A Dileucine Motif and a Cluster of Acidic Amino Acids in the Second Cytoplasmic Domain of the Batten Disease-related CLN3 Protein Are Required for Efficient Lysosomal Targeting*

Received for publication, September 22, 2004
Published, JBC Papers in Press, October 5, 2004, DOI 10.1074/jbc.M410930200

Stephan Storch, Sandra Pohl, and Thomas Braulke‡
From the Department of Biochemistry, Children’s Hospital, University of Hamburg, D-20246 Hamburg, Germany

The juvenile form of ceroid lipofuscinosis (Batten disease) is a neurodegenerative lysosomal storage disorder caused by mutations in the CLN3 gene. CLN3 encodes a multimembrane-spanning protein of unknown function, which is mainly localized in lysosomes in non-neuronal cells and in endosomes in neuronal cells. For this study we constructed chimeric proteins of three CLN3 cytoplasmic domains fused to the luminal and transmembrane domains of the reporter proteins LAP-1 and lysosomal acid phosphatase to identify lysosomal targeting motifs and to determine the intracellular transport and subcellular localization of the chimera in transfected cell lines. We report that a novel type of dileucine-based sorting motif, EEEXL1, present in the second cytoplasmic domain of CLN3, is sufficient for proper targeting to lysosomes. The first cytoplasmic domain of CLN3 and the mutation of the dileucine motif resulted in a partial mis-sorting of chimeric proteins to the plasma membrane. At equilibrium, 4–13% of the different chimera are present at the cell surface. Analysis of lysosome-specific proteolytic processing revealed that lysosomal acid phosphatase chimeras containing the second cytoplasmic domain of CLN3 showed the highest rate of lysosomal delivery, whereas the C terminus of CLN3 was found to be less efficient in lysosomal targeting. However, none of these cytosolic CLN3 domains was able to interact with AP-1, AP-3, or GGA3 adaptor complexes. These data revealed that lysosomal sorting motifs located in an intramolecular cytoplasmic domain of a multimembrane-spanning protein have different structural requirements for adaptor binding than sorting signals found in the C-terminal cytoplasmic domains of single- or dual-spanning lysosomal membrane proteins.

The neuronal ceroid lipofuscinoses (NCLs)† are a group of autosomally inherited neurodegenerative lysosomal storage disorders of childhood (1). Mutations in the CLN3 gene result in the juvenile form of ceroid lipofuscinosis (Batten disease, MIM 204200), which is clinically characterized by initial loss of vision at the age of 5–7 years, seizures, and motoric and mental retardation finally leading to premature death (2). The lysosomal storage material consists mainly of autofluorescent lipids and the mitochondrial ATP synthase subunit c (3). The storage occurs in all organs, but only neuronal tissues are functionally affected. The CLN3 gene product encodes a 438-amino acid residue membrane glycoprotein (CLN3) with 4–8 predicted transmembrane domains (4–6). Although the C terminus is directed to the cytoplasm, the orientation of the N terminus is a matter of debate. In overexpressing non-neuronal cells, CLN3 has been reported to be localized in the late endosomal/lysosomal compartment (7) and in neuronal cells in synaptosomes and endosomes (6, 8). Small amounts of CLN3 appear to be present at the plasma membrane (7, 9). CLN3 is highly conserved in vertebrates and has a yeast homologue Bt1p (10). The function of CLN3 is unknown, but it has been reported that it plays a role in the regulation of the vacuolar/lysosomal pH (10, 11), apoptosis (12), and arginine transport (13).

Lysosomal membranes are composed of groups of highly glycosylated lysosomal associated membrane protein-1 (LAMP-1), LAMP-2, LAMP-3 (or CD63, LIMP-1), and lysosomal integrated membrane protein-2 (LIMP-2), representing more than 50% of the total membrane proteins (14). In addition, several other less abundant proteins have been identified, such as lysosomal acid phosphatase (LAP), the lysosomal cystine transporter cystinosin, or the cholesterol transporter NPC-1 (15). The targeting of newly synthesized membrane proteins to lysosomes and to lysosome-related organelles relies on either tyrosine- or dileucine-based sorting motifs located in the cytoplasmic domains (15, 16). Tyrosine-based sorting signals have been identified in LAMP-1, LAMP-2, LAMP-3, LAP, and cystinosin (17–21). In the cytoplasmic domains of LIMP-2 and melanosomal tyrosinase, a leucine-isoleucine- and a dileucine-based motif, respectively, are important for sorting to lysosomes/lysosome-related organelles (22, 23). The sorting motifs are used as recognition sites for cytosolic heterotetrameric adaptor complexes mediating the incorporation of the respective cargo proteins in clathrin-coated vesicles. Four adaptor complexes AP-1, AP-2, AP-3, and AP-4 have been described and are thought to function in the transport of cargo proteins into the endocytic and biosynthetic pathways (24). The targeting of LAMP-1, LAMP-2, LAMP-3, and LIMP-2 from the trans-Golgi network (TGN) to lysosomes and lysosome-related organelles is mediated by the AP-3 adaptor complex (25–28). Dileucine motifs and the preceding acidic glutamate and aspartate (D/E)
residues forming the consensus sequence (DE)XXL(LI) have often been shown to interact in vitro with heterotetrameric AP-1, AP-2, and/or AP-3, and the acidic cluster dileucine DXXL-type sorting signals in the cytosolic tails of MPRs bind to the monomeric Golgi localized γ/ear-containing ARF-binding proteins (GGAs) (29–36). Furthermore, trafficking of LAP to lysosomes follows an indirect route that includes recycling between early endosomes and the plasma membrane before delivery to lysosomes (37). It has been shown that the tyrosine motif in the cytoplasmic domain of LAP determines the specificity of adaptor binding required for the transport in the recycling loop (19, 39).

