CLN3 is a transmembrane protein with a predominant localization in lysosomes in non-neuronal cells but is also found in endosomes and the synaptic region in neuronal cells. Mutations in the CLN3 gene result in juvenile neuronal ceroid lipofuscinosis or Batten disease, which currently is the most common cause of childhood dementia. We have recently reported that the lysosomal trafficking of CLN3 is facilitated by two targeting motifs: a dileucine-type motif in a cytoplasmic loop domain and an unusual motif in the carboxyl-terminal cytoplasmic tail comprising a methionine and a glycine separated by nine amino acids (Kytta¨la¨, A., Ihrke, G., Vesa, J., Schell, M. J., and Luzio, J. P. (2004) Mol. Biol. Cell 15, 1313–1323).

In the present study, we investigated the pathways and mechanisms of CLN3 sorting using biochemical binding assays and immunofluorescence methods. The dileucine motif of CLN3 bound both AP-1 and AP-3 in vitro, and expression of mutated CLN3 in AP-1- or AP-3-deficient mouse fibroblasts showed that both adaptor complexes are required for sequential sorting of CLN3 via this motif. Our data indicate the involvement of complex sorting machinery in the trafficking of CLN3 and emphasize the diversity of parallel and sequential sorting pathways in the trafficking of membrane proteins.

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CLN3 is a polytopic membrane protein mostly resident in lysosomes (1, 2). Mutations in the CLN3 gene cause an accumulation of autofluorescent lipofuscin in all tissues, affecting the central nervous system most severely, and result in a severe neurodegenerative disorder, juvenile neuronal lipofuscinosis (JNCL) or Batten disease (3). CLN3 is highly conserved from yeast to human, and therefore it has been suggested to have a fundamental role in cellular metabolism. Although sequence comparisons have shown a distant relationship between CLN3 and a family of equilibrative nucleoside transporters (4), experimental studies on CLN3 function have implied that CLN3 could have a role in the regulation of intraorganellar pH (5, 6), in arginine transport (7), or in membrane trafficking (8, 9). However, the exact function of the protein has remained unclear.

We have recently reported that the lysosomal trafficking of CLN3 in non-neuronal and neuronal cells is mediated by two targeting motifs located in the last cytoplasmic loop domain and in the long carboxyl-terminal cytoplasmic tail of the protein (2) (Fig. 1). Whereas the motif in the loop domain is a traditional dileucine-type motif LI, with an upstream acidic patch in an unusual position, the motif in the tail represents a novel type of targeting motif consisting of methionine and glycine residues separated by nine other amino acids (Fig. 1). CLN3 has been suggested to have a role also outside the lysosomes, especially in neurons, because endogenous CLN3 was located to the synaptic region in a subcellular fractionation analysis of the mouse brain (10), and transfected CLN3 was shown to localize to early endosomes in cultured rat hippocampal neurons by immunostaining methods (2). Deletion of both lysosomal targeting motifs of CLN3 abolished the lysosomal trafficking of the protein in neurons but did not detectably affect its targeting to early endosomes (2).

Trafficking of newly synthesized lysosomal membrane proteins from the trans-Golgi network (TGN)1 to lysosomes may occur indirectly, via the plasma membrane followed by endocytosis, or directly, via the endosomal system. Two major types of signal motif present in short cytoplasmic tails facilitate the targeting of many lysosomal membrane proteins: the GYXXΦ motif, in which Φ can be any amino acid residue and Φ is a bulky hydrophobic residue, and dileucine motifs (11). Unusual lysosomal targeting motifs have also been reported in a small number of other lysosomal membrane proteins (2, 12, 13). The correct sorting of proteins destined for late endocytic organelles is most often facilitated by specific recognition of their signal motifs by heterotetrameric adaptor proteins, AP-1, -2, -3, and -4, or by monomeric Golgi-localized, ω-ear-containing, ARF-binding proteins (GOAφs) (14–16). Although the recognition and binding of the heterotetrameric adaptors to tyrosine-based signal motifs via their μ-subunits is well characterized (17–19), much less is known about the recognition of the dileucine-based motifs. Conflicting data have been presented about which of the four adaptor subunits recognize(s) and bind(s) to dileucine motifs (20, 21). Recent data have suggested that the γ5( of the AP-1 and AP-3, respectively) and σ-subunits may both be involved in recognition of the dileucine-type motifs (22). While AP-2 has been reported to function mainly in clathrin-mediated endocytosis at the plasma membrane, the sites of action of

