Defective Endoplasmic Reticulum-resident Membrane Protein CLN6 Affects Lysosomal Degradation of Endocytosed Arylsulfatase A*

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Variant late infantile neuronal ceroid lipofuscinosis, a lysosomal storage disorder characterized by progressive mental deterioration and blindness, is caused by mutations in a polytopic membrane protein (CLN6) with unknown intracellular localization and function. In this study, transient transfection of BHK21 cells with CLN6 cDNA and immunoblot analysis using peptide-specific CLN6 antibodies demonstrated the expression of a 27-kDa protein that does not undergo proteolytic processing. Cross-linking experiments revealed the presence of CLN6 dimers. Using double immunofluorescence microscopy, epitope-tagged CLN6 was shown to be retained in the endoplasmic reticulum (ER) with no colocalization with the cis-Golgi or lysosomal markers. The translocation into the ER and proper folding were confirmed by the N-linked glycosylation of a mutant CLN6 polypeptide. Pulse-chase labeling of fibroblasts from CLN6 patients and from sheep (OCL6) and mouse (nclf) models of the disease followed by immunoprecipitation of cathepsin D indicated that neither the synthesis, sorting nor the proteolytic processing of this lysosomal enzyme was affected in CLN6-defective cells. However, the degradation of the endocytosed index protein arylsulfatase A was strongly reduced in all of the mutant CLN6 cell lines compared with controls. These data suggest that defects in the ER-resident CLN6 protein lead to lysosomal dysfunctions, which may result in lysosomal accumulation of storage material.

Neuronal ceroid lipofuscinoses (NCLs),† also collectively called Batten disease, are a group of autosomal recessively inherited neurodegenerative diseases that affect both children and adults. The clinical features include loss of vision, seizures, and mental and motor deterioration. Depending on the affected gene, the clinical signs become visible at various developmental stages (1). Currently, eight forms of human NCLs (CLN1–8) can be differentiated for which six underlying genes have been identified (2). CLN1 and CLN2 encode for soluble lysosomal enzymes, palmitoyl protein thioesterase 1 and tripeptidylpeptidase 1, respectively (3–5). CLN3, CLN5, CLN6, and CLN8 represent transmembrane proteins of still unknown function (6–10). Although the nature of these CLN genes differs, defects in most of them lead to massive storage of autofluorescent material in lysosomes that has been identified as the subunit c of the mitochondrial ATP synthase (2, 11, 12).

Newly synthesized palmitoyl protein thioesterase 1 and tripeptidylpeptidase 1 are transported to the lysosome in a Man-6-P-dependent manner (3, 5). Binding to Man-6-P-specific receptors, MPR46 and MPR300, allows the segregation from the secretory pathway and transport from the trans-Golgi network to the endosomal compartment. Because of the low pH, the receptor-ligand complexes dissociate and the enzymes are delivered to the lysosome. Soluble enzymes that escape binding to the MPR in the trans-Golgi network are secreted but can be partially endocytosed after binding to MPR localized at the cell surface and transported to the lysosome (13). CLN3, CLN5, and CLN8 have been localized in lysosomes and the endoplasmic reticulum (ER)/ER-Golgi-intermediate compartment, respectively (14–16). The subcellular localization of CLN6 is not known. The transport of lysosomal membrane proteins requires tyrosine- or dileucine-based sorting signals (17). Recently, lysosomal targeting motifs have been identified in CLN3 protein (18), whereas the localization of CLN8 in the ER is mediated by a KIXX-ER-retrieval signal (16).

The CLN6 gene encodes a highly conserved 311 amino acid protein with six to seven predicted membrane-spanning domains. The deduced amino acid sequence contains no putative N-glycosylation sites or classical lysosomal sorting signals. Furthermore, no sequence homologies to other proteins have been described (9, 10).

Various mutations of the CLN6 gene have been identified, leading to the variant late infantile form of NCL (9, 10, 19–21). Naturally occurring NCLs in South Hampshire and Merino sheep (OCL6) and nclf mice have been localized to CLN6 (22–24). The severe and progressive neurodegeneration of the cerebral cortex is accompanied by astrocytosis and elevated expression of the radical scavenger protein Mn-SOD (24, 25). Furthermore, differences in the fatty acid profiles of brain phosphatidylethanolamine have been observed in OCL6-affected sheep (26).

In this study, we expressed human CLN6 in BHK21 cells to investigate its subcellular localization. Furthermore, the transport of lysosomal enzymes via the biosynthetic and endocytic pathway was examined in fibroblasts from CLN6 patients,
OCCL, and nclf mice. The data indicate that CLN6 is an ER-resident protein.