To identify domains in CLN3 and to define the lysosomal targeting signals, we used chimeric proteins consisting either of the lumenal and transmembrane domains of rat LAMP-1 or human LAP fused to the individual intact and mutated cytoplasmic domains (CD) of CLN3. The subcellular localization of the chimera was determined by double immunofluorescence microscopy and cell surface biotinylation. Furthermore, the proteolytic processing of newly synthesized LAP-CLN3 chimera occurring in lysosomes has been proven to document the transport to lysosomes. Finally, pull-down assays were carried out to analyze the ability of cytoplasmic domains of CLN3 to interact with adaptor molecules. We found a novel type of dileucine-based sorting signal in the context of preceding acidic glutamate residues in the second cytoplasmic domain of CLN3 essential and sufficient for the transport to the lysosome.

**EXPERIMENTAL PROCEDURES**

[35S]Methionine, the prestained Rainbow marker, ultrapure dNTPs, the bacterial expression vector pGEX-4T-1, and the Escherichia coli strain BL21 were purchased from Amersham Biosciences. The following reagents were obtained commercially as indicated: restriction enzymes and T4 DNA ligase from New England Biolabs (Schwalbach, Germany); calf intestinal alkaline phosphatase (Roche Applied Science); Ffj Turbo polymerase and QuikChange™ site-directed mutagenesis kit (Stratagene Europe Amsterdam, The Netherlands); Dulbecco’s minimal essential medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, bacitracin, trypsin/EDTA, Lipopolysaccharide 2000, Opti-MEM, expression vectors pcDNA3.1 (+), pcDNA6-V5-His, and cloning the resulting product into EcoRI and XhoI sites of vector pcDNA3.1 (+). Alanine substitutions were introduced into the LAMP-1-CLN3-CD2 cDNA using the QuikChange™ site-directed mutagenesis kit with the following oligonucleotides: CD2-Leu253Ala,CD2-Ile254Ala-For,GCCAGCGCGCGGAGCC, and Rev, CGGGGCCATGGGCTGTGGTCCGGCACCGCGAGCGGCCGCGCCG

**Preparation of GST Fusion Proteins**—The individual CLN3-CD3 domains were amplified with Ffl polymerase using the following primers: CD1-For, GCCGCTAGCGGCGGAGGCCCAGAGCGCCCGAGCCCGAGAGAGGAGGAG; (B) CD1-Rev,GCCAGCGCGCGGAGGCCCAGAGCGCCCGAGCCCGAGAGAGGAGGAG; (B) CD2-For,GGATCCGCATCTGCGCTGCCCGAGGCC, and CD2-Rev,GCAGCGCGCGGAGGCCCAGAGCGCCCGAGCCCGAGAGAGGAGGAG; (B) CD3-For,GGATCCGCATCTGCGCTGCCCGAGGCC, and CD3-Rev,GCAGCGCGCGGAGGCCCAGAGCGCCCGAGCCCGAGAGAGGAGGAG.

**LAP-CLN3 chimera**—The preparation of cytosolic fractions from rat and pig brain was performed as described previously (42). For pull-down assays, 200 µg of GST or GST-CLN3-CD3 fusion proteins were bound to 50 µl of glutathione-agarose in a final volume of 500 µl of PBS for 1 h at 4 °C. After washing, the beads were incubated with 500 µl of brain cytosol (1 mg/ml) in lysis buffer (25 mM HEPES-KOH, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mg/ml glucose, 0.1 mM EDTA containing protease inhibitor mixture) for 2 h at 4 °C. After washing twice with 500 µl of lysis buffer containing 0.5% Triton X-100, the beads were solubilized, and one-third of the supernatants was subjected to SDS-PAGE, transferred onto nitrocellulose, and subjected to AP-1, AP-3, and GGA3 Western blotting.

**Antibodies**—Polyclonal antibody to human CLN3 (antibody 242) was raised in rabbits (Eurogentec, Belgium), using as immunogen keyhole limpet hemocyanin coupled to the synthetic peptide corresponding to human CLN3 amino acid sequence 242–258. The antibody was affinity-purified on columns containing GST-CLN3 aa 235–280 fusion protein coupled to Affi-Gel 10 (Bio-Rad). The monoclonal anti-hamster LAMP-2 (UH3) and anti-human LAMP-1 (H4A3) antibodies developed by Thomas August (The John Hopkins University, Baltimore), which were obtained from the Developmental Studies Hybridoma Bank developed with the help of the NICH, National Institutes of Health, and maintained by the University of Iowa (Iowa City), were used for immunofluorescence and Western blot analyses, respectively. The anti-rat LAMP-1 antibody (43) was kindly provided by Dr. Y. Tanaka (Kyushu University, Fukuoka, Japan). The polyclonal antibody against human LAP was a kind gift from Dr. von Figura (University of Göttingen). Antibodies used for double immunofluorescence stainings were diluted
as follows: polyclonal rabbit anti-rat LAMP-1 (1:200), monoclonal anti-protein-disulfide isomerase (PDI, 1:800, StressGen, Victoria, Canada), and monoclonal anti-GM130 (1:100, Transduction Laboratories). Secondary antibodies conjugated to horseradish peroxidase for Western blot analysis and fluorochrome-conjugated antibodies used for immunofluorescence were purchased from Dianova (Hamburg, Germany). Secondary antibodies coupled to fluorochrome were applied using the following dilutions: anti-mouse Cy3 (1:2000), anti-rabbit fluorescein isothiocyanate (1:100).

