

# Neuronal Ceroid Lipofuscinoses Are Connected at Molecular Level: Interaction of CLN5 Protein with CLN2 and CLN3

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Neuronal ceroid lipofuscinoses (NCLs) are neurodegenerative storage diseases characterized by mental retardation, visual failure, and brain atrophy as well as accumulation of storage material in multiple cell types. The diseases are caused by mutations in the ubiquitously expressed genes, of which six are known. Herein, we report that three NCL disease forms with similar tissue pathology are connected at the molecular level: CLN5 polypeptides directly interact with the CLN2 and CLN3 proteins based on coimmunoprecipitation and *in vitro* binding assays. Furthermore, disease mutations in CLN5 abolished interaction with CLN2, while not affecting association with CLN3. The molecular characterization of CLN5 revealed that it was synthesized as four precursor forms, due to usage of alternative initiator methionines in translation. All forms were targeted to lysosomes and the longest form, translated from the first potential methionine, was associated with membranes. Interactions between CLN polypeptides were shown to occur with this longest, membrane-bound form of CLN5. Both intracellular targeting and posttranslational glycosylation of the polypeptides carrying human disease mutations were similar to wild-type CLN5.

## INTRODUCTION

Neuronal ceroid lipofuscinoses (NCLs) are the most common neurodegenerative diseases in childhood. The incidence of these diseases is highest in Northern Europe and the United States, being ~1:10,000, whereas elsewhere the frequency is much lower (Santavuori, 1988; Uvebrant and Hagberg, 1997). The hallmark of all NCL forms is the accumulation of autofluorescent material in multiple tissues, but the ultrastructure of inclusion bodies differs in different NCL subtypes (Rapola, 1993). The classification of NCL disorders is based on clinical symptoms, the age of onset, and neuropathology.

The gene defects behind six NCL diseases are known. Two NCL genes encode soluble, lysosomal enzymes: palmitoyl protein thioesterase 1 defective in CLN1 (Vesa *et al.*, 1995; Hellsten *et al.*, 1996; Verkruse and Hofmann, 1996) and

tripeptidyl peptidase 1 defective in CLN2 (Sleat *et al.*, 1997). Recent evidence indicates that in neurons, palmitoyl protein thioesterase 1 is localized into synaptosomes and synaptic vesicles (Heinonen *et al.*, 2000; Lehtovirta *et al.*, 2001). CLN3 encodes a lysosomal transmembrane protein, which is targeted to lysosomes in nonneuronal cells (Jarvela *et al.*, 1998), whereas in mouse primary neurons it is targeted to neuronal synapses (Jarvela *et al.*, 1999; Luiro *et al.*, 2001). CLN8 is a novel transmembrane protein that resides in the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment (Lonka *et al.*, 2000). The fifth NCL gene discovered, CLN6, has recently been cloned, and it codes for a transmembrane protein with unknown localization and function (Wheeler *et al.*, 2001). The sixth known NCL gene is CLN5, predicted to code for a novel protein with two putative transmembrane domains (Savukoski *et al.*, 1998). Very little is known about the biosynthesis and the effect of disease mutations of CLN5.

Herein, we have investigated biosynthesis, processing, intracellular localization, and molecular interactions of both wild-type (WT) and mutant CLN5 proteins in transiently transfected COS-1 cells. WT CLN5 was synthesized as 47-

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44-, 41-, and 39-kDa polypeptides due to usage of alternative initiator methionine in translation. Based on immunofluorescence microscopy, all four forms, as well as FIN<sub>M</sub> and EUR polypeptides get targeted to lysosomes. Coimmunoprecipitation and *in vitro* binding assays revealed that CLN5 interacts directly with CLN2 and CLN3 polypeptides. Although all mutant forms of CLN5 retained their ability to interact with CLN3, none was able to interact with CLN2. Our findings reveal, for the first time, that CLN proteins are connected at the molecular level. This will have an impact on the concept of final common pathway in neuronal death in NCL diseases.

## MATERIALS AND METHODS

### *Construction of Expression Plasmids and In Vitro Translation Assay*

WT and FIN<sub>M</sub> cDNAs were cloned as described previously (Isomppi *et al.*, 2002). The EUR and SWE cDNA constructs were generated by QuickChange site-directed *in vitro* mutagenesis kits, according to manufacturer's protocols (Stratagene, La Jolla, CA). The CLN3 expression plasmid has been described previously (Jarvela *et al.*, 1998). For *in vitro* translation, AIRE (Ramsey *et al.*, unpublished data), CLN2, CLN3, and CLN5 cDNAs were cloned to pGEM4Z (Amersham Biosciences AB, Uppsala, Sweden) by polymerase chain reaction with linker primers. Coupled *in vitro* transcription/translation was performed according to manufacturer's protocols (Promega, Madison, WI) and analyzed on 10% SDS-PAGE. For the *in vitro* binding assay, the coding region of CLN5 was cloned in the pGEX-6P vector (Amersham Biosciences AB) as described above. The constructs were confirmed by sequencing.

### *Cell Culture, Transfections, and Metabolic Labeling*

COS-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in DMEM (Cellgro, Herndon, VA), supplemented with 10% fetal bovine serum (Cellgro) and antibiotics (Invitrogen, Carlsbad, CA) in 5% CO<sub>2</sub> at 37°C. Cells (2 × 10<sup>5</sup>/well) were plated in six-well plates 1 d before transfections. Transfections were carried out using LipofectAMINE PLUS reagent (Invitrogen), following the manufacturer's guidelines. Cells were metabolically labeled 48 h posttransfection by starving them in methionine- and cysteine-free medium (Invitrogen) for 1 h and thereafter labeling with 50 μCi/ml of both [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, United Kingdom) for 10 min (glycosylation assay) or 1 h (detection of the usage of N-terminal methionines). After the labeling, cells were harvested and lysed with radioimmunoprecipitation assay buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% ipegal, 0.5% deoxycholic acid, and 0.1% SDS) supplemented with protease inhibitors (Complete; Roche Applied Science, Indianapolis, IN). Lysed cells were immunoprecipitated with N-terminal, anti-CLN5 antibodies and protein A/G-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were separated on 10% SDS-PAGE and visualized by fluorography (Amplify; Amersham Biosciences UK, Ltd.). Endoglycosidase H (EndoH) and peptide N-glycosidase F (PNGaseF) digestions of immunocomplexes were performed as described previously (Jarvela *et al.*, 1998). Western blotting of transiently transfected COS-1 cells was performed using the N-terminal antibody.

### *Antibodies*

To obtain the CLN5 protein for immunization, the cDNA sequence corresponding to 75 N-terminal amino acids of CLN5 was subcloned into the pGEX-6P-1 vector (Amersham Biosciences AB). The CLN5 polypeptides were expressed in the *Escherichia coli* strain

BL21-DE as glutathione S-transferase (GST) fusion proteins and were purified with Glutathione-Sepharose 4B (Amersham Biosciences AB). Rabbits were immunized by subcutaneous injection with 500 μg of the CLN5 fusion protein in Freund's complete adjuvant. Immunization was repeated four times over a span of 3 wk, and the blood was collected 1 wk after the last immunization. To obtain a CLN2-specific peptide antibody, rabbits were immunized with a synthetic peptide corresponding to amino acids 368–383 of the CLN2 polypeptide coupled to keyhole limpet hemocyanin by using 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester as a coupling reagent. The immunizations were performed three times, as described above, by using peptide-keyhole limpet hemocyanin conjugate elusified in complete Freud's adjuvant. The CLN5-specific peptide antibody was similarly raised against amino acids 258–273 of the human CLN5 polypeptide (Isomppi *et al.*, 2002). The CLN3-specific peptide antibody was raised against amino acids 242–258 of human CLN3 (Jarvela *et al.*, 1998). The lysosome/late endosome-specific lysosome-associated membrane protein (lamp) 1 antibody H4A3, developed by Thomas August (Johns Hopkins University, Baltimore, MD), under auspices of the NICHD, was obtained from Developmental Studies Hybridoma Bank and maintained by the Department of Biological Sciences at the University of Iowa (Iowa City, IA). Secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO).