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1 The abbreviations used are: TGN, trans-Golgi network; GST, glutathione S-transferase; NRK, normal rat kidney; PFA, paraformaldehyde; GFP, green fluorescent protein.
FIG. 1. Lysosomal targeting motifs of CLN3. A schematic picture showing the localization of the lysosomal targeting motifs of the human CLN3 protein. The dileucine-type motif, LI (gray box) is located in the last cytoplasmic domain of the protein. The second potential targeting motif consists of methionine and a glycine (gray boxes) separated by nine amino acids and is located within the long carboxyterminal cytoplasmic tail of CLN3.

other heterotetrameric adaptor proteins have remained controversial. Originally, AP-1, -3, and -4 were thought to facilitate trafficking of proteins from the TGN, but studies in adaptor-deficient cell lines with wild type, mutated, and chimeric membrane proteins (23, 24) together with analyses of the localization of the adaptor proteins (25–28) have suggested that, in addition to the TGN, AP-1 and AP-3 also have sorting functions at the level of endosomes.

In the present study, we have addressed the trafficking and sorting of CLN3. We have examined the trafficking of different CLN3 polypeptides, carrying mutations in either one or both targeting motifs, in cell lines deficient for the adaptor proteins AP-1 or AP-3. We show that the dileucine motif of CLN3 binds both AP-1 and AP-3 in vitro, and a deficiency of either one of these adaptor results in mistargeting of CLN3 when sorting solely depends on its dileucine motif. The current data provide novel information toward understanding the cellular pathways and organelles in which CLN3 could function and emphasize the diversity of sorting mechanisms utilized in trafficking of membrane proteins.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Normal rat kidney (NRK) cells stably expressing wtCLN3 have been described earlier (2). Stable cell lines in AP-3-deficient mocha (29) and AP-1-deficient Δ41A mouse fibroblasts (23) were established by transfecting with different CLN3 cDNA constructs in ΔMP4 (see below) using FuGENE6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions. All stable cell lines were established and maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, 0.5 mM t-glutamine, 50 μg/ml streptomycin, 100 U/ml penicillin, and 100 μg/ml hygromycin B (Clontech). For transient expression in mocha and AP-3ΔβA-deficient pearl cells (30), the cells were transfected as above and were analyzed 40–72 h later.

Recombinant cDNA Constructs—Human CLN3 cDNA was expressed in pCMV5 vector in all transient expression experiments (2) in mocha and pearl cells. The alanine substitutions of the targeting motifs (L253A/I254A (≈ MX3G only) and M409A + G419A (≈ LI only) were made in vitro using a QuikChange site-directed in vitro mutagenesis kit (Stratagene) as described earlier by Kytta et al. (2). For stable expression in mocha and Δ41A cells, the different CLN3 cDNAs were cloned into ΔMP4 vector, which contains a promoter inducible with divalent cations (31). The green fluorescent protein (GFP)-tagged mutated Eps15 cDNA construct, GFP-EPS15/ΔMP4 (32), used for inhibition of clathrin-mediated endocytosis in NRK cells, was kindly provided by Dr. A. Benmerah (Paris, France). The monoclonal antibody to c-MYC (9E10) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lysosomes of mocha, pearl, and Δ41A cells were detected with monoclonal rat anti-mouse LAMP-1 antibody (1D4B) obtained from the Developmental Studies Hybridoma Bank (University of Iowa). The Golgi complex was stained with a monoclonal antibody against the Golgi matrix protein 130 kDa (GM130, PharMingen). Monoclonal antibodies to AP-1γ and AP-2α were from BD Biosciences (Pharmingen). AP-3 was detected either with a monoclonal mouse antibody against the δ-subunit (SA4) from the Developmental Studies Hybridoma bank (University of Iowa) or the purified rabbit antibody recognizing δ-adaptin (34), kindly provided by Dr. A. Peden (Genentech). All secondary conjugated antibodies to mouse, rabbit, or rat IgG used in the immunofluorescence analyses were from Jackson ImmunoResearch (West Grove, PA). The hors eradish peroxidase-conjugated secondary antibodies were from DAKO (Denmark).

