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Proteolytic cleavage of the disease-related lysosomal membrane glycoprotein CLN7

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ABSTRACT

CLN7 is a polytopic lysosomal membrane glycoprotein of unknown function and is deficient in variant late infantile neuronal ceroid lipofuscinosis. Here we show that full-length CLN7 is proteolytically cleaved twice, once proximal to the used *N*-glycosylation sites in lumenal loop L9 and once distal to these sites. Cleavage occurs by cysteine proteases in acidic compartments and disruption of lysosomal targeting of CLN7 results in inhibition of proteolytic cleavage. The apparent molecular masses of the CLN7 fragments suggest that both cleavage sites are located within lumenal loop L9. The known disease-causing mutations, p.T294K and p.P412L, localized in lumenal loops L7 and L9, respectively, did not interfere with correct lysosomal targeting of CLN7 but enhanced its proteolytic cleavage in lysosomes. Incubation of cells with selective cysteine protease inhibitors and expression of CLN7 in gene-targeted mouse embryonic fibroblasts revealed that cathepsin L is required for one of the two proteolytic cleavage events. Our findings suggest that CLN7 is inactivated by proteolytic cleavage and that enhanced CLN7 proteolysis caused by missense mutations in selected luminal loops is associated with disease.

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1. Introduction

Lysosomal membrane proteins have numerous functions including acidification of the lysosomal lumen, mediating fusion with other organelles, self-protection against proteolytic digestion, export of degradation products into the cytosol, protein import from the cytosol and motion of organelles [1]. The precise functions, however, of the majority of the lysosomal membrane proteins are unknown [2,3]. Defects in genes encoding lysosomal membrane proteins have been linked to a growing number of lysosomal storage disorders [4]. The neuronal ceroid lipofuscinoses (NCL) represent a group of lysosomal storage diseases which are characterized by the accumulation of lipofuscin-like ceroid lipopigments and the selective damage and loss of neurons [5]. NCL-causing mutations have been identified in eleven different genes encoding the soluble cysteine-string protein alpha (CLN4/DNAJC5), soluble lysosomal proteins (CLN1/palmitoyl-protein thioesterase 1, CLN2/tripeptidyl peptidase I, CLN5 and CLN10/cathepsin D), lysosomal membrane proteins (CLN3, CLN7/MFSD8, CLN12/ATP13A2 and ClC-7) and polytopic membrane proteins localized in the endoplasmic reticulum (CLN6 and CLN8) [5–8]. CLN7 disease, late infantile variant phenotype (MIM# 610951) is caused by defects in the *CLN7/MFSD8* gene with 31 different mutations known to date [9–14]. The *CLN7/MFSD8* gene encodes a polytopic lysosomal membrane protein of unknown function with 12 transmembrane domains, cytosolic N and C termini and two confirmed *N*-glycosylation sites in positions N371 and N376 [15]. The *CLN7/MFSD8* gene product is localized in lysosomes in non-neuronal and neuronal cells [9,15,16]. Its lysosomal targeting along the indirect pathway via the plasma membrane is mediated by an N-terminal dileucine-based E^{9} QEP $L^{13}L^{14}$ sorting signal [15]. CLN7 is a member of the major facilitator superfamily (MFS) of transporter proteins which are capable of transporting a wide range of low-molecular-mass substrates including carbohydrates and amino acids [17]. CLN7 is therefore believed to be a lysosomal transporter, but its substrate has not been identified.

We have previously shown that the intensity of CLN7-GFP immunoreactive bands increased after inhibiting cysteine and aspartic proteases [15]. In the present study we describe the proteolytic cleavage of the CLN7 membrane protein in endosomal/lysosomal compartments. CLN7 is asymmetrically cleaved twice in a cell-type independent manner. Inhibitor studies and expression analyses in gene-targeted mouse embryonic fibroblasts (MEF) demonstrated that the cysteine protease cathepsin L (Ctsl) is required for one of the two proteolytic cleavage

Abbreviations: BFA, brefeldin A; CHX, cycloheximide; Ctsb, cathepsin B; Ctsl, cathepsin L; DMEM, Dulbecco's minimal essential medium; endo H, endoglycosidase H; ER, endoplasmic reticulum; GFP, green fluorescent protein; HEK293, human embryonic kidney 293; HGSNAT, heparin sulfate acetyl-CoA α-glucosaminide N-acetyltransferase; IC50, half maximal inhibitory concentration; LAP, lysosomal acid phosphatase; MEF, mouse embryonic fibroblast; MFS, major facilitator superfamily; ML1, mucolipin-1; NCL, neuronal ceroid lipofuscinosis; PNGase F, peptide:*N*-glycosidase F; TLR9, Toll-like receptor 9; TM, transmembrane domain; vLINCL, variant late-infantile NCL

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events. In addition, two missense mutations identified in patients with CLN7 disease, p.T294K and p.P412L, resulted in increased proteolytic cleavage of CLN7 in lysosomes.