EXPERIMENTAL PROCEDURES

Materials—Sodium $^{131}$I (> 15 Ci/mg iodine), $^{35}$S-methionine (> 1000 Ci/mmol), and Rainbow$^{TM}$-colored protein molecular mass marker were obtained from Amersham Biosciences. Opti-MEM, LipofectAMINE$^{TM}$ 2000, and antibiotics (penicillin/streptomycin) were purchased from Invitrogen. Protease inhibitor mixture (P-2714), transferrin, protein A-agarose, 2-mercaptopethanesulfonic acid (MESNA), and Man-6-P were obtained from Sigma, Bradford reagent and Trans-Blot nitrocellulose membrane (0.2 μm) were purchased from Bio-Rad, and bis(sulfosuccinimidy)suberate (BS3), disuccinimidylsuberate (DSS), sulfosuccinimyd-2-biotinamidoethyl-1,3-dithio propionate, and IODO-GEN® were obtained from Pierce. Restriction endonucleases, T4 DNA ligase, and other molecular biology reagents were from Invitrogen, New England Biolabs, Qiagen, and Stratagene (La Jolla, CA). Recombinant human aroylsulfatase A (ASA) purified from ASA-overexpressing mouse embryonic fibroblasts deficient for MPFs (27) was kindly provided by Dr. T. Diersk (University of Gottingen, Gottingen, Germany).

CLN6 Cloning—Full-length CLN6-cDNA was amplified using forward primer 5'-CGGAGATCCCGATGAGGGCGGCGGAGG-3' and reverse primer 5'-CCCAAGCTTGGTGCTGACGGTACG-3' with IMAGE clone IMAG8986179643 as template in vector pCMVSPORT6. The PCR product was subcloned in-frame with the C-terminal Myc-His tag of vector pCdNA3.1/Myc-His$^{\text{TM}}$ (Invitrogen) into BamHI/HindIII restriction sites. The untagged CLN6 wild type construct was amplified with the reverse primer 5'-CCCAAGCTTGGTGCTGACGGTACG-3' and subcloned into EcoRI restriction sites of vector pGEX-4T-1 (Amersham Biosciences).

The mutation p.Ile153Ser was introduced by PCR using the Quick-Change site-directed mutagenesis kit (Stratagene) and the primers 5'-ATTCTTGATGCTGGGGTTCTC-3' and 5'-CGGGATCCGCGATGGAGGCGACGCGGAGG-3' and subcloned into EcoRI restriction sites of vector pGEX-4T-1. Overexpression of CLN6 in BHK21 cells. CLN6-overexpressing BHK21 cells were transfected with pcDNA3.1/Myc-His$^{\text{TM}}$, A (Invitrogen) into BamHI/HindIII restriction sites. The untagged CLN6 wild type construct was amplified with the reverse primer 5'-CCCAAGCTTGGTGCTGACGGTACG-3' and subcloned into EcoRI restriction sites of vector pGEX-4T-1 (Amersham Biosciences).

Isolation and Culture of Mouse Fibroblasts—Fibroblasts from OCL6, and nclf mice were used as controls. Western blot analysis using the peptide-specific anti-CLN6 antibody revealed a single prominent immunoreactive band of ~27 kDa in media as described previously (33) and analyzed by SDS-PAGE and fluorography.

Cell Surface Biotinylation and MPR300 Immunoprecipitation—Cell surface proteins were biotinylated with sulfo-NHS-S-s-biotin at 4 °C as described previously (34). After washing, the cells were incubated in the presence or absence of 50 mM MESNA in 50 mM Tris buffer, pH 8.6, containing 100 mM NaCl, 1 mM MgCl$_2$, and 0.1 mM CaCl$_2$ followed by immunoprecipitation of MPR300 from cell extracts as described previously (29).

Ligand Internalization—Internalization of $^{125}$I/ASA was examined as described previously (33). The internalized $^{125}$I/ASA was detected after 3 h of incubation at 37 °C. Cells were washed, and cell surface-bound $^{125}$I/ASA was displaced by an acidic wash (35). Cells were solubilized and analyzed by SDS-PAGE and autoradiography.

SDS-PAGE—Solubilized proteins from transfected and non-transfected cells were separated by SDS-PAGE, transferred to nitrocellulose membrane, and examined for CLN6- and Myc immunoreactivity as described previously (25).

Cross-link Experiments—CLN6-overexpressing BHK21 cells were permeabilized with 0.25% saponin in 50 mM MES buffer, pH 6.5, containing 150 mM NaCl and 0.5% bovine serum albumin for 40 min at 4 °C and then cross-linked with BS3 and DSS at the indicated concentrations. The proteins were analyzed by Western blotting (36, 37).

