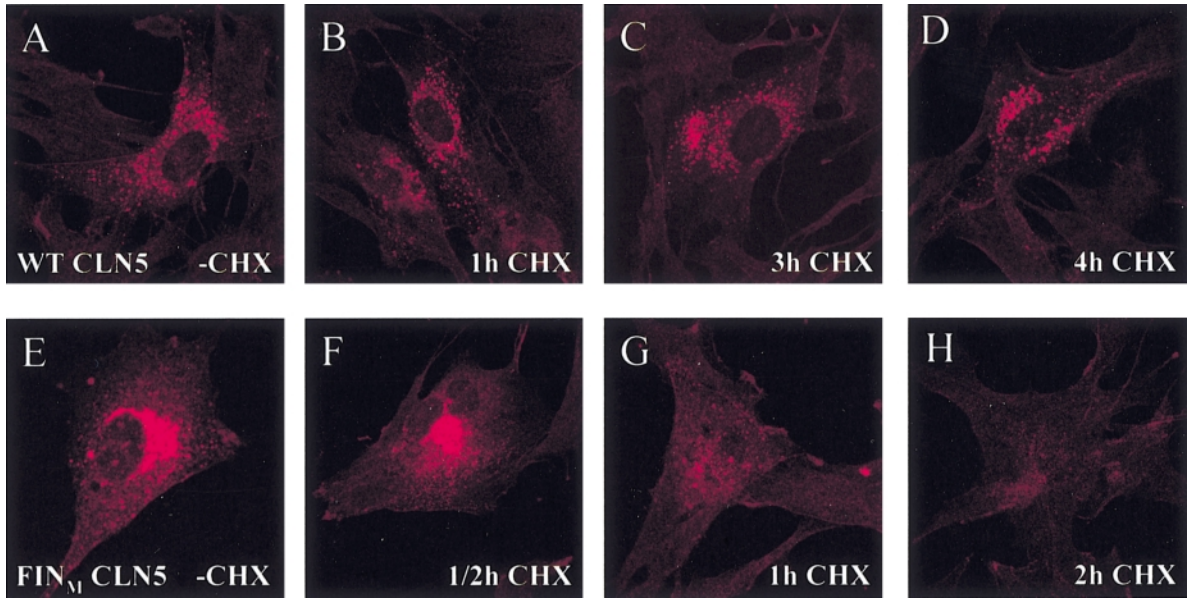

Figure 4. Effect of cycloheximide treatment on the subcellular localization of WT and mutant CLN5 proteins in BHK-21 cells. Cells were transiently transfected either with WT (A–D) or FIN$_M$ (E–H) CLN5 cDNAs. Cells were incubated for different times in the presence of 50 μg/ml of cycloheximide. Thereafter, fixed, permeabilized and blocked cells were stained with CLN5-specific antibody (5289). The secondary antibody was conjugated with TRITC.

CLN5 was not detectable in the Golgi complex after a 1 h cycloheximide treatment, and the ER-like staining became weaker when the treatment time exceeded 3 h. Vesicular-like staining still remained after 4 h of cycloheximide treatment (Fig. 4A–D). These results suggest that WT CLN5 is predominantly targeted to the lysosomal compartment in BHK-21 cells.

To determine the effect of the FIN$_M$ mutation on the intracellular targeting of the CLN5 protein, we performed localization studies in BHK-21 cells transiently transfected with the FIN$_M$ cDNA construct. The FIN$_M$ mutant results in a premature stop codon that leaves the last 16 amino acids of the polypeptide untranslated. Immunostaining of this mutant polypeptide showed co-localization with the medial Golgi marker CTR433 (Fig. 3D–F). In a fraction of cells, ER staining and some minor vesicular-like staining was also observed in addition to Golgi staining. However, these vesicles did not overlap with the lgp120 marker, suggesting that they do not represent lysosomes or late endosomes (data not shown). After 1 h of cycloheximide treatment, the Golgi staining had almost completely disappeared and only some minor vesicular staining was detectable (Fig. 4G). After 2 h of cycloheximide treatment, CLN5-specific staining had completely disappeared. FIN$_M$ polypeptides either become degraded or were secreted into the culture medium (Fig. 4H).