**Cell Culture and Transfection**—HeLa cells (ATCC, Manassas, VA) were cultured in DMEM containing 10% FCS and antibiotics at 37 °C and 5% CO₂. Cells were seeded on 35-mm plates at a density of 4 × 10⁴ cells/plate and transiently transfected with 0.4 µg of pcDNA3.1(+) LAMP-1-CLN3 chimERIC constructs and 10 µl of Effectene. Forty eight hours after the start of transfection, cells were lysed in 100 µl of lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1 mM EDTA, protease inhibitor mixture) and incubated for 30 min on ice. After centrifugation for 10 min at 21,000 × g, the supernatants were analyzed by Western blotting. BHK cells (4.8 × 10⁶ cells/plate) were transfected with 2 µg of wild type LAP and LAP chimera in vector pcdNA6V5 using 4 µl of Lipofectamine 2000. Twenty four hours after the start of transfection, cells were either pulse-labeled with [35S]methionine or processed for cell surface biotinylation. For stable transfections, 4.8 × 10⁵ CHO cells (ATCC) were transfected with 4 µg of each linearized pcDNA6-V5-LAMP-1-CLN3-chimeric constructs and 8 µl of Lipofectamine 2000 in Opti-MEM medium. Twenty four hours after transfection, cells were trypsinized and diluted 1:10 on 60-mm plates with DMEM containing 10% FCS and 6 µg/ml blasticidin. Ten days after transfection, single colonies were picked, and expression of LAMP-1-CLN3 chimera was tested by immunofluorescence microscopy. At least two different cell clones of two independent transfections were tested in each experiment.

**Immunofluorescence Microscopy**—CHO cells stably expressing LAMP-1 and LAMP-1-CLN3-CD constructs were grown on glass coverslips in 24-well chambers and cultivated in DMEM containing 10% FCS, antibiotics, and 6 µg/ml blasticidin at 37 °C and 5% CO₂. The cells were washed with 10 mM PBS, 5 mM glucose, fixed with methanol for 5 min on ice, washed three times with PBS, and blocked in PBS containing 2% albumin (PBS/BSA). Subsequently, cells were incubated with primary antibodies against LAMP-1, LAMP-2, PDI, and GM130 diluted in PBS/BSA as indicated for 1 h at room temperature. After washing with PBS/BSA, cells were incubated with secondary antibodies conjugated to the fluorochromes Cy3 and fluorescein isothiocyanate for 1 h at room temperature as indicated. After washing with PBS, the cells were mounted in Fluoromount (DAKO) and analyzed using a Zeiss Axiovert S100 microscope (Carl Zeiss, Göttingen, Germany) equipped with an Olympus dp50 digital camera.

**Cell Surface Biotinylation**—BHK cells expressing wild type LAP and LAP-CLN3-CD chimera were washed four times with PBS and incubated with the non-membrane-permeable NHS-LC biotin (1.3 mg/ml PBS) for 45 min at 4 °C to specifically biotinylate proteins localized at the cell surface. After washing, the cells were scraped in PBS, sedimented by centrifugation, and lysed in 250 µl of PBS containing 1% Nonidet P-40 and protease inhibitors at 4 °C. The cell extract was centrifuged at 21,000 × g for 10 min and the supernatant removed. Equal amounts of total protein (300 µg) were mixed with streptavidin-agarose (50% w/v) at 4 °C for 1 h with constant rotation. Subsequently, the beads were washed five times with 500 µl of PBS containing 0.1% SDS. After solubilization, the precipitates were resolved by SDS-PAGE and transferred onto nitrocellulose, followed by LAMP Western blotting. To quantify the amount of LAP localized at the cell surface, 6.5% of the total cell lysate was analyzed in parallel. Immunoreactive bands were quantified by densitometric scanning (Hewlett-Packard Scan Jet 4c IT) using the AIDA 2.11-software (Raytest, Deisenhofen, Germany).

**Metabolic Labeling and Immunoprecipitation**—For pulse-chase experiments, 35-mm dishes of BHK cells expressing wild type and LAP-CLN3-CD chimera were starved for 1 h in methionine-free DMEM and labeled for 2 h with [35S]methionine (0.1 µCi). After a chase of 17 h in complete medium supplemented with 0.25 mg/ml methionine, cells were harvested, and cell extracts were subjected to immunoprecipitation using LAMP-specific antiserum LS-4 (39, 44). Immunoprecipitates were solubilized and subjected to SDS-PAGE followed by fluorography. The radiolabeled polypeptides were quantified by densitometry.