### *Membrane Fractionation and Western Blotting*

COS-1 cells were transiently transfected with CLN3 and CLN5 constructs subcloned to the pCMV5 vector (Andersson *et al.*, 1989) and were subjected to membrane fractionation. CLN2 was detected as an endogenously expressed protein. The fractionation was performed as described previously (Dell'Angelica *et al.*, 1997). Briefly, transfected cells were resuspended in membrane fractionation buffer A (10 mM HEPES pH 7.0, 0.15 M KCl, 1 mM EGTA, 0.5 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol) supplemented with protease inhibitors (Complete; Roche Applied Science) and mechanically lysed with a syringe and 26-gauge needle. Lysed cells were first centrifuged with 2000 and 10,000 × *g* for 5 min at 4°C to remove any unbroken cells, aggregates, and potential inclusion bodies, respectively. Thereafter, supernatants were subjected to ultracentrifugation at 120,000 × *g* for 90 min at 4°C. The fractions were analyzed by Western blotting with the N-terminal antibody.

Triton X-114 fractionation was performed as described previously (Roseblat *et al.*, 1994). In brief, transiently transfected cells were lysed with TX114 lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, and 1% Triton X-114) supplemented with protease inhibitors. Then 200 μl of cell lysate and 200 μl of sucrose solution (0.5 M sucrose, 10 mM Tris-HCl pH 7.4, and 150 mM NaCl) were mixed, incubated at 37°C for 5 min, and centrifuged at 10,000 × *g* for 5 min. Fractions (pellet and supernatant) were analyzed by Western blotting.

### *Immunofluorescence Microscopy*

To determine the subcellular localization of CLN5, COS-1 cells were plated on coverslips and transfected as described above. Forty-eight hours posttransfection, cells were incubated in DMEM without fetal bovine serum for 1 to 3 h, in the presence of 50 μg/ml cycloheximide (Sigma-Aldrich), to halt the protein synthesis. Thereafter, cells were fixed with methanol and blocked with 0.5% bovine serum albumin (fraction V; Sigma-Aldrich)/0.2% saponin (Sigma-Aldrich). The cells were then double labeled with the CLN5-specific peptide antibody and the lamp1-specific H4A3 antibody. Cells were washed with 0.5% bovine serum albumin/0.2% saponin and incubated with fluorescein isothiocyanate- and tetramethylrhodamine B isothiocyanate-conjugated anti-rabbit and anti-mouse secondary antibodies. After washing with phosphate-buffered saline (PBS), the cells were mounted in glycerol and viewed with a DMR immunofluorescence microscope (Leica Microscope and Scientific Instruments Group,

Solms, Germany) by using Quips fluorescence in situ hybridization image capture system (Applied Imaging, Santa Clara, CA).

### Coimmunoprecipitation Assay

For coimmunoprecipitation assay, COS-1 cells were transfected with CLN1, CLN3, or CLN5 cDNAs cloned into the pCMV5 expression vector (Andersson *et al.*, 1989), and the expression of lamp1, lamp2, and CLN2 was detected from endogenously expressed proteins. Cells were lysed with lysis buffer A (50 mM Tris pH 7.4, 300 mM NaCl, 1% Triton X-100, and 0.1% bovine serum albumin) by incubating them at 4°C for 20 min with shaking, centrifuging, and transferring the supernatant to a fresh tube. Equal expression levels of each construct were confirmed by Western blotting by using the N-terminal antibody. Cell lysates were immunoprecipitated with a CLN1- (Hellsten *et al.*, 1996), CLN2-, or CLN5-specific antibody. Immunocomplexes were analyzed by Western blotting by using lamp1, lamp2, CLN3-, or CLN5-specific antibodies.

### In Vitro Binding Assay

For the in vitro binding assay, 100  $\mu$ l of Glutathione-Sepharose beads (Amersham Biosciences AB) were washed twice with PBS and resuspended in 200  $\mu$ l of binding buffer A (40 mM HEPES pH 7.4, 0.2 mM EDTA, 10% glycerol, 1% bovine serum albumin, 1.5 mM dithiothreitol, 100 mM KCl, 0.1% Igepal, and 5% glycine). Beads were first combined with 3  $\mu$ g of GST or a GST/CLN5 fusion protein produced in *E. coli* and incubated at 4°C for 1 h with rocking. Thereafter, 20  $\mu$ l of radioactively labeled AIRE, CLN2, or CLN3 produced by in vitro transcription/translation was added and the coupling reaction was performed at 4°C for 2 h with rocking. Formed complexes were washed three times with binding buffer A and two times with the same buffer without bovine serum albumin and glycine. Samples were resuspended in 100  $\mu$ l of 2 $\times$  Laemmli buffer and analyzed by SDS-PAGE and fluorography.

### Tripeptidyl-Peptidase I Activity Assay

The tripeptidyl-peptidase I (TPP-I, CLN2) activities were measured from a control subject's and CLN5-patient's fibroblasts as described previously (Sohar *et al.*, 2000). Briefly, cells were washed twice with PBS and harvested by scraping with TPP-1 lysis buffer (50 mM sodium acetate pH 4.0 and 0.1% Triton X-100). Then 10  $\mu$ l of cell sample and 40  $\mu$ l of substrate solution (250  $\mu$ M Ala-Ala-Phe 7-amido-4-methylcoumarin [Sigma-Aldrich] in dimethyl sulfoxide) were incubated at 37°C for 1 h with shaking. The reaction was terminated with 100  $\mu$ l of TPP-I stop solution (0.1 M monochloroacetic acid, 0.13 M NaOH, and 0.1 M acetic acid) and results were analyzed by fluorometry with 355-nm excitation, 460-nm emission, and 420-nm cutoff.

## RESULTS

### Expression of WT and Mutant CLN5 Polypeptides

The CLN5 gene codes for a polypeptide of 407 amino acids with the predicted molecular weight of 46.3 kDa (Savukoski *et al.*, 1998) (Figure 1A). To monitor the biosynthesis of the WT and naturally occurring disease mutants, we transiently transfected COS-1 cells with corresponding cDNA constructs. The crude cell lysates were analyzed by Western blotting by using an N-terminal antibody raised against amino acids 1–75 of the full-length CLN5. The expression of WT and EUR (changing Asp 279 to Asn) CLN5 resulted in polypeptides of 47 kDa, whereas FIN<sub>M</sub> (changing Tyr 392 to Stop), Fin<sub>m</sub> (changing Trp 75 to Stop), and SWE (changing Glu 253 to Stop) mutants generated polypeptides with the molecular weights of 46, 12, and 34 kDa, respectively (Figure

1B). Interestingly, the SWE mutant produced two additional polypeptides with higher molecular weights (37 and 40 kDa), which became visible during longer exposure time (see "Further Analyses"). The molecular weights of observed polypeptides correspond to the predicted ones, suggesting that in Western blotting the N-terminal antibody recognizes the unglycosylated forms of the polypeptides.

The sequence of the CLN5 polypeptide has eight potential N-glycosylation sites, three encoded by sequences of exon 3 and five by those of exon 4. To test the utilization of the predicted glycosylation sites, we metabolically labeled transiently transfected COS-1 cells and digested the immunoprecipitated proteins with enzymes removing either high-mannose type sugars (EndoH) or any N-linked sugar chains (PNGaseF). The expression of both WT and EUR polypeptides resulted in a smear of up to 75 kDa in addition to the 47-kDa band. The disappearance of the high molecular weight smear after EndoH suggested that CLN5 polypeptides were modified with high-mannose type sugars (Figure 1C). After EndoH treatment, two bands with molecular weights differing in ~2 kDa were visible. This observation suggests that in addition to high mannose sugars, complex sugars are also added to the CLN5 polypeptides. This was confirmed by PNGaseF treatment, which produced only one 47-kDa band. The expression of the FIN<sub>M</sub> mutant, lacking the 16 carboxy-terminal amino acids, resulted in a major 46-kDa band and a smear of up to 74 kDa. The results of EndoH and PNGaseF treatments of FIN<sub>M</sub> polypeptides were similar to the results obtained with the WT and EUR constructs, suggesting that FIN<sub>M</sub> also gets modified by both high-mannose and complex-type sugars. The SWE mutant has only two potential N-glycosylation sites, both of which are located in the region encoded by exon 3. The transient expression of the SWE polypeptide resulted in three bands with the molecular weights of 34, 37, and 40 kDa, of which 37- and 40-kDa polypeptides were sensitive to EndoH and PNGase F, rendering two bands after EndoH treatment and one band after PNGaseF treatment. These findings indicate that the observed molecular weight heterogeneity is due to differential glycosylation and is not caused by proteolytic trimming of the CLN5 protein. Furthermore, the polypeptides affected by FIN<sub>M</sub>, EUR, and SWE mutations can, at least to some extent, pass the quality control of the ER because they got posttranslationally glycosylated by complex-type sugars. Additionally, these results reveal that the N-terminal antibody is also able to recognize glycosylated forms of CLN5 in immunoprecipitation experiments.