DISCUSSION

Mutations in the CLN5 gene result in a severe neurodegenerative disorder, vLINCL, with the most drastic clinical symptoms being caused by the rapidly progressing death of cortical neurons. Currently, nothing is known about the biosynthesis and intracellular targeting of the CLN5 polypeptide and the function of CLN5 also remains uncharacterized. As an essential step towards the understanding of the pathogenesis of vLINCL, we have here analyzed the biosynthesis and intracellular localization of CLN5 in transiently transfected BHK-21 cells. Our results demonstrate that WT CLN5 is a lysosomally targeted glycoprotein, which is partially secreted into the culture medium. Further, immunofluoresence-based localization of the naturally occurring disease mutant lacking 16 C-terminal amino acids indicated that defective intracellular targeting of the mutant polypeptide is part of the etiology of vLINCL.

Evidence for the lysosomal localization of CLN5 was obtained by double immunofluorescence studies in transiently transfected BHK-21 cells. Our CLN5-specific antibody showed an almost complete overlap with the endogenous lysosomal protein lgp120 in most of the transfected cells. Transient localization of CLN5 in the ER and Golgi reflects intracellular trafficking of CLN5 to lysosomes through the ER and Golgi compartments. Like most soluble lysosomal proteins, WT CLN5 was shown to be N-glycosylated, and the glycosylated polypeptides were also secreted into the culture medium. This would imply that CLN5 is a soluble lysosomal glycoprotein, not an integral transmembrane protein as predicted earlier. On the initial analyses of the nucleotide sequence, CLN5 was predicted to have two transmembrane helices. This prediction was based on the results of the TMpred transmembrane prediction program and a Kyte–Doolittle hydrophobicity plot (26). However, recent studies have shown that most of the transmembrane prediction programs perform poorly in distinguishing transmembrane proteins from soluble proteins (28). For example, the error rate for TMpred was shown to be 55%, while for the Kyte–Doolittle method it was as high as 77%. The best performing programs have been TMHMM (29) (available at http://www.cbs.dtu.dk/services/TMHMM-2.0/) and SOSUI

(30) (http://sosui.proteome.bio.tuat.ac.jp/), with error rates of 1% and 3%, respectively. For the CLN5 protein, neither TMHMM nor SOSUI predicted any transmembrane helices. These predictions do not confirm, but further support the idea that CLN5 is a soluble protein.

The polypeptides with the most common naturally occurring CLN5 disease mutant FIN_M were not targeted to the lysosomes. This mutation represents a premature stop codon that leaves the 16 C-terminal amino acids of the protein untranslated (26). When the mutant polypeptides were transiently expressed in BHK-21 cells, they became normally glycosylated. However, no co-localization with lysosomal marker protein was observed. Instead, a distinct overlap with medial Golgi protein (CTR433) was seen. After cycloheximide treatment, this staining disappeared. In the immunoprecipitation analysis, FIN_M was still observed in the culture media samples after a chase period of 2 h. These results suggest that the mutant CLN5 polypeptides are initially sufficiently correctly folded to pass the quality control of the ER but are not recognized as a lysosomal protein by the intracellular sorting system in the Golgi. Based on the results of pulse chase experiments, the mistargeted mutant CLN5 polypeptides are not degraded, but rather secreted into the culture medium. These data are in agreement with the concept that the CLN5 is a soluble lysosomal protein.