2. Materials and methods

2.1. Antibodies and reagents

The following antibodies were used for Western blot analyses: mouse anti-GFP (1:500 dilution; Roche Applied Science, Mannheim, Germany), mouse anti-FLAG (clone M2, 1:1000 dilution; Sigma-Aldrich, Taufkirchen, Germany), and mouse anti-Myc (clone 9B11, 1:1000 dilution; Cell Signaling Technologies, Danvers, MA). Antibodies used for immunofluorescence microscopy were as follows: polyclonal anti-human cathepsin D (1:100 dilution, [18]), monoclonal anti-human LAMP-1 (clone H4A3, 1:150 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA), polyclonal anti-Myc (1:100 dilution; Sigma-Aldrich), monoclonal anti-FLAG (clone M2, 1:100 dilution) and polyclonal anti-FLAG (1:100 dilution, Sigma-Aldrich). Secondary goat anti-mouse antibodies coupled to horse radish peroxidase were from Dianova (Hamburg, Germany) and were used at a 1:5000 dilution. Fluorochrome-coupled secondary antibodies goat anti-rabbit IgG AlexaFluor 488, goat anti-rabbit IgG Cy5, goat anti-rabbit IgG AlexaFluor 546, goat anti-mouse IgG AlexaFluor 488 and goat anti-mouse IgG AlexaFluor 546 were from Invitrogen (Karlsruhe, Germany) and used at a 1:1000 dilution.

The following reagents were obtained commercially as indicated: Normocin from Invivogen (San Diego, CA), fast digest restriction enzymes from Fermentas (St. Leon-Rot, Germany), Quick Start Bradford Protein Assay from Bio-Rad (Munich, Germany), peptide *N*-glycosidase F (PNGase F) from Roche Applied Sciences (Mannheim, Germany), endoglycosidase H (endo H) from New England Biolabs (Ipswich, MA), Pfu Turbo polymerase and QuikChange site-directed mutagenesis kit from Stratagene Europe (Amsterdam, the Netherlands), Dulbecco's minimal essential medium (DMEM), fetal calf serum, Glutamax, penicillin, streptomycin, trypsin/EDTA, Lipofectamine 2000 and Optimem from Invitrogen, Phusion® High-Fidelity DNA polymerase from Finnzymes (Espoo, Finland), GeneJET™ Plasmid Mini Kit and gel extraction kit from Fermentas (St. Leon-Rot, Germany), Plasmid Midi Kit from QIAGEN (Hilden, Germany), medium for cultivating *Escherichia coli* from Roth (Karlsruhe, Germany), prestained Rainbow marker, and ultrapure dNTPs from GE Healthcare Life Sciences (Freiburg, Germany). Oligonucleotides used for cloning and sequencing were synthesized by MWG Biotech (Munich, Germany). Cycloheximide, brefeldin A, leupeptin, pepstatin A, E-64, SID26681509, and protease inhibitor cocktail were purchased from Sigma-Aldrich, Ctsl inhibitor Z-FY-CHO from Calbiochem (Nottingham, UK), Albumin standard and enhanced chemiluminescence reagents from Thermo Fisher Scientific (Rockford, IL). GFP-TRAP® agarose beads were purchased from ChromoTek (Planegg-Martinsried, Germany).