Inhibition of Clathrin-mediated Endocytosis and Transferrin Uptake—NRK-wtCLN3ΔMP4 cells (2) were grown on coverslips and were transfected with 4% FPA 45–72 h after transfection with different CLN3ΔMP4 constructs. In stably transfected cells, the expression of the ΔMP4 construct was induced by adding 5 μM CdCl2 to the culture medium. Twenty-four hour after transfection, the cells were starved in serum-free Dulbecco's modified Eagle's medium for 2–4 h and then were incubated for 20 min in serum-free Dulbecco's modified Eagle's medium containing 25 μg/ml Alexa594-conjugated human serum transferrin (Molecular Probes). The cells were then washed and subsequently fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline for immunofluorescence analysis.

Immunofluorescence Labeling and Microscopy—For immunofluorescence analysis, transiently transfected mocha and pearl cells were grown on coverslips and were fixed with 4% PFA 45–72 h after transfection with different CLN3ΔMP4 constructs. In stably transfected cells, the expression of the ΔMP4 construct was induced by adding 3 μM CdCl2 to the culture medium 16–48 h before fixation or before inhibiting protein synthesis with cycloheximide to allow chasing of proteins to their sites of residence. The cytochrome b was performed by adding 50 μg/ml cycloheximide 20 h before fixation. The PFA-fixed cells were then permeabilized with 0.2% saponin, which was then washed once in all solution during the labeling process. The labeled coverslips were mounted in GelMount (Biomeda Corp., Foster City, CA) and were visualized with a Zeiss Axioplan microscope equipped with a CCD camera (Fig. 2) or confocal microscopes Bio-Rad MRC 1024 or Leica DMR. Adobe Photoshop software was used for image processing.

GST Pull-down Experiments—For in vitro assays, the wild type and mutated (L253A/I254A) CLN3loop domains (see above) were produced as GST fusion proteins in Ε. coli and immobilized by binding to glutathione-Sepharose 4B beads (Amersham Biosciences) overnight at 4 °C. The cytosolic extract of HeLa cells was prepared by lysing the cells in a lysis buffer (25 mM Hepes, pH 7.4, 0.5 mM MgCl2, 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100) and removing the cell debris by centrifugation. The mouse liver extract was prepared by homogenization in 8 M urea, 0.1% sodium dodecyl sulfate, 0.5 mM Hepes-KOH, pH 7.4, 100 mM NaCl, 5 mM MgCl2, and 0.5% Triton X-100 followed by centrifugation at 100,000 × g for 30 min in a Beckman TL-A100.3 rotor. GST pull-down experiments were performed by incubating the GST fusion proteins either with 2 ml of cytosolic extract of HeLa cells (~2 mg/ml) or with 0.5 ml (~10 mg/ml) of mouse liver extract (diluted to 2 ml with extraction buffer) overnight at 4 °C. The beads were washed five times with lysis or extraction buffer, and samples were separated by 10% SDS-PAGE, immunoblotted, and probed with antibodies to different adaptor protein subunits (see above).