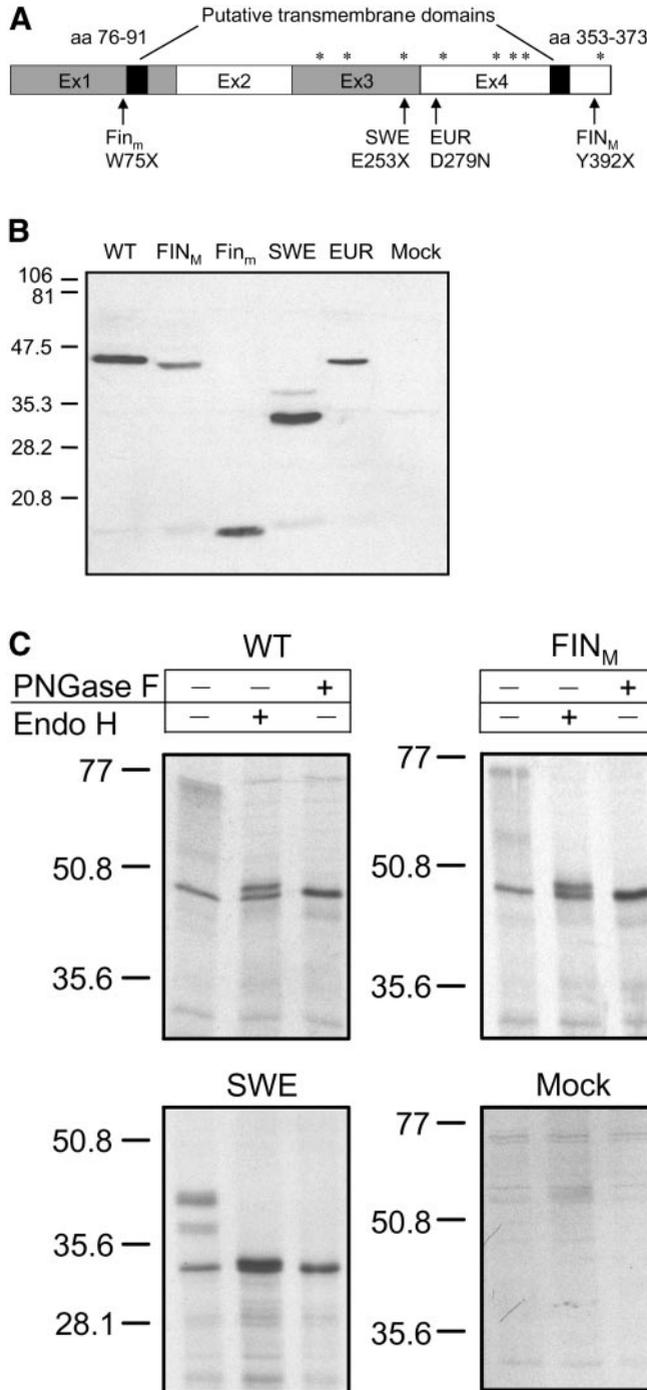
### CLN5 Is Translated as Four Different Isoforms Due to Alternative Initiator Methionines

CLN5 has four methionines at amino acid positions 1, 30, 50, and 62 that can potentially serve as an initiator for the translation of the polypeptide. The usage of these methionines would result in polypeptides with predicted molecular masses of 46.3, 43.4, 41.5, and 40.3. To clarify whether more than one of these methionines is used as a translational start residue, we performed a cell-free in vitro translation assay for CLN5 cDNA containing all four N-terminal methionines. The assay produced one major protein band of 47 kDa (Figure 2A). In addition to this, three fainter bands with molecular weights of 44, 41, and 39 kDa were also detectable on the gel, suggesting that CLN5 gets translated in more

than one polypeptide form. The molecular weights of the observed protein bands were in accordance with the predicted molecular weights of polypeptides initiating from different N-terminal methionines. This phenomenon was also observed in a cellular system by using transiently trans-

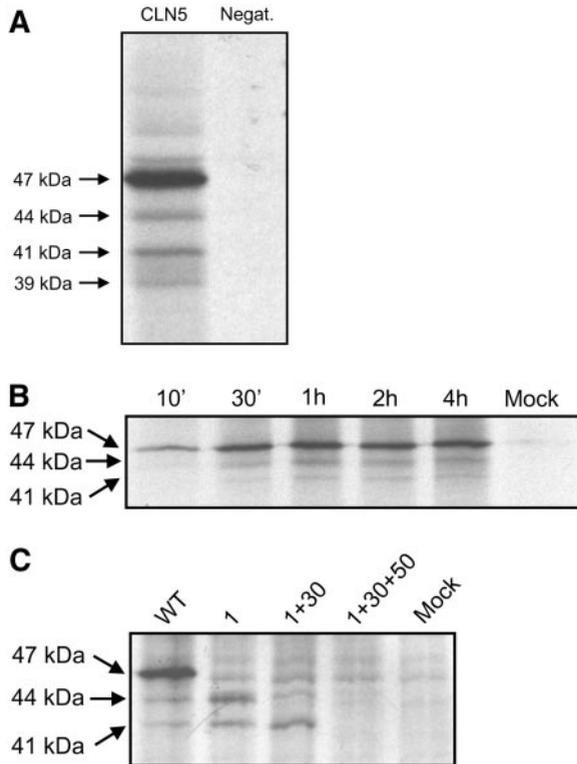
ected COS-1 cells. In this analysis, cells were metabolically labeled for different time periods and immunoprecipitated with the N-terminal antibody. SDS-PAGE of the immunocomplexes showed similar results to in vitro translation assay, the major protein band being 47 kDa and the minor bands 44 and 41 kDa (Figure 2B). The proteins of 44 and 41 kDa were not detectable after a 10-min labeling time but became visible after the labeling time of 30 min. The fourth band of 39 kDa, observed in in vitro translation assay, was not visible even with a labeling time of 4 h in these conditions. This observation is most likely due to the specificity of the CLN5 antibody. The N-terminal antibody used in this experiment was raised against amino acids 1–75 of WT CLN5, and the 39-kDa form uses the initiator methionine, which is located at position 62. In all likelihood, these overlapping 14 amino acids were not sufficient to guarantee the immunodetection with this antibody.

To confirm that the polypeptides, observed by both in vitro translation assay and transient COS-1 cell expression of the WT CLN5, are due to the usage of alternative initiating methionines in translation, we mutagenized the first three methionines one by one, in the expression vector containing the CLN5 cDNA. WT CLN5, as well as cDNA constructs having mutagenized methionines to isoleucine at amino acid position 1, 1 and 30, or at positions 1, 30, and 50 were expressed in COS-1 cells and immunoprecipitated with the N-terminal antibody. When the construct, lacking the first methionine was expressed, the major band of 47 kDa disap-



**Figure 1.** (A) Schematic presentation of the CLN5 polypeptide. Putative transmembrane domains are indicated by black boxes. Gray and white boxes indicate amino acid regions encoded by distinct exons within the CLN5 gene. The positions of disease mutations are indicated by arrows and putative N-glycosylation sites by asterisks. aa, amino acid. (B) Expression of WT and mutant CLN5 polypeptides. COS-1 cells, transfected with WT and mutant CLN5 constructs, were analyzed by Western blotting with a CLN5-specific antibody. cDNA constructs used are shown above and molecular weights of marker bands on the left. Mock, cells transfected with vector without insert. (C) N-Glycosylation of WT and mutant CLN5. COS-1 cells were transiently transfected with WT,  $Fin_m$ , EUR, and SWE constructs, metabolically labeled with [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine, immunoprecipitated with CLN5-specific antibody, and incubated in the absence or

presence of glycosidases EndoH and PNGaseF. Proteins were separated on SDS-PAGE and analyzed by fluorography. Mock-transfected COS-1 cells were used to show unspecific background. Molecular weights of marker bands are indicated on the left.