**RESULTS**

**LAMP-1-CLN3-CD Chimera Are Expressed in HeLa Cells**—In non-neuronal cells, CLN3 is localized in lysosomes (7). The CLN3 protein contains three or four proposed cytoplasmic domains (CD) accessible for interaction with cytoplasmic proteins involved in lysosomal trafficking (4–6). To study the importance of the isolated CLN3-CDS, we constructed chimera between the luminal and the transmembrane domain of rat LAMP-1 and the CLN3-CDS (Fig. 1). LAMP-1 is a major component of the lysosomal membrane and carries a tyrosine-based sorting signal critical for lysosomal sorting in its C-terminal domain (45). To investigate expression, stability, and correct glycosylation of the chimeric constructs, we transiently transfected HeLa cells. When wild type rat LAMP-1 cDNA was transfected, a 100-kDa immunoreactive band was detected in Western blot analyses which was absent in nontransfected cells (Fig. 2). Although the nonglycosylated LAMP-1 has a molecular mass of 45 kDa (45), the appearance of the 100-kDa immunoreactive protein demonstrates its glycosylation and transport through the Golgi. These data also indicate that the anti-rat LAMP-1 antibody shows no cross-reactivity with endogenous human LAMP-1. After transfection of LAMP-1-CLN3-CD1, -CD2, -CD3 constructs, proteins with similar molecular masses as the wild type LAMP-1 were detected indicating expression, translocation into the ER, and correct glycosylation of the chimeric proteins. The reduced immunoreactivity of LAMP-1-CLN3-CD3 suggests a reduced stability compared with wild type LAMP-1 (Fig. 2A). The antibody 242 raised against amino acid residues 242–258 of CLN3 only detected the LAMP-1-CLN3-CD2 fusion protein (Fig. 2B). This antibody did not bind CLN3 in permeabilized fixed cells nor could the antibody be used for immunoprecipitation. Western blot analyses with an antibody against the endogenous human LAMP-1 (Fig. 2C) confirmed equal loading.

**The Cytoplasmic CLN3-CD2 Domain Contains Lysosomal Sorting Motifs**—CHO cells were stably transfected with cDNAs of wild type LAMP-1 and the LAMP-1-CLN3 chimera (Fig. 1), and the subcellular localization of the proteins was studied by double immunofluorescence microscopy (Fig. 3). As expected, wild type LAMP-1 colocalized with the endogenous lysosomal marker protein LAMP-2, indicating correct sorting to the lysosome in transfected CHO cells. Mock-transfected CHO cells showed no LAMP-1 staining, indicating no cross-reactivity of the antibody with endogenous hamster LAMP-1 (not shown). When the cytoplasmic domain of rat LAMP-1 was substituted with either CLN3-CD1 or CLN3-CD3, an increase in cell surface staining was observed in addition to the punctate intracellular localization of the constructs (Fig. 3). The surface localization of the chimera CD1 and CD3 was also verified in immunofluorescence experiments with fixed CHO cells in the absence of the permeabilizing detergent saponin (not shown). In contrast, the LAMP-1-CLN3-CD2 chimera colocalized almost completely with LAMP-2, indicating a lysosomal localization of this protein (Fig. 3). No co-staining was observed with the ER marker PDI and the cis-Golgi marker GM130. We conclude that the CLN3-CD2 is sufficient to direct a chimeric protein to lysosomes. To define the lysosomal sorting signal in CD2 the potential leucine-isoleucine motif Leu-253, Ile-254 and the cluster of glutamate residues in positions 242, 243, 244, and 246 were replaced for alanine (Fig. 1). Both expressed mutant chimeric constructs did not colocalize with the lysosomal marker LAMP-2 but showed cell surface staining, indicating a misrouting to the plasma membrane (Fig. 4). No colocalization was observed with the ER marker PDI ruling out a possible retention in the ER (not shown). Thus, both the dileucine and the preceding glutamate residues in position 242–244 are required for effective and correct sorting. In contrast, another mutant CLN3-CD2 construct with alanine substitution of residues Glu-246 and Ser-247 colocalized with LAMP-2 (Fig. 4). To further define amino acid residues in the C-terminal part of
CLN3-CD2 critical for sorting, residues 255–280 (R255) were deleted. The truncated CD2 domain still contains both the dileucine signal and the cluster of glutamate residues (Fig. 1). The truncated mutant CD2 R255 showed colocalization with LAMP-2 suggesting correct sorting to lysosomes (Fig. 4). These results indicate that amino acids 235–254 of the CLN3-CD2 domain, which harbor both the dileucine-based motif and the preceding acidic glutamate residues, are essential and sufficient for sorting LAMP-1-CD2 chimera to lysosomes.

Detection of CLN3-CD Chimera at the Plasma Membrane—To quantify the fraction of CLN3-CD chimera at the cell surface at steady state, biotinylation experiments were performed. BHK cells expressing various CLN3-CD chimera fused to the lumenal and transmembrane domain of LAP, illustrated in Fig. 5, were chilled to 4 °C and cell surface-biotinylated. Biotinylated proteins were then precipitated from cell extracts by means of immobilized streptavidin, separated by SDS-PAGE, and LAP was visualized by Western blotting. Aliquots (6.5%) of the cell extracts prior to streptavidin precipitation were processed in parallel to quantify the total amount of cellular LAP by Western blotting. Human LAP can be detected as diffusely appearing polypeptide with an apparent molecular size of 67 kDa, which is absent in nontransfected control cells (Fig. 6). About 8% of total wild type LAP were located at the cell surface. Both LAP-LN3-CD1 and the dileucine motif mutant LAP-LN3-CD2 L253A, I254A showed an increased expression at the plasma membrane (12 and 13%), respectively. The fraction of the LAP chimera with CLN3-CD2, -CD2 E246A, S247A, and CD2 R255 were significantly reduced at the plasma membrane compared with the wild type LAP. In BHK cells expressing LAP-LN3-CD3, and C-terminally truncated CLN3-CD3-L428X and -L418X chimera, a fraction of 6% was localized at the plasma membrane. These data confirmed the following observations made by immunofluorescence microscopy: (i) CLN3-CD2 is efficiently sorted to the lysosome and the amino acid residues 235–255 are sufficient for effective sorting of CD2 chimera to lysosomes; (ii) alanine substitution of the dileucine motif in CLN3-CD2 results in an increased expression at the plasma membrane; (iii) obviously the CLN3-CD1 contains no lysosomal targeting signal compared with CLN3-CD2. The cytoplasmic tail of the MPR46 fused to the LAP domains was used as a control because the tail contains all information for recycling between TGN, endosomes, and plasma membranes and prevents the transport to the lysosome. About 4–10% of total MPR46 and of mutant MPR46CT E59X are localized at the cell surface, whereas the
majority is found in intracellular, nonlysosomal membranes (46–48), supporting the present data of the LAP-MPR46 CT chimera.