RESULTS

Determination of the Intracellular Trafficking Route(s) of CLN3—Newly synthesized lysosomal membrane proteins traffic to lysosomes either directly from the TGN or indirectly via the plasma membrane. Investigation of the possible indirect route taken by the CLN3 protein by antibody uptake experiments was not possible, because all the antibodies we have produced so far recognize only cytoplasmic (or intracellular) domains of the protein (2, 10, 35). Trials to make use of intramolecular tags in luminal (extracellular) loop domains of CLN3 failed because all inserted tags caused defects in normal trafficking of CLN3 such as retention of the protein in the ER.2

2 A. Kytta et al., unpublished results.
Thus, to determine the lysosomal trafficking route(s) of CLN3, we tested whether the protein could be arrested at the plasma membrane in cells in which the clathrin-mediated endocytosis was inhibited. NRK cells stably expressing wtCLN3 (2) were transiently transfected with GFP-tagged mutant Eps15-GFP-EΔ95/295, or myc-tagged AP180pCMV, both well characterized inhibitors of clathrin-mediated endocytosis (32, 33). The transfected cells were fed with Alexa594-conjugated transferrin for 20 min and then were fixed, permeabilized, and stained for CLN3 and/or the MYC tag (in AP180-transfected cells). Immunostaining of CLN3 is shown in GFP-EΔ95/295-transfected cells (A) and in AP180pCMV-transfected cells (C) (transfected cells indicated by asterisk). The distribution of Alexa594-conjugated transferrin in corresponding cells is shown in B and D, respectively. The accumulation of transferrin to the plasma membrane in GFP-EΔ95/295- and in AP180-transfected cells is indicated by arrows (B and D). Non-transfected cells accumulated transferrin in a perilnuclear recycling compartment. Scale bar, 10 μm.

![Image](image_url)

**FIG. 2.** Localization of CLN3 in NRK cells with inhibited endocytosis. NRK-wtCLN3ΔMEP4 cells (see “Experimental Procedures”) were transiently transfected with GFP-tagged mutant Eps15-GFP-EΔ95/295 (A and B) or the myc-tagged AP180pCMV (C and D), which both effectively inhibit clathrin-mediated endocytosis. At 24 h after transfection, the cells were fed with 5 μg/ml Alexa594-conjugated transferrin for 20 min and then were fixed, permeabilized, and stained for CLN3 and/or the MYC tag (AP180-transfected cells). Immunostaining of CLN3 is shown in GFP-EΔ95/295-transfected cells (A) and in AP180pCMV-transfected cells (C) (transfected cells indicated by asterisk). The distribution of Alexa594-conjugated transferrin in corresponding cells is shown in B and D, respectively. The accumulation of transferrin to the plasma membrane in GFP-EΔ95/295- and in AP180-transfected cells is indicated by arrows (B and D). Non-transfected cells accumulated transferrin in a perilnuclear recycling compartment. Scale bar, 10 μm.

Thus, to determine the lysosomal trafficking route(s) of CLN3, we tested whether the protein could be arrested at the plasma membrane in cells in which the clathrin-mediated endocytosis was inhibited. NRK cells stably expressing wtCLN3 (2) were transiently transfected with GFP-tagged mutant Eps15-GFP-EΔ95/295, or myc-tagged AP180pCMV, both well characterized inhibitors of clathrin-mediated endocytosis (32, 33). The transfected cells were fed with Alexa594-conjugated transferrin for 20 min and then inhibition of endocytosis in the transfected cells was confirmed by monitoring the uptake of transferrin by fluorescence microscopy. Expression of the GFP-EΔ95/295 or AP180 effectively inhibited internalization of transferrin, which was predominantly located on the plasma membrane (Fig. 2, B and D). Assuming that the trafficking of CLN3 occurs by the indirect route, some of the protein should have been arrested at the plasma membrane under these conditions. However, the stably expressed CLN3 protein was not detected on the plasma membrane in the cells with restricted endocytosis (Fig. 2, A and C), suggesting that in stably transfected NRK cells, CLN3 favors the direct intracellular trafficking route from the TGN to the lysosomes.