Lysosomal targeting of soluble lysosomal enzymes occurs mainly via the mannose-6-phosphate (M-6-P) receptor pathway. The specificity of this pathway is determined by the Golgi-resident enzyme UDP-N-acetylglucosamine 1-phosphotransferase (phosphotransferase), which transfers N-acetylglucosamine-1-phosphate from UDP-N-acetylglycosamine to the mannose residues of high mannose-type oligosaccharide side-chains of lysosomal enzymes. In a second reaction, the N-acetylglucosamine is removed by another intra-Golgi enzyme (N-acetylglucosamine 1-phosphodiester α-N-acetylglucosaminidase), generating M-6-P residue on the oligosaccharide side-chains. In the late Golgi compartments, lysosomal enzymes bind to M-6-P receptors, which mediate their vesicular transport to the lysosomes (31). In transient expression systems, protein production may exceed the capacity of the phosphotransferase, and oligosaccharides may be further processed to contain complex or hybrid forms that are resistant to Endo H. Unphosphorylated molecules are secreted, as are a small portion of phosphorylated polypeptides that escape capture by the M-6-P receptor. Endo H treatment showed that at least some secreted CLN5 polypeptides contained complex-type oligosaccharides. This raises the possibility that CLN5 could represent a soluble protein targeted to lysosomes via the M-6-P receptor-mediated pathway, perhaps even a lysosomal enzyme. Further studies are needed to determine whether the high mannose-type oligosaccharides of the CLN5 protein are phosphorylated and what is the exact lysosomal targeting mechanism and the function of CLN5.

There are four in-frame AUG codons in the 5′ end sequence of the CLN5 gene (26). Since none of these translation initiation codons is in an optimal context, a leaky scanning mechanism may allow translation to be initiated at downstream AUG codons (32). The cell-free reticulocyte system analysis of WT CLN5 showed evidence that all of these alternative start codons can potentially be used and result in four different sized translation products. In a recent report, it was shown that translation initiation from upstream AUGs is more common than generally believed (33). Thus, it is difficult to predict which one(s) of these start methionines is used for the translation initiation of the CLN5 protein in vivo. In the 5′ end of the mouse cln5 gene there is only one AUG codon. The position of this AUG codon in the mouse gene is comparable to the position of the fourth AUG in the human CLN5 gene, emphasizing the biological significance of the fourth initiation methionine of human CLN5. Moreover, in the immunoprecipitation analysis, our CLN5-specific antibody recognized a single polypeptide, with a molecular weight of 38 kDa after deglycosylation. The theoretical molecular weight of the protein translated from the first start methionine is substantially more: 46.3 kDa. Interestingly, a SignalP V2.0 program (34) (available at http://www.cbs.dtu.dk/) predicts that the CLN5 polypeptide undergoes proteolysis of the N-terminal signal peptide, if the CLN5 protein is translated from the fourth initiation methionine, resulting in a theoretical molecular weight of 36.6 kDa. Thus, it is possible that the CLN5 protein is translated from the fourth start methionine and the N-terminal signal sequence is cleaved from this polypeptide. Cleavage of the N-terminal signal peptide is one of the characteristic features of soluble lysosomal proteins. The CLN5 polypeptide could represent such a lysosomal protein, many of which are enzymes participating in the lysosomal degradation of macromolecules. So far no functional data exist to support this hypothesis, but the molecular character of two other previously characterized NCL gene products, CLN1 and CLN2, both of which are lysosomal enzymes should encourage further analyses of potential enzymatic functions of CLN5. However, it should be emphasized that our in vitro translation resulted in the synthesis of four polypeptide chains with molecular weights of 47, 44, 42 and 40 kDa. It cannot be excluded that this differential usage of the initiator methionine also occurs in vivo. The use of the first methionine would leave a predicted membrane-spanning domain intact, and thus membrane-bound isoforms of CLN5 polypeptides might exist in some cells or tissues.

MATERIALS AND METHODS

Construction of expression plasmids

The coding region of CLN5, including start and stop codons, was amplified by RT–PCR from human fibroblast RNA and cloned into a mammalian expression vector: pCMV5 or pGEM-3-vector (Promega, Madison, WI). The mutant cDNA constructs were generated by the QuickChange site-directed in vitro mutagenesis kit, according to the manufacturer's protocols (Stratagene, La Jolla, CA). The constructs were confirmed by sequencing.

Cell culture and transfections

BHK-21 cells (CCL-10; ATCC, Manassas) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. For transfection, the cells were seeded on 3 cm plates at a density of

1.5×10^5 cells per well. Transfection was performed with the FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) following the guidelines supplied by the manufacturer. Experiments were performed 48 h post transfection.