2.2. Plasmids and transgenic cDNA constructs

The generation of the GFP-CLN7 cDNA construct has been described previously [15]. A cDNA construct with a triple FLAG tag fused to the N-terminus of CLN7 was generated by amplifying the cDNA coding for human CLN7 (NM_152778, RZPD clone IRATp970E0532D6; imaGenes, Berlin, Germany) by PCR using primers 3xFLAG pCMV10-CLN7 F/R and by cloning the resulting PCR product into *Hind*III and *Bam*HI sites of expression vector p3xFLAG-CMV10 (Sigma-Aldrich). The pcDNA3.1 (+) CLN7-3xMyc cDNA construct was generated by amplifying a PCR product with primers pcDNA3.1. (+) CLN7-3xMyc F/R using GFP-CLN7 as a DNA template. The resulting PCR product was subcloned into the pCR®-BluntII-TOPO® vector (Invitrogen), the fragment subsequently excised with restriction enzymes *Hind*III and *Bam*HI and cloned into the corresponding sites of expression vector pcDNA3.1 Hygro (+)-3xMyc [19]. The GFP-CLN7-3xMyc expression construct was generated by amplifying a PCR product with primers pcDNA3.1 GFP-CLN7 3xMyc F/R using the GFP-CLN7 cDNA as template. The PCR product was cloned into pcDNA3.1D/V5-His-TOPO® vector (Invitrogen). For the cloning of the 3xFLAG-mucolipin-1 construct a cDNA clone (IMAGE clone p998L049490Q) containing the human mucolipin-1 cDNA (NM_020533) was amplified by PCR with primers 3xFLAG pCMV10-mucolipin-1F/R. The resulting PCR product was subcloned into the pCR®-BluntII-TOPO® vector and recombinant vectors were subsequently incubated with restriction enzymes *Hind*III and *Sac*I. The fragment was finally cloned into the *Hind*III/*Sac*I sites of vector pCMV10 3xFLAG (Sigma).

All primers used for cloning and site-directed mutagenesis are described in the Supplementary material (Table S1). Mutagenesis was performed using the QuikChange® site-directed mutagenesis kit. All constructs were sequenced by Seqlab (Göttingen, Germany). The cDNAs encoding mouse cathepsin L (Ctsl, NM_009984) and cathepsin B (Ctsb, NM_007798) cloned into expression vector pcDNA3.1 (+) were kindly provided by Dr. Schröder (University of Kiel, Germany).

2.3. Cell culture and transfections

COS-7, HEK293 cells, wild-type and $Ctsl^{(-/-)}$- and $Ctsb^{(-/-)}$ mouse embryonic fibroblasts (MEF) were cultivated in DMEM containing 10% fetal calf serum, 1× Glutamax, 100 µg/ml Normocin, 100 IU/ml penicillin, and 50 mg/ml streptomycin. $Ctsb^{(-/-)}$ and $Ctsl^{(-/-)}$ MEF were described previously [20,21]. Cells were transfected with Lipofectamine 2000 (Invitrogen) and Optimem according to the manufacturers' instructions and cells were analyzed 24 h after the start of transfection. Where indicated, media were supplemented with brefeldin A (BFA) or protease inhibitors during the complete course of transfection.

2.4. Western blotting

Cells grown on 35 mm dishes were scraped in ice-cold phosphate-buffered saline, pH 7.4, and centrifuged for 5 min at 1000×*g*. Cell pellets were lysed in 100 µl ice-cold lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% TX-100, 1% sodium deoxycholate) supplemented with protease inhibitors. After incubation for 30 min on ice, extracts were centrifuged at 15,000×*g* for 10 min followed by determination of protein concentrations with a Bradford assay. Aliquots of total cell extracts were solubilized in Laemmli sample buffer containing 1% beta-mercaptoethanol at 50 °C for 10 min. Samples were separated by SDS-PAGE (10% acrylamide unless otherwise specified). Proteins were transferred to PVDF membranes (GE Healthcare, Munich, Germany). ECL-detection was performed using the manufacturer's instructions (Thermo Fisher Scientific) and blots were imaged on a Molecular Imager (Model Chemi Doc XRS, Bio-Rad). For complete removal of all *N*-linked oligosaccharides from CLN7 50 µg of total cell extracts was denatured for 10 min at 50 °C in 0.3% SDS and protease inhibitors, followed by the addition of NP-40 (final concentration: 1%) and 3 units PNGase F. For removal of *N*-linked high-mannose and hybrid oligosaccharides 50 µg of total cell extracts was denatured in denaturation buffer (New England Biolabs) for 10 min at 50 °C, followed by the addition of buffer G5 and 1000 units endo H. Samples were incubated in the presence of endo H or PNGase at 37 °C for 30 to 60 min, followed by analysis by SDS-PAGE and immunoblotting.

2.5. Co-immunoprecipitations

HEK293 cells were co-transfected with GFP-CLN7 and 3xFLAG-CLN7 or GFP-CLN7 and CLN7-3xMyc and harvested 24 h after the start of transfection. Cell pellets were lysed using 200 µl of a low-detergent lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP40, protease inhibitors) to leave protein complexes intact. Subsequently, GFP-CLN7

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