**FIG. 3.** Trafficking of CLN3 in AP-3-deficient mocha cells. The AP-3-deficient mocha cells were stably transfected with various CLN3 ΔMEP4 constructs carrying mutations in the lysosomal-targeting motifs. The expression of the proteins was induced by adding 3 μM CdCl2 to the culture medium 16–20 h before fixation with 4% PFA. The fixed cells were permeabilized with 0.2% saponin, stained for CLN3 and organelle-specific markers, and analyzed by confocal microscopy. The wtCLN3 and the CLN3 carrying the mutation in its dileucine motif (CLN3 L253A/I254A = MXG only) colocalized extensively with a lysosomal marker, LAMP-1 (A–C and D–F, respectively). The CLN3 carrying mutations in the tail targeting motif (CLN3 M409A = MXG, G only) did not show an overlapping signal with LAMP-1 (G–I) but, instead, colocalized significantly with the Golgi marker GM130 (J–L). Scale bar, 10 μm.

**Trafficking of CLN3 in AP-3-deficient mocha and pearl Cells**—We have identified two lysosomal targeting motifs located in two different cytoplasmic domains of CLN3 (2) (Fig. 1). The MXG motif in the long cytoplasmic tail of the CLN3 protein represents a novel type of sequence motif among lysosomal targeting signals, and its recognition by different adapters cannot be predicted. However, the second motif is a dileucine-type LI signal located in a cytoplasmic loop domain of CLN3, preceded by an acidic patch in an unusual position (Fig. 1). Although the location of the acidic residues in the CLN3 loop does not favor the positional criteria reported for recognition by AP-3 (11), the role of this adapter in the trafficking of CLN3 was suggested by studies in which the function of AP-3 was inhibited by antisense oligonucleotides (36). Therefore, we expressed (stably or transiently) wtCLN3 and CLN3 carrying mutations in either one or both targeting motifs in AP-3-deficient mocha cells. The steady state localization of the stably expressed wtCLN3 was in lysosomes, judged by its colocalization with LAMP-1 (Fig. 3, A–C). Similarly, when the dileucine motif was mutated (L253A/I254A = MXG, G only), the protein was also present in lysosomes targeted by the tail motif (Fig. 3, D–F). Interestingly, when we expressed CLN3 carrying alanine substitutions in the tail targeting motif (M409A = G419A = LI only), so that trafficking of the protein was completely dependent on its dileucine motif, the protein was not detected in the lysosomes of the mocha cells (Fig. 3, G–I). Instead, the trafficking of the mutated protein was mainly arrested at the Golgi complex (Fig. 3, J–L). After chasing the mutated protein for 16 h in the presence of cycloheximide, some of the cells also showed mutated CLN3 on the plasma membrane (data not shown). Similarly, when this mutated CLN3 construct was expressed transiently under the strong cytomegalovirus promoter (in pCMV5 vector) and chased in the presence of cycloheximide, some of the transfected cells showed mutated CLN3 on the plasma membrane and in vesicular structures, which, however, did not significantly colocalize with the lysosomal marker LAMP-1 (Fig. 4, A–C). We also transiently expressed CLN3 carrying mutations in both targeting motifs (double mutant) (2) in mocha cells. The double-mutated CLN3 did not accumulate at the Golgi complex but, similarly to what was...
observed earlier in HeLa cells (2), was mainly detected on the plasma membrane and in vesicular structures with hardly any colocalization with LAMP-1 (Fig. 4, D–F). To confirm that the detected accumulations were due to AP-3 deficiency, we also expressed all the different CLN3 constructs transiently in pearl cells deficient for the β3A-subunit of the AP-3. Similarly to what was seen in mocha cells, the localization of both wtCLN3 and CLN3 carrying the alanine substitution in the dileucine motif (MX_{2}G only) was in the lysosomes of pearl cells (data not shown). The mutated protein with only the functional dileucine motif (and mutations in the tail motif) was mostly localized to the perinuclear region (G–I). Chasing for 20 h with cycloheximide led to the clearance of the perinuclear accumulation in most of the transfected pearl cells (J–L). Scale bar, 10 μm.