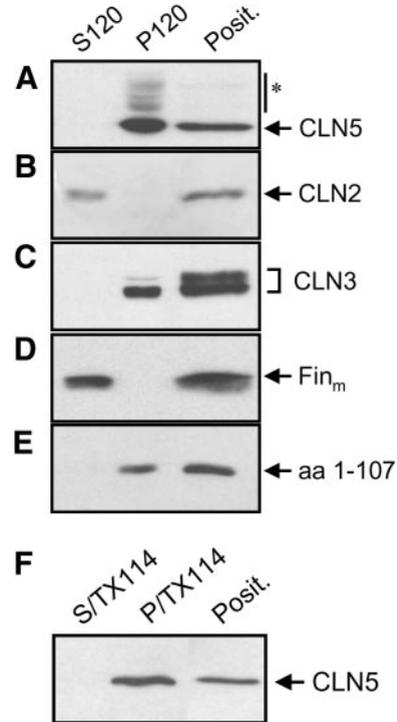


**Figure 2.** (A) Cell-free expression analysis of CLN5. CLN5 cDNA subcloned to pGEM4Z was produced by in vitro translation with [<sup>35</sup>S]methionine. Proteins were separated on SDS-PAGE and analyzed by fluorography. pGEM4Z was used as a negative control. Molecular weights of the protein bands produced are shown on the left. (B) Metabolic labeling of transiently transfected COS-1 cells. COS-1 cells were transiently transfected with WT CLN5 cDNA construct and labeled with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine. Cell lysates were immunoprecipitated with CLN5-specific antibody. Results were obtained from SDS-PAGE and fluorography. Labeling time used is shown above and the molecular weights of the bands observed are shown on the left. (C) In vitro mutagenesis of N-terminal methionines. COS-1 cells were transiently transfected with either WT or mutant constructs and metabolically labeled with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine. Cell lysates were immunoprecipitated with CLN5-specific antibody and subjected to SDS-PAGE and fluorography. Transfected constructs, in which numbers indicate the position of the mutagenized methionines, are shown above. Molecular weights of the protein bands observed are indicated on the left.

peared, whereas the two bands with lower molecular weights were still visible and became stronger in density (Figure 2C). The construct without methionines at positions 1 and 30 resulted in only one band of 41 kDa, and no specific protein bands were visible on the gel when the methionine 50 was also mutagenized to isoleucine, in addition to methionines 1 and 30. These observations demonstrate that the translational machinery of COS-1 cells is able to use at least three different methionines, located at positions 1, 30, and 50, in the initiation of translation of the CLN5 polypeptide.

**The 47-kDa CLN5 Form Is a Membrane Protein**

The analyses of the primary amino acid sequence have revealed that CLN5 contains two hydrophobic regions, at



**Figure 3.** Membrane fractionation of CLN5. COS-1 cells were transiently transfected with CLN5 (A and F), CLN3 (C), Fin<sub>m</sub> (D), or CLN5 cDNA construct coding for amino acids 1–107 (E), CLN2 (B) expression being detected endogenously. (A–E) Cells were lysed and centrifuged at 120,000 × g, and fractions were analyzed by Western blotting with either CLN5-specific (A, D, and E), CLN2-specific (B), or CLN3-specific (C) antibody. (F) Cells were lysed and subjected to Triton X-114 fractionation. Fractions were analyzed by Western blotting with CLN5-specific antibody. \*, glycosylated forms; S120, supernatant after 120,000 × g centrifugation; P120, pellet after 120,000 × g centrifugation; S/TX114, supernatant after Triton X-114 fractionation; P/TX114, pellet after Triton X-114 fractionation; Posit., crude COS-1 cell lysate transfected with corresponding cDNA construct.

positions 76–91 and 353–373 (Figure 1A), suggesting two transmembrane domains for the polypeptide (Savukoski *et al.*, 1998). However, TMHMM (Krogh *et al.*, 2001), and SOSUI (Hirokawa *et al.*, 1998) prediction programs were not able to identify any potential transmembrane regions in CLN5. To test whether the 47-kDa form of CLN5 is attached to membranes, we performed membrane fractionation of the transiently transfected COS-1 cells. Before membrane fractionation, cell lysates were centrifuged at 2,000 and 10,000 × g to remove any unbroken cells and aggregates or inclusion bodies, respectively. Based on the density of the protein bands in Western blotting, ~10–30% of proteins were in inclusion bodies or formed aggregates (our unpublished data). The results obtained from Western blotting indicated that the full-length CLN5 is located in the pellet after centrifugation of postnuclear supernatants at 120,000 × g, suggesting that the 47-kDa form of CLN5 resides in membranes (Figure 3A). As a control, we performed similar membrane fractionation assays for two other NCL proteins, CLN2 and CLN3, of which the former is known to be a soluble lysosomal enzyme (Sleat *et al.*, 1997), and the latter is a resident of

the lysosomal membrane in nonneuronal cells (Jarvela *et al.*, 1998). As expected, CLN2 was found associated with the soluble fraction and CLN3 with the membrane fraction (Figure 3, B and C). To clarify whether CLN5 is a transmembrane protein Triton X-114 fractionation assay was performed. In this experiment, CLN5 was found in the Triton X-114 fraction, suggesting that the longest form of CLN5 represents a transmembrane protein.

To test whether the more amino terminal of two putative transmembrane domains, consisting of amino acids 76–91, is real, we used two mutant constructs in membrane fractionation experiments. The first construct mimics the naturally occurring disease mutant *Fin<sub>M</sub>* (Trp 75Stop), resulting in the truncated polypeptide of 12 kDa. The second artificial construct codes for 107 amino-terminal amino acids containing the first putative transmembrane domain. The Western blotting of the synthesized polypeptides revealed that *Fin<sub>M</sub>* remains in the soluble fraction, whereas the construct encoding the first 107 amino acids produces a polypeptide that is associated with the membrane fraction (Figure 3, D and E). These findings would imply that the hydrophobic region encompassing amino acids 76–91 represents a real transmembrane domain. The status of the second putative transmembrane domain remains elusive, because, despite of several attempts, we have not been able to raise functional antibodies against the C-terminal part of the CLN5 polypeptide.

#### **All Four Isoforms of WT CLN5, as Well as *FIN<sub>M</sub>* and EUR Mutants Are Targeted to Lysosomes**

To determine the subcellular location of different isoforms of CLN5 as well as disease mutants in COS-1 cells, we applied a CLN5-specific peptide antibody, raised against amino acids 258–273. In addition, the cells transfected with WT construct were stained with the N-terminal antibody. The staining with the peptide antibody showed almost complete colocalization with lysosomal membrane protein lamp1, suggesting lysosomal localization for the WT protein (Figure 4A). In contrast, the immunostaining with the N-terminal antibody overlapped with a Golgi-specific marker 58K, but also showed very strong reticular-like staining resembling the ER. This would imply that the N-terminal antibody recognizes mainly the ER and Golgi forms of the polypeptide in immunofluorescence. To determine the effect of the most common CLN5 mutation, *FIN<sub>M</sub>* (Tyr392Stop), and that of EUR CLN5 (Asp279Asn), on intracellular targeting of the CLN5 polypeptide, we also performed localization studies with the mutant CLN5 constructs by using the peptide antibody. Similar to WT CLN5, *FIN<sub>M</sub>* and EUR colocalized with the lamp1 antibody, suggesting that these polypeptides are also targeted to lysosomes. Both mutant proteins were also detectable after 3 h of cycloheximide treatment, suggesting that they owe similar stability to the WT protein in COS-1 cells. The intracellular location of the SWE mutant remains elusive due to the lack of functional antibody in immunofluorescence experiments recognizing this truncated polypeptide.