**Lysosome-specific Processing of LAP Chimera**—Lysosomal targeting signals in the cytoplasmic domains of CLN3 have been examined in a third experimental approach using LAP-CLN3-CD chimera transiently expressed in BHK cells. LAP is synthesized as a 63-kDa membrane-bound precursor glycoprotein. After delivery to lysosomes, the precursor is proteolytically processed at the C terminus in two steps by generating the mature, soluble 52-kDa form with a half-time of ~14 h (44, 49). Thus, the sorting of the LAP chimera constructed with different CLN3-CDs to lysosomes can be monitored and quantified by the appearance of the soluble 52-kDa form of LAP. As negative control for nonlysosomal delivery, LAP fused to the cytoplasmic tail of the MPR46 was chosen. To analyze the molecular forms, BHK cells expressing wild type LAP and various LAP chimera were metabolically labeled followed by immunoprecipitation using anti-human LAP antibodies. Under these conditions the majority of wild type LAP could be immunoprecipitated as diffusely appearing precursor form and 8% as mature 52-kDa protein (Fig. 7). The different molecular masses of the immunoprecipitated precursor LAP-CLN3-CD chimera reflect the different lengths of the fused CLN3 cytoplasmic domains. When immunoprecipitated wild type LAP and LAP chimera with CLN3-CD1, -CD2, or -CD3 were treated with endoglucoaminidase H (endo H), the precursor forms of all fusion proteins with the exception of CD3 revealed almost complete endo H resistance (endo H), the precursor forms of all fusion proteins with the exception of CD3 revealed almost complete endo H resistance, indicating the presence of complex-type oligosaccharide chains and the transport to or exit from the Golgi. LAP-CLN3-CD3 exhibited both endo H-sensitive (high mannose-type oligosaccharides most likely retained in the ER) and endo H-resistant forms (not shown). When LAP-CLN3-CD2 was expressed, 30% of the newly synthesized chimer could be immunoprecipitated as mature form indicating that the CD2 domain contributed to an efficient transport to lysosomes. In addition, neither the alanine substitution of the dileucine motif or residues Glu-246 and Ser-247 (L253A,I254A and E246A,S247A) nor the C-terminal truncation of CD2 (R255X) significantly reduced the efficiency of lysosomal delivery and proteolytic processing. In contrast, when the LAP-CLN3-CD3 chimera and C-terminally truncated CD3 mutant (L428X) were expressed, 15 and 13% of total synthesized fusion proteins could be precipitated as mature form. The recovery of the newly synthesized CD3 chimera C-terminally truncated by 20-amino acid residues (L428X) was strongly reduced, suggesting a decreased stability. In BHK cells expressing LAP-MPR46CT chimera, only small amounts of mature forms of LAP could be immunoprecipitated, thus supporting data demonstrating the retention of the MPR46 in nonlysosomal membranes depending on both cytosolic palmitoylation (50) and a diaromatic Phe-18, Trp motif in the cytoplasmic tail (51).

**LAP-CLN3-CD3 Chimeras Are Partially Degraded by Proteasomes**—Western blot analysis of LAMP-1 CLN3-CD3 constructs in HeLa cells (Fig. 2), immunoprecipitation of newly synthesized LAP-CLN3 CD3 chimera (Fig. 7), and the presence of endo H-sensitive N-linked oligosaccharides in LAP-CD3 chimera (not shown) suggest a partial retention and increased degradation proximal to the Golgi. Incubation of BHK cells expressing three LAP-CLN3-CD3 chimera for 15 h in the presence of the peptide aldehyde inhibitor N-acetyl-Leu-Leu-nor-leucinal and the irreversible proteasome inhibitor lactacycin dramatically increased the amounts of immunoreactive fusion proteins (Fig. 8). These results indicate that the degradation of the majority of LAP-CD3 chimera is mediated by proteasomes sensitive to proteasomal inhibitors early after synthesis.