Trafficking of CLN3 in AP-1-deficient Cells—To determine other possible adaptor proteins involved in lysosomal sorting of CLN3, we stably expressed all the different CLN3 constructs also in Δδ1A mouse fibroblasts deficient for AP-1 (23). In these cells, the steady state localization of the two mannose 6-phosphate receptors is shifted to endosomes (23). Proteins in which targeting motifs are normally recognized by AP-1 are mis sorted to the plasma membrane and transported to lysosomes after endocytosis (37). In contrast, the distribution and trafficking of LAMP-1 is not altered in AP-1-deficient cells (23). When the localization of the stably expressed CLN3 constructs was analyzed in Δδ1A cells by confocal microscopy, the wild type protein was found to colocalize well with the lysosomal marker protein LAMP-1 (Fig. 5, A–C). CLN3 carrying mutations in its dileucine motif (MX_{2}G only) was mostly located to the lysosomes, although, as seen in transiently transfected HeLa cells (2), a significant amount was also present at the plasma membrane (Fig. 5D). Interestingly, the steady state distribution of the CLN3 protein carrying mutations in the tail targeting motif (M409A + G419A = LI only) was again dramatically changed. Most of the protein was detected on the plasma membrane, and no significant colocalization with the LAMP-1 marker protein was seen (Fig. 5, G–I). Additionally, the mutated protein accumulated in the perinuclear region, which, however, had no overlapping localization with the Golgi markers (data not shown). Instead, when the cells were fed with fluorescent transferrin for 20 min, the perinuclear accumulation of mutated CLN3 colocalized well with the endocytosed transferrin, implying localization at the early endosomal/recycling compartment (Fig. 5, J–L).

Interactions of CLN3 Cytoplasmic Loop Domain with Adaptor Proteins—The steady state localization of the different CLN3 constructs expressed in AP-1- and AP-3-deficient cell lines suggested that both of these heterotetrameric adaptors are involved in lysosomal sorting of the CLN3 protein, most probably by recognizing the dileucine type LI motif. Therefore, we investigated interactions of the cytoplasmic loop domain of CLN3 containing the LI motif with different adaptor protein complexes. The cytoplasmic loop domain of human CLN3 (amino acids 232–280) was expressed as a GST fusion protein, and GST pull-down experiments of adaptor proteins were performed either from HeLa cell cytosol or from mouse liver extracts. Binding of different adaptors to Sepharose-bound GST-CLN3loop was detected by immunoblotting with antibodies specific for adaptor protein subunits. As suggested by the expression studies, the CLN3loop domain specifically interacted with both AP-1 and AP-3 originating from HeLa cytosol (Fig. 6A) or from mouse liver (Fig. 6B). To test whether the observed binding was specifically dependent on the dileucine motif in the loop domain of CLN3, we substituted two alanines for the LI motif.
sequence of the GST-CLN3loop (L253A/I254A) and repeated the pull-down experiments. The interaction of both AP-1 and AP-3 was completely abolished by the alanine substitutions (Fig. 6), indicating that the interactions were mediated by the LI sequence motif. To answer the question as to why CLN3 containing only the LI-targeting motif was not efficiently targeted from the plasma membrane to the lysosomes in AP-3- and AP-1-deficient cells (Figs. 4 and 5), we tested whether the CLN3 containing only the functional LI motif (LI only) was mistargeted from the lysosomes, the cells expressing this mutated construct were fed with 25 μg/ml Alexa594-conjugated transferrin for 20 min before fixation and were analyzed by confocal microscopy. Colocalization of the wtCLN3, the CLN3 carrying mutation in the LI motif (CLN3 L253A/I254A = MXG only), and the CLN3 carrying the mutated tail targeting motif (CLN3 M409A+G419A = LI only) with LAMP-1 are shown in merged images C, F, I, and I', respectively. Because the CLN3 containing only the functional LI motif (LI only) was mistargeted from the lysosomes, the cells expressing this mutated construct were fed with 25 μg/ml Alexa594-conjugated transferrin for 20 min before fixation and were analyzed by confocal microscopy. Colocalization of CLN3 with the mutated tail motif (LI only, J) and endocytosed transferrin (K) is shown in L and L'. Scale bar, 10 μm.