Antibodies

To obtain the CLN5-specific peptide antibody (5289), rabbits were immunized with a synthetic peptide (CYETWNVKASPEKGAET) corresponding to amino acids 258–273 of the CLN5 polypeptide. The keyhole limpet hemocyanin (KLH)-coupled peptide was purchased from Genosys Biotechnologies (Europe) Ltd (London). Rabbits were immunized by subcutaneous injection with 200 μg of KLH-conjugated synthetic peptide in Freund's complete adjuvant. Injections were repeated 2, 6 and 10 weeks after the first injection. The blood was collected 1 week after the last booster and serum was isolated by centrifugation. The antiserum was IgG-purified using Protein A Sepharose CL-4B (Amersham Pharmacia Biotech). The medial Golgi-specific antibody CTR433 and the lysosome/late endosome-specific antibody lgp120 were kind gifts from Dr Michel Bornens (Institute CURIE, Paris) and Dr Jean Gruenberg (Department of Biochemistry, Geneva), respectively. Secondary antibodies were purchased from Jackson's Immunoresearch Laboratories (Bar Harbor, ME).

Metabolic labelling and immunoprecipitation

For immunoprecipitation analysis, transfected BHK-21 cells were metabolically labeled by starving them in methionine- and cysteine-free medium (Lifetechnologies, Rockville, MD) for 1 h and thereafter labeling with 50 μCi/ml of both [^{35}S]methionine and [^{35}S]cysteine (Amersham, Buckinghamshire, UK) for 2 h. After the pulse labeling, cells were either harvested and lysed with RIPA-buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% IGEPAL, 0.5% deoxycholic acid and 0.1% SDS) supplemented with protease inhibitors (Complete, Roche Diagnostics, Indianapolis, IN) or subjected to chase in DMEM without FBS. Lysed cells and culture media samples were immunoprecipitated with the 5289 anti-CLN5 antibody and Pansorbin cells (Calbiochem). Culture media samples were centrifuged for 10 min (13 000 rpm, $+4^\circ$C) prior to immunoprecipitation. Immunocomplexes were separated on 12% SDS–PAGE and visualized by fluorography (Amplify, Amersham, Buckinghamshire, England). Endo H and PNGase F digestions of immunocomplexes were performed as recommended by the manufacturer (New England BioLabs Inc., Beverly, MA).

Confocal microscopy

To determine the subcellular localization of CLN5, BHK-21 cells were plated on cover slips and transfected as described above. Forty-eight hours post transfection, cells were incubated in DMEM, in the presence of 50 μg/ml of cycloheximide (Sigma, St Louis, MO), for 0.5–4 h. Thereafter, cells were fixed with 4% paraformaldehyde (PFA) for 20 min, permeabilized for 2 min with -20°C methanol and blocked with 0.5% bovine serum albumin (BSA) (Fraction V, Sigma, St Louis, MO). PFA-fixed cells were permeabilized with 0.2% saponin (Sigma, St Louis, MO) or 1% Triton X-100 (Sigma, St Louis, MO). The cells were double labeled with the 5289 CLN5 antibody and either with CTR433 or lgp120. Cells were washed with 0.5% BSA/0.2% saponin and incubated with FITC- or TRITC-conjugated anti-rabbit or anti-mouse secondary antibodies. After washing with phosphate-buffered saline (PBS) and water, the cells were mounted in Gel/Mount (Biomeda, Foster City, CA) and analyzed using Leica DMR confocal immunofluorescence microscopy with TCS NT software.

In vitro translation

In vitro translation of WT CLN5 in the pGEM3Z plasmid was performed using the TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI). One-half of a microgram of plasmid DNA and 10 μCi of Redivue L-[^{35}S]methionine (Amersham Pharmacia Biotech) were incubated for 90 min at 30°C in a final volume of 25 μl. Control reactions were performed without plasmid DNA. Fixed (50% methanol, 10% acetic acid, 40% water, 30 min) and dried gels were exposed directly to Kodak BioMax MR film for visualization.

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