**The CLN3-CD Domains Do Not Interact with the Adaptor Proteins AP-1, AP-3, and GGA3**—The sorting of several integral lysosomal and melanosomal membrane proteins is mediated by the adaptor complex AP-3 (25, 36, 53). Additionally, the brain-specific AP-3 isomorf is required for synaptic vesicle formation from endosomes (54). To investigate the binding of the
different adaptor proteins to the cytoplasmic CLN3 domains, in vitro pull-down assays were performed using individual CLN3-CD domains fused to GST, which were expressed in and purified from E. coli (Fig. 9B). In the pull-down experiments, a GST fusion protein containing the proposed N-terminal domain (CD0) of CLN3 was included. The fusion products were either incubated with cytosolic fractions derived from rat brain (detection of AP-1) or pig brain (detection of AP-3 and GGA3) and followed by analysis for the presence of precipitated adaptor proteins. As a positive control for AP-1, AP-3, and GGA3 binding, the cytoplasmic LIMP-2 domain and the MPR46-CT, respectively, were used. None of the CLN3-CD domains bound significantly to AP-1 and AP-3, whereas the cytoplasmic LIMP-2 domain strongly bound to AP-3 and weakly interacted with AP-1 as expected (Fig. 9A) (42). We also tested interactions with the neuronal specific \( \mu \)3B-subunit of the AP-3 adaptor complex and the CLN3-CDs, but none of the domains interacted with this subunit (not shown). Furthermore, none of the CLN3-CDs bound to GGA3, which could be specifically precipitated by the MPR46-CT. These results indicate that individual cytoplasmic domains of CLN3 do not directly interact with the heterotetrameric adaptor complexes AP-1 and AP-3 or monomeric GGA3 despite their capability to direct fusion proteins to lysosomes.

**DISCUSSION**

The results presented in this study demonstrate that the sorting signal of the multispanning membrane protein CLN3 mediating the most efficient transport of chimeric proteins to lysosomes is localized in the second cytoplasmic domain (CD2). Within this domain both the leucine-isoleucine motif Leu-253, Ile-254 and the preceding acidic glutamate residues Glu-242, Glu-243, and Glu-244 are important for lysosomal targeting. Mutation of these residues increases the steady state concen-
targeting of newly synthesized integral LAP chimera precursor forms of −68 kDa was followed by immunoprecipitation of the radiolabeled polypeptides and the lysosome-specific proteolytic cleavage of the C terminus resulting in the formation of the soluble 52-kDa mature LAP form. The first experimental approach led to the identification of a major dileucine-based lysosomal sorting motif in CD2 of CLN3. Two types of dileucine-based sorting signals have been identified in C-terminal cytoplasmic domains of several membrane proteins so far. The DXXLL motif present in the cytoplasmic tails of the mannose 6-phosphate receptors is essential for sorting in the TGN and endosomes and is recognized by the cytosolic monomeric Golgi-localized, γ-ear-containing, ARF-binding proteins (GGA) (48, 55). A second dileucine motif, (DE)XXXL(LI), is critical for targeting transmembrane proteins, such as LIMP-2, tyrosin-
ase, TRP-1, and QNR71, to lysosomes and lysosome-related organelles (22, 23, 56, 57). The lysosomal sorting signal identified in CD2 of CLN3 differs from the former by an absence of aspartate or glutamate residues at position H11002 relative to the dileucine motif, its localization to a cytosolic loop, and the distance to the transmembrane domain. Although the acidic residue of H11002 is not always essential for correct sorting (see this work and Ref. 56), the three essential glutamate residues at positions H11002 to H11002 relative to the first leucine in CLN3-CD2 suggest the presence of a third type of dileucine motif, EEEEX8L(LI), that is conserved in the CLN3 sequence among all vertebrate species (58). A similar motif, EDEEXEEXXXXLL, has been identified in the cytoplasmic domain of the insulin-regulated aminopeptidase that is, however, required for retention in a slowly endosomal recycling compartment (59). Therefore, it is likely that the lysosomal sorting of the multispanning CLN3 requires different signal structures than membrane proteins spanning the membrane once or twice (Fig. 10) or requires more than one signal. During the course of this work, Luzio and co-workers (6) reported the identification of the same dileucine motif in the second cytoplasmic domain of CLN3 as a lysosomal targeting signal by using double immunofluorescence microscopy. Additionally, the authors described a second unconventional motif in the C-terminal CD3 domain consisting of a methionine (Met-409) and a glycine (Gly-419) separated by nine amino acids, M(X)9G, sufficient to mediate lysosomal targeting, which is completely abolished by the C-terminal deletion of 20 amino acid residues (L418X). However, the analysis of our LAP chimera revealed a delay in transport kinetics of the CLN3-CD3 constructs to the lysosome monitored by the proteolytic maturation compared with CD2 chimera accompanied by an increased steady state concentration at the plasma membrane. Furthermore, all CLN3-CD3 constructs showed reduced stability resulting in low ER exit rates and proteasome-mediated degradation, which has also been suggested for the CDS-CLN3-CD3 chimera expressed in normal rat kidney cells (6).

The full-length CLN3 protein with an intact dileucine motif and the substitution of both Met-409 and Gly-419 by alanine was mostly found in lysosomes (6), indicating the marginal role of the CLN3-CD3 domain in lysosomal targeting. Furthermore, the substitution of the dileucine-based motif in the full-length

![FIG. 6. Cell surface biotinylation of wild type and chimeric LAP. BHK cells expressing wild type LAP and the indicated LAP-CLN3-CD chimera were cell surface-biotinylated for 30 min at 4 °C. After lysis, 6.5% of the cell extracts were removed to quantify the total amount of LAP. From the remaining lysates biotinylated proteins were precipitated using immobilized streptavidin and analyzed by Western blotting. Immuno-reactive bands were quantified densitometrically, and the fraction of surface LAP was expressed as percentage of total LAP.](image)

![FIG. 7. Proteolytic processing of LAP chimera. BHK cells expressing wild type and mutant LAP-CLN3-CD were labeled for 2 h with [35S]methionine (0.15 mCi/ml) followed by a chase for 17 h. Cell extracts were prepared, and equal amounts of radioactivity incorporated in cellular proteins were used for immunoprecipitation with the LS-4 antiserum raised against the luminal domain of LAP. As control, LAP fused to the C-terminally truncated cytoplasmic domain of the MPR46-CT (LAP-MPR46-CT E59X) was processed in parallel. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. Radioactive bands were quantified densitometrically, and the mature, lysosomal forms of LAP (M) were expressed as percentage of the total amount of immunoprecipitated precursor (P) and mature LAP.](image)