DISCUSSION

In the present study, we have investigated the trafficking of CLN3. Although the lysosomal localization of the protein in non-neuronal cells is well established (1, 2) and is shown to be mediated by two targeting motifs (2) (Fig. 1), little is known about trafficking routes and mechanisms involved in the sorting of the protein. Because CLN3 is expressed at low levels in most cell types, limiting the detection of the endogenous protein, we prepared a NRK cell line stably expressing the human CLN3 (2) for analysis of its trafficking route(s). Although the uptake of transferrin could be efficiently blocked in NRK-CLN3 cells by inhibiting clathrin-mediated endocytosis, the cells did not show any plasma membrane accumulation of CLN3 under these conditions. Therefore our results suggest that CLN3 favors the direct route from the TGN to lysosomes in NRK cells.

Both AP-1 and AP-3 are good candidate adaptors to facilitate sorting on the direct route. We therefore expressed full-length CLN3 carrying alanine substitutions in either one of the targeting motifs in cell lines defective for AP-1 or AP-3 sorting function. Interestingly, the trafficking of the mutated CLN3, carrying only the dileucine motif, was arrested at the level of the Golgi in the AP-3-deficient mocha and pearl cells. This is very unusual because, typically, proteins for which trafficking is dependent on AP-3 are mistargeted to the plasma membrane in the absence of AP-3 and, in most cases, can be transported from there to lysosomes after endocytosis (29, 38–40). The observed retention of CLN3 carrying only the dileucine targeting motif in the Golgi of AP-3-deficient cells could be dependent on a specific retention signal, because such signals have been found in proteins trafficking in intracellular pathways (41, 42). However, the retention of the mutated CLN3 was not seen in any other cell type in our previous study (2) nor in this one, and thus it is likely to be specific for the AP-3 deficiency. In AP-1-deficient cells, CLN3, sorted via its dileucine motif, was mislocalized into transferrin-positive endosomes as well as to the plasma membrane, indicating that, in addition to AP-3, AP-1 also plays an important role in lysosomal sorting of CLN3. Further evidence for the involvement of both of these adaptor complexes in specific recognition of the dileucine motif of CLN3 was obtained in GST pull-down experiments, in contrast to data from Storch et al. (43).
The molecular machinery recognizing the unconventional targeting motif MXG in the cytoplasmic tail of CLN3 still remains to be clarified. In our experiments, we found that expression of the carboxyl-terminal tail of CLN3 (amino acids 384–438 of human CLN3) as a GST fusion was toxic in bacteria, precluding its use in the search for binding partners in pull-down experiments. In the AP-3-deficient cells, the steady state localization of the wild type protein and of CLN3 carrying mutations in the dileucine motif was in lysosomes, suggesting that sorting via the tail motif of CLN3 can also occur independently of AP-3. In AP-1-deficient cells, the CLN3 carrying only the tail targeting motif was detected both in lysosomes and at the plasma membrane. This two-site localization is similar to the distribution of the mutated protein in transiently transfected HeLa cells and may therefore also result from the lack of the dileucine targeting motif per se. The lysosomal localization of CLN3, sorted via the tail targeting motif both in AP-1- and AP-3-deficient cells, and the unusual amino acid composition of the motif (Fig. 1) suggest that this unusual motif may be recognized and sorted by a novel cargo-specific adaptor(s) (for review, see Ref. 16).