Other Methods—Protein concentrations were determined using the Bradford protein assay with bovine serum albumin as a standard. Proteins (0.5–1 μg) were loaded on IODO-GEN® as described previously (38). For deglycosylation experiments, total BHK21 extracts were solubilized and incubated in the presence or absence of 1 milliunit of peptide N-glycosidase F (PNGase F) or 5 milliunits of endoglycosidase H (endo H, Roche Applied Science) according to the manufacturer’s protocol. The samples were separated by SDS-PAGE followed by Western blot analysis. Double immunofluorescence microscopy was performed with transfected BHK21 cells as described previously (25) using anti-Myc (1:50), anti-PDI (1:300), or anti-mouse CtsD antibodies (1:50) as indicated. β-Hexosaminidase and ASA activities were determined as described previously (39, 40).

RESULTS

Expression of Wild Type and Myc-tagged CLN6—Wild type and C-terminally Myc-tagged CLN6 were transiently expressed in BHK21 cells. Non-transfected and pcDNA3.1 vector-transfected BHK21 cells were used as controls. Western blot analysis using the peptide-specific anti-CLN6 antibody revealed a single prominent immunoreactive band of ~27 kDa in

FIG. 1. Overexpression of CLN6 in BHK21 cells. A, cell extracts of vector-transfected wild type CLN6 and wild type CLN6/Myc/His tag-expressing BHK21 cells were separated by SDS-PAGE, transferred to nitrocellulose membrane, and examined for CLN6- and Myc immunoreactivity as described previously (25).
cell extracts of wild type CLN6-transfected cells (Fig. 1). No specific cross-reacting material was found in cells transfected with the vector only. An immunoreactive band of ~30 kDa was observed in cells expressing the CLN6Myc construct. After stripping of the nitrocellulose membrane and reprobing with anti-Myc antibodies, only the ~30-kDa Myc-tagged CLN6 was detectable.

CLN6 Is an ER-resident Protein—The deduced amino acid sequence and the predicted protein structure of CLN6 revealed neither potential N-glycosylation site sequences (NX(S/T), where X = any amino acid) nor classical lysosomal sorting motifs. On the other hand, the N-terminal RRR sequence (amino acids residues 5–7) has been detected in the ER type II membrane protein glucosidase I (41), whereas the N-terminal segment of CLN6 encoded by exon 1 (amino acids residues 1–28) has been suggested to function as a putative targeting signal for mitochondria (10). To investigate its subcellular localization, an N-glycosylation site was introduced in the predicted second luminal loop. Ile residue 153 was substituted for Ser (p.Ile153Ser) by site-directed mutagenesis of the CLN6 cDNA changing the sequence Asn-X-Ile to Asn-X-Ser. When the p.Ile153Ser was expressed in BHK21 cells, Western blot analysis revealed an additional band of ~33 kDa, which disappeared after deglycosylation with PNGase F or endo H (Fig. 2A). These data demonstrate that the nascent CLN6 polypeptide had been translocated to the lumen of the ER and became accessible to the glycosylation machinery. This was confirmed
by double immunofluorescence microscopy. The CLN6Myc protein colocalized with the ER marker protein PDI but not with the lysosomal marker enzyme cathepsin D (Fig. 2B, CtsD).

**Defective CLN6 Mice Do Not Impair Sorting and Processing of Newly Synthesized Cathepsin D**—To examine whether CLN6 is critical for the transport and processing of Man-6-P-containing lysosomal proteins, the secretion and proteolytic modifications of newly synthesized CtsD was determined in fibroblasts from CLN6 patients, OCL6 sheep, nclf mice, and the corresponding control cells. In human fibroblasts, CtsD was synthesized as a 53-kDa precursor form (28). A small fraction of the precursor protein binds to MPRs in the trans-Golgi network and is transported to prelysosomal/endoosomal compartments where it is processed to the intermediate form within 2 h after synthesis (42). The intermediate CtsD forms are delivered to lysosomes and are processed to the 47-kDa form within 6 h after synthesis (43), mediated uptake because the amounts of internalized [125I]ASA detectable in control cells. The experiment was repeated twice with similar results.

**Accumulation of Endocytosed Arylsulfatase A in CLN6-defective Fibroblasts**—To examine whether defective CLN6 affect the endocytic pathway to the lysosome, fibroblasts from CLN6 patients, OCL6 sheep, nclf mice, or from nclf mice. Additionally, the specific activities of two other lysosomal enzymes, β-hexosaminidase and ASA, were comparable in control and human, sheep, and mouse CLN6-defective cells.

**Degradation of endocytosed [125I]ASA is reduced in CLN6-defective fibroblasts.** Fibroblasts from control (Co) and affected (CLN6) patients were incubated with [125I]ASA (800,000 cpm/ml) for 3 h at 37 °C. After removal of the media, the cells were either harvested or chased for an additional 20 h at 37 °C. The cells were solubilized and separated by SDS-PAGE, and internalized [125I]ASA was visualized by autoradiography. The autoradiograph was quantified by densitometric scanning and expressed as a percentage of total [125I]ASA detectable in control cells. The experiment was repeated twice with similar results.