![FIG. 8. Peptide-aldehyde and lactacystin inhibit the degradation of LAP-CLN3-CD3 chimera. Twenty four hours after the start of transfection, BHK cells expressing LAP-CLN3-CD3 and C-terminally truncated CD3-L428X and CD3-L418X chimeras were incubated in the presence (+) or absence (−) of 50 μM N-acetyl-Leu-Leu-norleucinal (ALLN) or 10 μM lactacystin, respectively, for 15 h. After cell lysis, 20 μg of total protein extract were separated by SDS-PAGE, transferred to nitrocellulose, and processed for LAP Western blotting.](image)
SDS-PAGE, transferred to nitrocellulose, and processed for Western blotting. Five and 30 μg of AP-1, or pig brain (for detection of AP-3 and GGA3). The beads were washed and solubilized. One-third of the supernatants were separated by SDS-PAGE, transferred to nitrocellulose, and processed for Western blotting. Five and 30 of AP-1) or pig brain (for detection of AP-3 and GGA3). The beads were washed and solubilized. One-third of the supernatants were separated by SDS-PAGE, transferred to nitrocellulose, and processed for Western blotting. Five and 30 μg of AP-1, or pig brain (for detection of AP-3 and GGA3).

Lysosomal Sorting Motif of CLN3

Fig. 9. GST-CLN3-CD constructs fail to interact with brain-derived adaptor complexes. A, 200 μg of GST and GST-CLN3-CD fusion protein were coupled to glutathione-agarose. GST-LIMP-2 and GST-MPR46-CT were used as positive controls for AP-1/AP-3 and GGA3-binding, respectively. The immobilized GST-fusion proteins were incubated for 2 h at 4 °C with cytosolic fractions derived either from rat brain (for detection of AP-1) or pig brain (for detection of AP-3 and GGA3). The beads were washed and solubilized. One-third of the supernatants were separated by SDS-PAGE, transferred to nitrocellulose, and processed for Western blotting. Five and 30 μg of rat and pig brain cytosol, respectively, were applied for comparison. B, aliquots of the GST fusion proteins purified from E. coli were separated by SDS-PAGE and stained with Coomassie Blue.

Fig. 10. Sequence alignment of lysosomal/melanosomal membrane proteins. The dileucine-based sorting motif of CLN3-CD2 differs from (DE)(XXL/LL) consensus motifs identified in cytoplasmic domains of other lysosomal and melanosomal membrane proteins. Dileucine-based motifs in shaded boxes and surrounding residues are shown including glutamate residues (cursive) located at position −4 relative to the first leucine.

CLN3 resulted only in a partial missorting to the plasma membrane, whereas the majority of mutant CLN3 was found in lysosomes (6), leading to the conclusion that additional signal structures mediate the lysosomal targeting. To our knowledge, there is only one well described example, the lysosomal cystine transporter cystinosin with seven transmembrane domains, which requires distinct sorting signals (21). Its targeting to the lysosome depends on a classical C-terminal tyrosine-based sorting motif, GYDQL, and a novel conformational motif, YF-

Some lysosomal membrane proteins such as LAMP-1, LIMP-2, QNR-71, and tyrosinase are sorted to endosomes/lysosomes via a direct transport route with a minor pathway via the plasma membrane (22, 23, 38, 39, 62), whereas others, e.g. LAP, are transported to lysosomes indirectly via the recycling pathway and the cell surface (37). From cell surface biotinylation experiments on cells expressing LAP-CLN3-CD2 or CD3 chimera, 3% and 5% of total cellular chimera, respectively, were detected at the cell surface. The concentration of LAP-CD1 or chimeric LAP with a mutated dileucine motif in CD2 was significantly increased at the cell surface. The relative amounts of the chimera at the plasma membrane are similar to the steady state concentration of LAP at the cell surface (8–10% of total) (37). In contrast to LAP, however, it is unclear at present whether the low number of CLN3-CD chimera proteins is transported to the lysosome via the plasma membrane or represents missorted proteins accumulating at the plasma membrane because of the lack of tyrosine-based signals in each of the predicted cytoplasmic domains of CLN3 (6) required for efficient internalization and transport along the endocytic pathway. Nevertheless, the substitution of the tyrosine 380 residue in a putative tyrosine-based sorting motif by alanine did not affect the lysosomal targeting of full-length CLN3 in HeLa cells [2] (6).

(DE)(XXL/LL) signals have been reported to interact with hemicomplexes of AP-1 and AP-3 subunits and intact AP-3 (15, 42, 52). The adaptor complex recognizing the EEEX_LI motif in CLN3-CD2 is still unknown. The present GST pull-down experiments have demonstrated that none of the CLN3 cytoplasmic domains interact directly with the brain-derived heterotetrameric adaptor complexes AP-1, AP-3, and GGA3, whereas the cytoplasmic domain of LIMP-2 containing a leucine-isoleucine-based sorting motif with acidic residues in positions −4, −5, and −9 relative to the first leucine strongly bound AP-3 and weakly bound AP-1 complexes, confirming BIAcore data with purified adaptor complexes (42). In preliminary experiments, the full-length wild type CLN3 colocalized completely with LAMP-1 in AP-3-deficient embryonic fibroblasts derived from mocha mice [3].