The present data suggest a complex and hierarchical sorting mechanism for CLN3. Whereas the molecular mechanism that recognizes the tail targeting motif of CLN3 remains unknown, the sorting of the protein via the dileucine motif requires at least two different adaptor complexes, AP-1 and AP-3. Our results suggest that the dileucine motif of CLN3 is an “intrinsically strong” lysosomal targeting motif (24), which is primarily recognized by AP-3 at the TGN, consistent with our earlier studies on the YXXΦ motifs of CD63 (40) and endolyn (24). However, the site of AP-1-dependent sorting of CLN3 is less clear. AP-1 has earlier been suggested to function in both

**FIG. 6. Interactions of dileucine motif of CLN3 with adaptor protein complexes.** The cytoplasmic loop domain of CLN3 (amino acids 232–258) containing the dileucine motif LI was produced as a GST fusion, and the GST pull-down experiments were performed by incubating the GST-CLN3loop protein with the cytosolic extract of HeLa cells (A) or with mouse liver extract (B). The bound adaptors were detected in immunoblotting analysis by staining with antibodies to γ-, α-, or δ-subunits, the specific markers for the AP-1, AP-2, and AP-3, respectively.

**FIG. 7. The possible sorting pathways mediated by the dileucine motif of CLN3.** A schematic picture presenting the possible sorting pathways (arrows) mediated by the dileucine motif of CLN3. Based on expression studies of mutated CLN3 carrying only the dileucine motif in AP-1- or AP-3-deficient cells, sorting of CLN3 can occur by at least two possible mechanisms. In the upper model (a–c), AP-3 mediates sorting of CLN3 from the trans-Golgi network (TGN) to early endosomes (EE), AP-3-dependent sorting is followed by AP-1-mediated sorting step from EE to late endosomes (LE), from which the protein can be targeted to lysosomes (L). In the lower model (d–f), AP-1-dependent sorting precedes the AP-3-mediated sorting in the endosomes. However, CLN3 carrying only the dileucine motif avoid efficient sorting by AP-3 from endosomes to lysosomes (cross-hatched lines in f) in AP-1-deficient cells. Crosses indicate the absence of sorting pathways in AP-1- and AP-3-deficient cells. Experimentally determined localization of CLN3 carrying only the dileucine motif in different cell types are illustrated below the separate boxes (a–f). Dotted lines (c, f) indicate possible mistargeting of the protein to the plasma membrane (PM) and possible endocytosis via AP-2-dependent route.
anterograde and retrograde transport of proteins between the TGN and early endosomes (23, 37, 44). AP-1 has also been found to mediate anterograde transport from post-Golgi organelles in Toxoplasma gondii (45), but such a sorting function has not been reported in mammalian cells. If AP-1 could also function in anterograde transport from endosomes in mammalian cells, the sorting of CLN3 via its dileucine motif could occur sequentially, first in the TGN using AP-3, followed by AP-1 sorting in endosomes (Fig. 7, a–c). However, earlier data have implied that when functioning sequentially in lysosomal sorting, AP-1 sorting precedes AP-3 (37, 46). Therefore, it is possible that in AP-3-deficient cells, CLN3 containing only the dileucine motif could reach their cellular destinations even in the absence or inhibition of the AP-1 and AP-3 machinery. Parallel and sequential sorting pathways via multiple targeting motifs may represent a compensatory mechanism for cells to ensure that proteins can reach their cellular destinations even in the absence or inhibition of one pathway. Multiple sorting pathways may also have evolved for strict control of protein localization and, accordingly, functional regulation. This view is especially interesting in the case of CLN3 because, in addition to lysosomes, the protein has been located in different cell types to many intracellular organelles and membrane domains including Golgi (47), endosomes (2), lipid rafts (48), and synaptic terminals (10, 49, 50). Bearing in mind that the most severe symptoms of Batten disease manifest in the central nervous system, a future challenge will be to elucidate the hierarchy and the role of parallel sorting pathways of CLN3 in neuronal cells.

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