**Extent of glycosylation.** These results show that the sorting and processing of newly synthesized CtsD was not affected in fibroblasts from a CLN6 patient, from OCL6 sheep, or from nclf mice. Additionally, the specific activities of two other lysosomal enzymes, β-hexosaminidase and ASA, were comparable in control and human, sheep, and mouse CLN6-defective cells.

**Accumulation of Endocytosed Arylsulfatase A in CLN6-defective Fibroblasts**—To examine whether defective CLN6 affect the endocytic pathway to the lysosome, fibroblasts from CLN6 patients, OCL6 sheep, nclf mice, and the respective controls were incubated with [125I]ASA for 16 h. Because the MPR46 does not function in endocytosis of lysosomal enzymes (43), the uptake of ASA is mediated by the MPR300. The ASA uptake is specific as shown by the complete inhibition in the presence of an excess of Man-6-P (Fig. 5). The densitometric evaluation of autoradiographs of several experiments (n = 3–4) showed that the average amount of [125I]ASA detectable in human CLN6, OCL6, and nclf cells is 2-, 51-, and 2-fold higher, respectively, than in control cells. This effect is specific for the MPR300-mediated uptake because the amounts of internalized [125I]transferrin are comparable in control and CLN6-defective cells (Fig. 5B).

The increased intracellular amounts of [125I]ASA in CLN6-defective cells could result from an increased rate of endocytosis of [125I]ASA caused by elevated concentrations of MPR300 at the cell surface or more rapid MPR300 kinetics of cycling or decreased degradation of endocytosed [125I]ASA. The concentration of MPR300 at the cell surface of control and OCL6 sheep fibroblasts was shown to be the same. This was determined on the control and affected sheep fibroblasts showing most significant difference in intracellular [125I]ASA content.
after endocytosis by cell surface biotinylation followed by immunoprecipitation of MPR300 and Western blot analysis (Fig. 6). Even in these cells, the amounts of MPR300 at the cell surface of control and OCL6 fibroblasts were similar, indicating that the increased amounts of intracellular \([125]I\)ASA in OCL6 cells is not the result of increased concentrations of MPR300 at the cell surface. The specificity of the procedure was shown by the effect of MESNA, which cleaved the biotin label linkage.

To examine whether the degradation of endocytosed \([125]I\)ASA was inhibited in CLN6-defective fibroblasts, cells were incubated with \([125]I\)ASA for 3 h at 37 °C. After removal of the radioactive media, the cells were either harvested or chased for a further 20 h. The solubilized cells were analyzed by SDS-PAGE and autoradiography (Fig. 7). Densitometry of the autoradiograph revealed that the amount of intracellular \([125]I\)ASA detected in cells from CLN6 patients was 1.5-fold more than in control cells after 3 h of endocytosis. During the following chase period of 20 h, the intracellular \([125]I\)ASA remaining in control cells was reduced to 32%, whereas 96% remained in fibroblasts from CLN6 patients. These data indicate that the degradation of endocytosed \([125]I\)ASA is strongly inhibited in CLN6-defective cells.

**DISCUSSION**

In the present study, we have examined the subcellular localization of the CLN6 protein. Mutations in the CLN6 gene cause the accumulation of storage material in lysosomes and the clinical manifestation of the variant late infantile neuronal ceroid lipofuscinosis. Here we report that CLN6 is an ER-resident protein capable of forming dimers. Furthermore, neither the synthesis, the sorting, nor the transport of newly synthesized CtsD, a lysosomal protease, was impaired in CLN6-defective cells, whereas the degradation of an exogenous protein, ASA, was reduced.

Several lines of evidence imply the translocation and localization of the CLN6 protein to the ER. First, the CLN6 antisense RNA inhibited in CLN6-defective fibroblasts, cells were incubated with \([125]I\)ASA for 3 h at 37 °C. After removal of the radioactive media, the cells were either harvested or chased for a further 20 h. The solubilized cells were analyzed by SDS-PAGE and autoradiography (Fig. 7). Densitometry of the autoradiograph revealed that the amount of intracellular \([125]I\)ASA detected in cells from CLN6 patients was 1.5-fold more than in control cells after 3 h of endocytosis. During the following chase period of 20 h, the intracellular \([125]I\)ASA remaining in control cells was reduced to 32%, whereas 96% remained in fibroblasts from CLN6 patients. These data indicate that the degradation of endocytosed \([125]I\)ASA is strongly inhibited in CLN6-defective cells.

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