Taken together, the efficient transport of CLN3 to lysosomes appears to require two sorting signals, a major dileucine motif EEEX_LI in the second cytoplasmic domain CD2 and a minor signal structure in the C-terminal CD3 domain. Although low amounts of chimeric CLN3-CD proteins are present at the cell surface, it is unclear whether CLN3 is transported to the lysosomal/vacuolar system by an AP-3-dependent route.

\[^{2}\] S. Storch, unpublished results.

\[^{3}\] S. Pohl and S. Storch, unpublished results.
some via the indirect pathway. The failure to document interactions of cytoplasmic domains of CLN3 with adaptor complexes AP-1, AP-3, or GGA3 in vitro and the localization of CLN3 in additional nonlysosomal compartments in neuronal cells suggest the existence of distinct/novel adaptor complexes or more complex structural requirements in CLN3 to mediate the directed transport to target compartments.

Acknowledgments—The anti-rat LAMP-1 antibody and the cDNA coding for rat LAMP-1 were kindly provided by Dr. Tanaka (Kyushu University, Fukuoka) and Dr. Saftig (University of Kiel), respectively. We thank Dr. von Figura (University of Go¨ttingen) for providing antibodies against the AP-3 alpha subunit and GGA3. The cDNA coding for the chimeric LAP-MPR46-CT E59 (thesda) for antibodies against the AP-3 was kindly provided by Dr. Tanaka (Kyushu University, Fukuoka) and Dr. Saftig (University of Kiel), respectively.

REFERENCES

1. Goebel, H. H., Male, S. E., and Lake, B. D. (eds) (1999) The Neuronal Ceroid Lipofuscinoses (Batten Disease): Biomedical and Health Research, Vol. 33, IOS Press, Amsterdam.

2. The International Batten Disease Consortium (1995) Cell 82, 949–957.

3. Palmer, D. N., Pearley, I. M., Walker, J. E., Hall, N. A., Lake, B. D., Wolfe, L. S., Haltia, M., Martinus, R. D., and Jelly, R. R. (1992) Am. J. Med. Genet. 42, 561–567.

4. Mao, Q., Foster, B. J., Xia, H., and Davidson, B. L. (2003) FEBS Lett. 541, 40–46.

5. Easaki, J., Takeda-Easaki, M., Kikue, M., Ohawa, Y., Taka, H., Mineki, R., Murayama, K., Uchiyama, Y., Ueno, T., and Kominami, E. (2003) J. Neurochem. 87, 1296–1308.

6. Kyt¨alla, A., Ihrke, G., Vesa, J., Schell, M. J., and Luzio, J. P. (2004) J. Biol. Chem. 279, 6622–6631.

7. Jarvela, I., Sainio, M., Rantamaki, T., Olkkonen, V. M., Carpen, O., Peltonen, L., and Jalkanen, A. (1998) Hum. Mol. Genet. 7, 85–90.

8. Lario, K., Krop, O., Lehtovirta, M., and Jalkanen, A. (2001) Hum. Mol. Genet. 10, 2123–2131.

9. Mao, Q., Xia, H., and Davidson, B. L. (2003) FEBS Lett. 555, 351–357.

10. Pearce, D. A., Ferea, T., Nosel, S. A., Das, B., and Sherman, F. (1999) Nat. Genet. 22, 55–58.

11. Golabek, A. A., Kida, E., Walus, M., Kaczmariski, W., Michalewski, M., and Wisniewski, K. E. (2000) Mol. Metab. 70, 203–213.

12. Persaud-Sawin, D. A., Van Dungen, A., and Boustany, R. M. (2002) Hum. Mol. Genet. 11, 2129–2142.

13. Kim, Y., Ramirez-Montaileague, D., and Pearce, D. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 15458–15462.

14. Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I., and Hellenius, A. (1987) J. Cell Biol. 104, 875–886.

15. Eskelinen, E. L., Tanaka, Y., and Saftig, P. (2003) Trends Cell Biol. 13, 137–145.

16. Bonifacio, J., and Traub, L. M. (2003) Annu. Rev. Biochem. 72, 395–447.

17. Williams, M. A., and Fukuda, M. (1990) J. Cell Biol. 111, 955–966.

18. Fukuda, M., Viitala, J., Matteson, J., and Carlsson, S. R. (1988) J. Biol. Chem. 263, 18920–18923.

19. Peters, C., Braun, M. Weber, B., Wendland, M., Schmidt, B., Pohlmann, R., Waheed, A., and Fukuda, V. (1999) EMBO J. 9, 3497–3506.

20. Metzelaar, M. J., Wijngaard, P. L., Peters, P. J., Sixma, J. J., Nieuwenhuis, H. K., and Clevers, H. C. (1991) J. Biol. Chem. 266, 3239–3245.

21. Cherqui, S., Kalaitzi, V., Trugnan, G., and Antignac, C. (2001) J. Biol. Chem. 276, 13314–13321.

22. Sandoval, I. V., Arredondo, J. J., Alcalde, J., Gonzalez Noriega, A., Vandekeereck, J., Jimenez, M. A., and Rico, M. (1994) J. Biol. Chem. 269, 6622–6631.

23. Simmen, T., Schmidt, A., Hunziker, W., and Beermann, F. (1999) J. Cell Sci. 112, 45–53.

24. Robinson, M. S., and Bonifacio, J. S. (2001) Curr. Opin. Cell Biol. 13, 444–453.