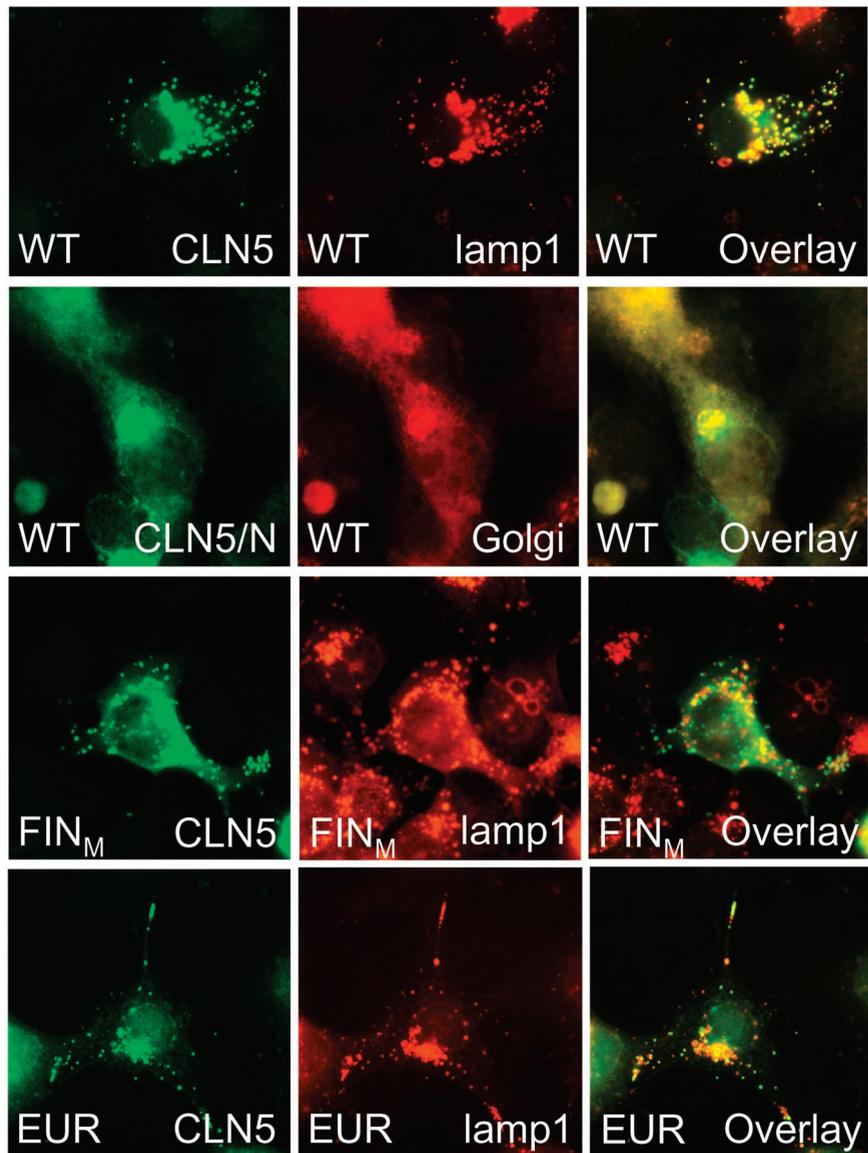
The observation that CLN5 is translated from several different start codons raised a question whether these forms are targeted to different intracellular organelles, or whether they all represent residents of lysosomes. To address this issue, we performed similar localization studies as described

above by using constructs with only one intact initiator methionine, residing either at position 1, 30, 50, or 62. In addition, we analyzed whether any other methionines are used in the initiation of translation, by using a construct lacking all four N-terminal methionine codons. The immunofluorescence staining of COS-1 cells transfected with constructs having an intact methionine at position 1, 30, 50, or 62 showed colocalization with lamp1 antibody. These results would suggest that all four forms of the CLN5 polypeptide are targeted to lysosomes (Figure 4B). The polypeptide lacking all four N-terminal methionines did not show any specific staining (our unpublished data), revealing that no other methionines beyond amino acid position 62 are used to start the translation.

#### **CLN5 Interacts with CLN2 and CLN 3**

To assess whether the CLN5 polypeptide would interact with other lysosomal CLN proteins, we examined all possible combinations of CLN1, CLN2, CLN3, and CLN5 by coimmunoprecipitation assay. For this purpose, COS-1 cells were transiently transfected with the combination of CLN1, CLN3, or CLN5 cDNA constructs, whereas the CLN2 was analyzed as the endogenously expressed protein. The cell lysates were immunoprecipitated and analyzed by Western blotting, by using two different antibodies specific to different CLN proteins of interest. CLN1 did not show any evidence for interactions with other CLN proteins (our unpublished data). The coimmunoprecipitation studies demonstrated that CLN2-specific antibody is able to coprecipitate the CLN5 polypeptide, which became visible in CLN5-specific antibody staining of Western blots (Figure 5A). The interacting capability of three naturally occurring mutants, *FIN<sub>M</sub>*, SWE, and EUR, was also examined by coimmunoprecipitation assay to evaluate the potential pathological mechanism behind CLN5. Interestingly, none of mutant polypeptides tested was able to bind CLN2, suggesting that these disease mutations underlying CLN5 interfere with the interacting capability with CLN2. To confirm the specificity of the interaction between CLN2 and CLN5, we performed a similar coimmunoprecipitation assay by using lamp1 and lamp2, both being lysosomal associated membrane proteins, as controls. Neither the CLN2 nor CLN5 antibody was able to pull down lamp1 or lamp2 (our unpublished data), suggesting that the observed interaction between CLN2 and CLN5 is specific. CLN1, which is a soluble lysosomal enzyme like CLN2, served as another control because the CLN5-specific antibody was unable to coimmunoprecipitate the CLN1 polypeptide.

Another putative interaction was observed in the coimmunoprecipitation assay between the CLN3 and CLN5 polypeptides. Both cDNA constructs were transfected into COS-1 cells because the antibodies used were unable to detect endogenous expression of either protein. The cell lysates were immunoprecipitated with the CLN5-specific antibody and Western blots were stained with CLN3-specific antibody. The results obtained from these experiments revealed that the WT CLN5 antibody coprecipitates the CLN3 polypeptide, suggesting that these proteins are interacting with each other (Figure 5B). Unlike CLN2, the *FIN<sub>M</sub>*, SWE, and EUR mutants of CLN5 were able to interact with CLN3. These observations imply that *FIN<sub>M</sub>*, SWE, and EUR mutants do not interfere with the binding capability of



**Figure 4.** (A) Subcellular localization of WT and mutant CLN5. Transiently transfected COS-1 cells were stained with CLN5-specific antibodies and a lysosome-specific lamp1 or Golgi-specific 58K antibody. (B) Subcellular localization of methionine mutants of CLN5. COS-1 cells were transfected with constructs having intact methionine at position 1, 30, 50, or 62 and stained with CLN5 and lamp1-specific antibodies. The secondary antibodies were conjugated with either fluorescein isothiocyanate or tetramethylrhodamine B isothiocyanate. Cells were viewed with an immunofluorescence microscope. The antibody used is shown in the lower right corner and the transfected construct in the lower left corner of each frame. The right-most figures show the overlay of both CLN5 and the organelle-specific staining. Colocalization is indicated by yellow. CLN5, peptide antibody; CLN5/N, N-terminal antibody.

CLN3. lamp1 and lamp2 showed negative results in the similar experiments (our unpublished data), revealing that the observed interactions are specific.

To confirm these results obtained from coimmunoprecipitation assays and to test whether the interactions between these CLN proteins are direct, we carried out in vitro binding assay for these proteins. CLN5 was expressed in *E. coli* as a fusion protein with GST, whereas the CLN2 and CLN3 proteins were produced by in vitro translation by using <sup>35</sup>S-labeled methionine. The coupling of these proteins was carried out in the presence of Glutathione-Sepharose, which was used to isolate the protein complexes. As a negative control, plain GST-protein was coupled with radioactively labeled CLN2 and CLN3. As another negative control, GST/CLN5 was coupled with the radioactively labeled AIRE-protein defective in an autoimmune disease, APECED (The Finnish-German APECED Consortium, 1997). CLN2 was

produced in in vitro translation as a precursor form of 63 kDa, which specifically interacted with GST-CLN5, showing no interaction when coupled with plain GST (Figure 5C). Similar results were obtained when radioactively labeled CLN3 was coupled with GST-CLN5, GST-coupling being negative. AIRE did not show any binding to GST nor to GST/CLN5. The results obtained from these experiments further confirm the coimmunoprecipitation results, also indicating that the interactions of CLN5 with CLN2 and CLN3 are direct and no other proteins are necessary for these interactions.

The observed interaction between CLN2 and CLN5 raised a question whether CLN5 interferes with the biological function of CLN2. Therefore, we measured the CLN2 activities in control fibroblasts and CLN5 patients' fibroblasts. CLN5 fibroblasts did not show any evidence about the defective function of CLN2. The activity of CLN2 was actually ~35%

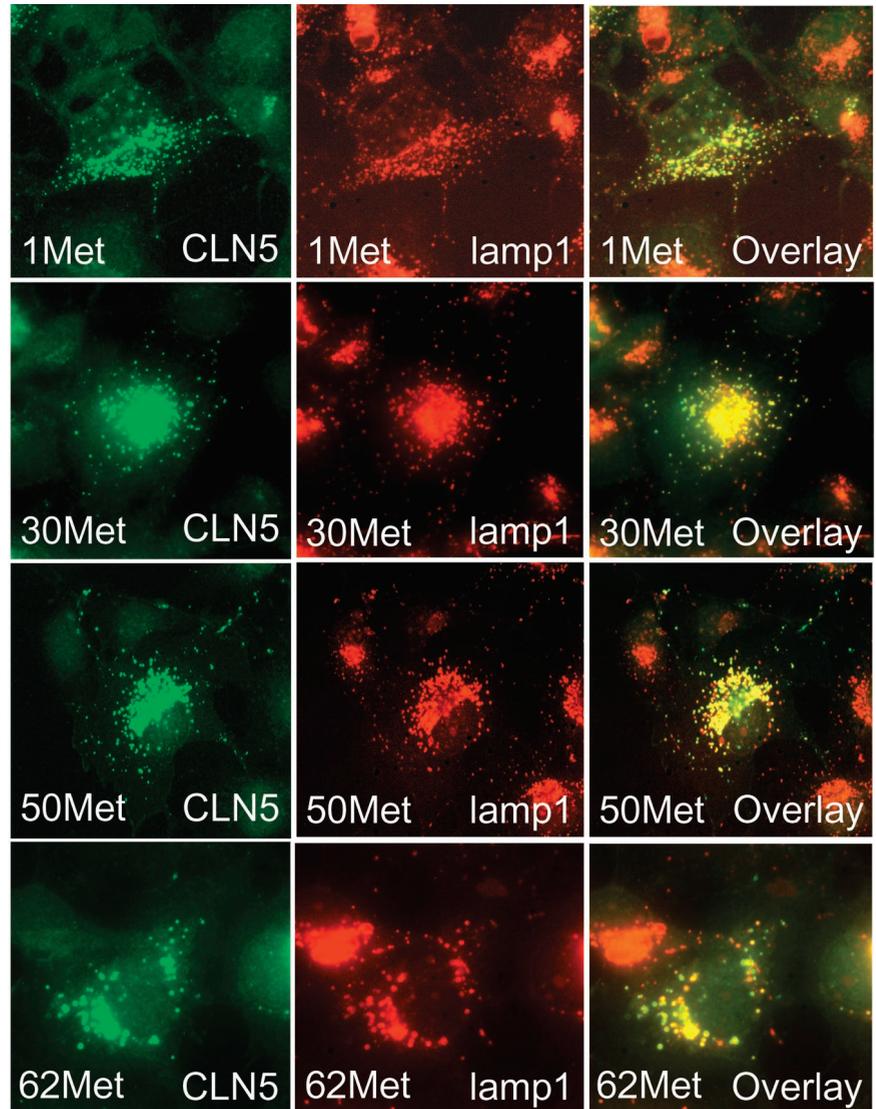


Figure 4 (continued).

higher than in the control sample (Figure 6). This observation suggests that in CLN5 patients' fibroblasts, CLN2 is targeted to its correct destination, lysosomes, where it gets activated in an acidic environment.

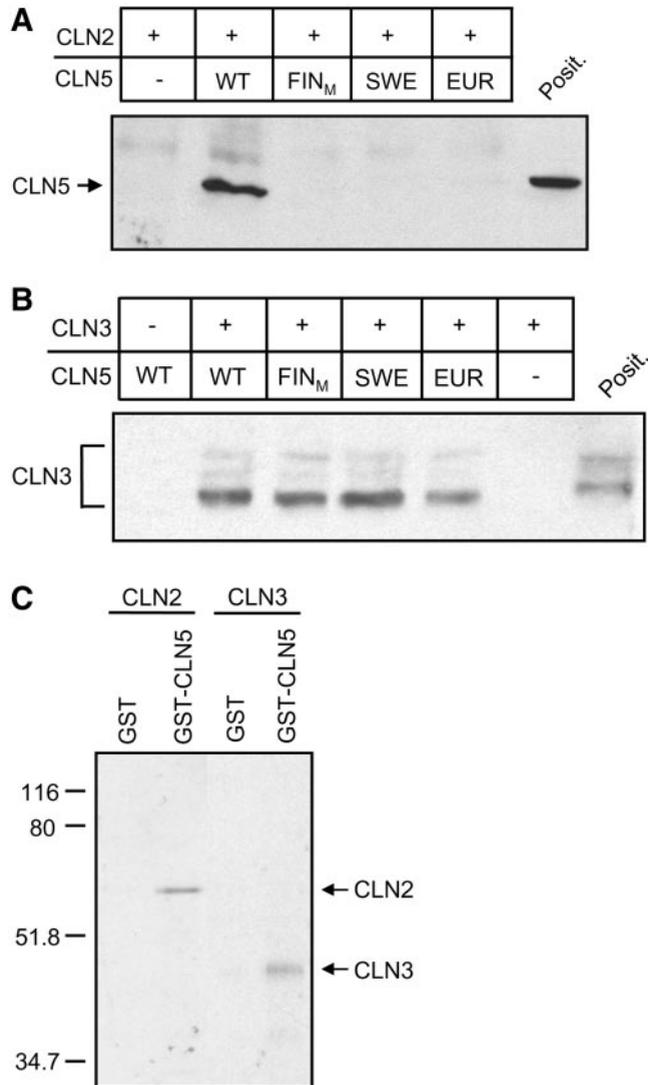
## DISCUSSION

Clinical subtypes of NCL disorders are all characterized by visual failure, mental retardation, and abnormalities in EEG. The CNS pathology in autopsy reveals a dramatic loss of cortical and cerebellar neurons. Most tissues and cell types show accumulation of autofluorescent material, in most subtypes consisting mainly of subunit c of the mitochondrial ATP synthase complex (Tynnela *et al.*, 1997). The causative genes, mutated in human disease, are established in six different NCL subtypes. However, considering the most recently identified CLN5, CLN6, and CLN8 genes, very little is known about the gene product and the actual molecular

pathogenesis of the diseases. Herein, we have evaluated the cellular consequences of different CLN5 disease mutations on corresponding polypeptides.

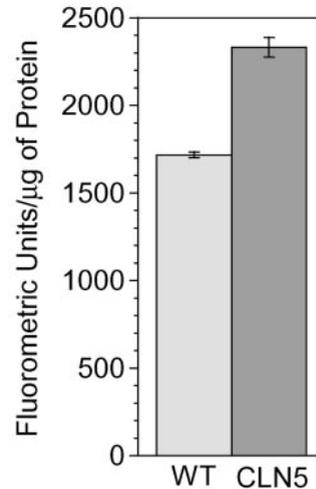
In Western blotting, the antibody against the amino-terminal part of CLN5 identified distinct bands of expected molecular weights for WT and mutant polypeptides. These observations suggest that the polypeptides recognized by the N-terminal antibody in Western blotting are not proteolytically processed or specifically glycosylated after translation. Further evidence for the relative stability of the mutated polypeptides emerged from the glycosylation analyses of the metabolically labeled polypeptides. Both WT and mutant polypeptides get modified by high-mannose type sugars and complex sugars. Furthermore, the glycosylation status of the SWE mutant suggests that the loop between two hydrophobic regions must be lumenally located.

The molecular weight of the 47-kDa polypeptide, observed in Western blotting, corresponds to the size of the



**Figure 5.** Interaction analyses of CLN-proteins. (A) COS-1 cells were transfected by either WT or mutant CLN5 cDNA constructs, and cell lysates were immunoprecipitated with CLN2-specific antibody. CLN2 was expressed endogenously. Results were obtained by Western blotting with CLN5-specific antibody. (B) COS-1 cells were transfected with CLN3 cDNA construct and with either WT or mutant CLN5 cDNA constructs. Cell lysates were immunoprecipitated with CLN5-specific antibody. Results were obtained by Western blotting with a CLN3-specific antibody. (C) Radiolabeled CLN2 or CLN3, produced by *in vitro* translation, was coupled with GST or GST-CLN5 produced in *E. coli* and pulled down with Glutathione-Sepharose. Results were obtained by SDS-PAGE and fluorography. Coupled proteins are indicated above, molecular weights of the marker bands are shown on the left, and the CLN-specific bands on the right. Posit., crude COS-1 cell lysate (A) expressing endogenous CLN2 (B) transfected with CLN5 cDNA.

translation product predicted from the first methionine in the open reading frame of the cDNA (Savukoski *et al.*, 1998). There are three other methionines, located downstream on the polypeptide at amino acid positions 30, 50, and 62.



**Figure 6.** TPP-I activity of control subject's and CLN5 patient's fibroblasts. Activities were measured using Ala-Ala-Phe 7-amido-4-methylcoumarin as a substrate. Results were obtained by fluorometry and are presented as fluorometric units per microgram of protein.

Herein, we demonstrated that all four methionines can be used in the initiation of translation of CLN5 and the localization studies performed for different methionine forms indicated that all four CLN5 forms are transported to lysosomes. The reason for four different CLN5 polypeptides with variable N termini, even in the same cells, is currently unknown, but the different forms may have some cell or tissue specificity in processing and targeting to better fulfill their function in different cellular backgrounds. Alternatively, different forms may have different, still unknown functions in cells. Of special interest are the polypeptides, which are synthesized from the third or fourth methionine. Based on the signal peptide prediction programs (SignalP; Nielsen *et al.*, 1997) these proteins have N-terminal signal peptides, which are cleaved off between amino acids 95–96, completely deleting the first transmembrane domain. It is possible that the usage of the third or fourth methionine results in a soluble protein, revealing that the second hydrophobic region is not sufficient for membrane association of the polypeptide. Evidence for a soluble CLN5 polypeptide has recently been obtained in transiently transfected BHK-21 cells (Isosomppi *et al.*, 2002), further suggesting a dual sub-cellular localization for the CLN5 protein. The sequence of the mouse CLN5 gene in the databases lacks the first three methionines located at positions 1, 30, and 50 in the human polypeptide. What the potential cell type or tissue specificity of the use of different initiator methionines, resulting in both soluble and membrane bound lysosomal proteins is in humans, remains to be clarified.

The computer-assisted predictions of the primary amino acid sequence of CLN5 have proposed two hydrophobic regions; suggesting two transmembrane domains (Savukoski *et al.*, 1998). The results obtained from our membrane fractionation analyses showed that the largest 47-kDa form of the CLN5 polypeptide is a transmembrane protein. The more detailed characterization of transmembrane domains, by using the expression of truncated CLN5 polypeptide,

revealed that the first hydrophobic region (amino acids 76–91) is sufficient for this membrane association.

To determine the intracellular localization of WT and naturally occurring disease mutants, we examined the cellular targeting of the CLN5 protein by immunofluorescence microscopy of transiently transfected COS-1 cells. The WT CLN5 showed extensive colocalization with lysosomal markers, which is in agreement with previous findings in BHK-21 cells (Isosomppi *et al.*, 2002). Interestingly, FIN<sub>M</sub> and EUR mutants are also targeted, at least to some extent, to lysosomes in COS-1 cells. Unlike in COS-1 cells, FIN<sub>M</sub> shows predominantly Golgi staining in BHK-21 cells, indicating that the trafficking of CLN5 may be cell specific (Isosomppi *et al.*, 2002).

Relatively uniform clinical manifestations and the fact that all NCL patients accumulate similar storage material in their cells suggest that the CLN proteins may be involved in the same pathological cascade of the diseases. Additionally, the major protein component of the accumulating material has been shown to be subunit c of mitochondrial ATP-synthase in all other CLN forms, except in CLN1, where the major protein component of the accumulating material consists of saposin A and D (Tyynela *et al.*, 1993). These observations suggest that the pathological defect of CLN patients other than CLN1 must be related to the catabolic cascade of subunit c of mitochondrial ATP-synthase. Previous studies have failed to observe physical interactions between CLN1, CLN2, and CLN3 with the yeast two-hybrid system (Zhong *et al.*, 2000). Based on this background we started evaluating the molecular interactions between the CLN5 and other lysosomal CLN proteins.

The coimmunoprecipitation assay revealed that CLN1 is not interacting with CLN2, CLN3, or CLN5. These results, as well as the observations that the accumulation material of CLN1 differs from that of other NCL diseases, suggest that the pathological cascade behind CLN1 is different from CLN2, CLN3, and CLN5. Unlike the results obtained with CLN1, the coimmunoprecipitation and *in vitro* binding assays revealed that CLN5 specifically interacts with WT CLN2 and CLN3. The observations that all CLN5 mutants studied are able to interact with CLN3 revealed that the interacting domain of CLN5 must locate amino terminally to the SWE mutation, terminating the CLN5 polypeptide at position 224. In addition, the pathological effect of these CLN5 mutants does not seem to interfere with the interactions with CLN3. The *in vitro* binding further confirmed the observed interaction between CLN3 and CLN5, also showing that the interaction between these two proteins occurs directly and assisting proteins are not necessary. The function of the observed interaction remains elusive. CLN3, like the longest form of CLN5, is a lysosomal membrane protein and is reportedly involved in the pH homeostasis of vacuoles of yeast cells (Pearce *et al.*, 1999). Therefore, CLN5 may collaborate with CLN3 to regulate the lysosomal pH in human cells. The recent study indicated that the lysosomal pH is increased in the fibroblasts of the CLN3 and CLN5 patients (Holopainen *et al.*, 2001), further supporting the hypothesis of the involvement of the CLN5 protein in pH homeostasis of lysosomes. Increased pH of lysosomes may, in turn, result in nonfunctional lysosomes, and eventually in accumulation of the subunit c of ATP synthase. Even although this accumulation can be observed in several tissues,

only cortical and cerebellar neurons are affected in CLN3 and CLN5. This observation suggests that cerebellar and cerebral neurons are the most sensitive to the accumulation of subunit c of ATP synthase.

Although both WT and disease mutants of CLN5 were interacting with CLN3, only the WT CLN5 polypeptide was able to bind with CLN2. Importantly, both the FIN<sub>M</sub> polypeptide, lacking only 16 C-terminal amino acids, and EUR polypeptide, having amino acid substitution at position 279, are not able to bind with CLN2. This would imply that the CLN2 interacting domain of CLN5 most likely resides in the C-terminal part of the protein. The detailed characterization of the protein domains participating in this interaction is under investigation. Recent studies revealed that CLN2 is synthesized as an inactive zymogen that is autocatalytically converted to an active serine protease at acidic pH (Lin *et al.*, 2001). The mutations in the CLN2 result in an inactive enzyme and, as in CLN3 and CLN5, accumulation of subunit c of ATP synthase, and eventually in death of cortical neurons. The pathological cascade from the gene mutation to the manifestation of the disease is still to be clarified. Also the *in vivo* substrates of the CLN2 enzyme are currently unknown. The *in vitro* binding assay revealed that the precursor form of CLN2 is able to interact with CLN5 most likely already in a prelysosomal compartment. Therefore, it is possible that CLN2 is needed for correct targeting of CLN5 or CLN5 may have domains with activity to modify CLN2. The observation that the TPP-I/CLN2 activity was increased in the CLN5 patients' fibroblasts is consistent with the previous observation, where TPP-I/CLN2 activity was increased in the brain lysates from CLN3 patients (Sleat *et al.*, 1998). This may be due to a functional redundancy, which is further confirmed by the interactions reported herein. Further structural analyses of these potential interactions between three CLN proteins should cast some light into the causes of the accumulation of the subunit c of mitochondrial ATP synthase in these diseases and the molecular pathogenesis of the severe destruction of CNS neurons.

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## REFERENCES

- Andersson, S., Davis, D.L., Dahlback, H., Jornvall, H., and Russell, D.W. (1989). Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* 264, 8222–8229.
- Dell'Angelica, E.C., Ohno, H., Ooi, C.E., Rabinovich, E., Roche, K.W., and Bonifacino, J.S. (1997). AP-3: an adaptor-like protein complex with ubiquitous expression. *EMBO J.* 16, 917–928.
- Heinonen, O., Kyttala, A., Lehmus, E., Paunio, T., Peltonen, L., and Jalanko, A. (2000). Expression of palmitoyl protein thioesterase in neurons. *Mol. Genet. Metab.* 69, 123–129.

- Hellsten, E., Vesa, J., Olkkonen, V.M., Jalanko, A., and Peltonen, L. (1996). Human palmitoyl protein thioesterase: evidence for lysosomal targeting of the enzyme and disturbed cellular routing in infantile neuronal ceroid lipofuscinosis. *EMBO J.* *15*, 5240–5245.
- Hirokawa, T., Boon-Chieng, S., and Mitaku, S. (1998). SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* *14*, 378–379.
- Holopainen, J.M., Saarikoski, J., Kinnunen, P.K., and Jarvela, I.I. (2001). Elevated lysosomal pH in neuronal ceroid lipofuscinoses (NCLs). *Eur. J. Biochem.* *268*, 5851–5856.
- Isosomppi, J., Vesa, J., Jalanko, A., and Peltonen, L. (2002). Lysosomal localization of the neuronal ceroid lipofuscinosis CLN5 protein. *Hum. Mol. Genet.* *11*, 1–7.
- Jarvela, I., Lehtovirta, M., Tikkanen, R., Kyttala, A., and Jalanko, A. (1999). Defective intracellular transport of CLN3 is the molecular basis of Batten disease (JNCL). *Hum. Mol. Genet.* *8*, 1091–1098.
- Jarvela, I., Sainio, M., Rantamaki, T., Olkkonen, V.M., Carpen, O., Peltonen, L., and Jalanko, A. (1998). Biosynthesis and intracellular targeting of the CLN3 protein defective in Batten disease. *Hum. Mol. Genet.* *7*, 85–90.
- Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E.L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* *305*, 567–580.
- Lehtovirta, M., Kyttala, A., Eskelinen, E.L., Hess, M., Heinonen, O., and Jalanko, A. (2001). Palmitoyl protein thioesterase (PPT) localizes into synaptosomes and synaptic vesicles in neurons: implications for infantile neuronal ceroid lipofuscinosis (INCL). *Hum. Mol. Genet.* *10*, 69–75.
- Lin, L., Sohar, I., Lackland, H., and Lobel, P. (2001). The human CLN2 protein/tripeptidyl-peptidase I is a serine protease that autoactivates at acidic pH. *J. Biol. Chem.* *276*, 2249–2255.
- Lonka, L., Kyttala, A., Ranta, S., Jalanko, A., and Lehesjoki, A.E. (2000). The neuronal ceroid lipofuscinosis CLN8 membrane protein is a resident of the endoplasmic reticulum. *Hum. Mol. Genet.* *9*, 1691–1697.
- Luiro, K., Kopra, O., Lehtovirta, M., and Jalanko, A. (2001). CLN3 protein is targeted to neuronal synapses but excluded from synaptic vesicles: new clues to Batten disease. *Hum. Mol. Genet.* *10*, 2123–2131.
- Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997). A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Int. J. Neural Syst.* *8*, 581–599.
- Pearce, D.A., Ferea, T., Nosel, S.A., Das, B., and Sherman, F. (1999). Action of BTN1, the yeast orthologue of the gene mutated in Batten disease. *Nat. Genet.* *22*, 55–58.
- Rapola, J. (1993). Neuronal ceroid-lipofuscinoses in childhood. *Percept. Pediatr. Pathol.* *17*, 7–44.
- Roseblat, S., Durham-Pierre, D., Gardner, J.M., Nakatsu, Y., Brilliant, M.H., and Orlow, S.J. (1994). Identification of a melanosomal membrane protein encoded by the pink-eyed dilution (type II oculocutaneous albinism) gene. *Proc. Natl. Acad. Sci. USA* *91*, 12071–12075.
- Santavuori, P. (1988). Neuronal ceroid-lipofuscinoses in childhood. *Brain Dev.* *10*, 80–83.
- Savukoski, M., Klockars, T., Holmberg, V., Santavuori, P., Lander, E.S., and Peltonen, L. (1998). CLN5, a novel gene encoding a putative transmembrane protein mutated in Finnish variant late infantile neuronal ceroid lipofuscinosis. *Nat. Genet.* *19*, 286–288.
- Sleat, D.E., Donnelly, R.J., Lackland, H., Liu, C.G., Sohar, I., Pullarkat, R.K., and Lobel, P. (1997). Association of mutations in a lysosomal protein with classical late-infantile neuronal ceroid lipofuscinosis. *Science* *277*, 1802–1805.
- Sleat, D.E., Sohar, I., Pullarkat, P.S., Lobel, P., and Pullarkat, R.K. (1998). Specific alterations in levels of mannose 6-phosphorylated glycoproteins in different neuronal ceroid lipofuscinoses. *Biochem. J.* *334*, 547–551.
- Sohar, I., Lin, L., and Lobel, P. (2000). Enzyme-based diagnosis of classical late infantile neuronal ceroid lipofuscinosis: comparison of tripeptidyl peptidase I and pepstatin-insensitive protease assays. *Clin. Chem.* *46*, 1005–1008.
- The Finnish-German APECED Consortium. (1997). An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat. Genet.* *17*, 399–403.
- Tyynela, J., Palmer, D.N., Baumann, M., and Haltia, M. (1993). Storage of saposins A and D in infantile neuronal ceroid-lipofuscinosis. *FEBS Lett.* *330*, 8–12.
- Tyynela, J., Suopanki, J., Santavuori, P., Baumann, M., and Haltia, M. (1997). Variant late infantile neuronal ceroid-lipofuscinosis: pathology and biochemistry. *J. Neuropathol. Exp. Neurol.* *56*, 369–375.
- Uvebrant, P., and Hagberg, B. (1997). Neuronal ceroid lipofuscinoses in Scandinavia. *Epidemiology and clinical pictures. Neuropediatrics* *28*, 6–8.
- Verkruyse, L.A., and Hofmann, S.L. (1996). Lysosomal targeting of palmitoyl-protein thioesterase. *J. Biol. Chem.* *271*, 15831–15836.
- Vesa, J., Hellsten, E., Verkruyse, L.A., Camp, L.A., Rapola, J., Santavuori, P., Hofmann, S.L., and Peltonen, L. (1995). Mutations in the palmitoyl protein thioesterase gene causing infantile neuronal ceroid lipofuscinosis. *Nature* *376*, 584–587.
- Wheeler, R.B., Sharp, J.D., Schultz, R.A., Joslin, J.M., Williams, R.E., and Mole, S.E. (2001). The gene mutated in variant late-infantile neuronal ceroid lipofuscinosis (CLN6) and in *nclf* mutant mice encodes a novel predicted transmembrane protein. *Am. J. Hum. Genet.* *70*, 2.
- Zhong, N.A., Moroziwicz, D.N., Ju, W., Wisniewski, K.E., Jurkiewicz, A., and Brown, W.T. (2000). CLN-encoded proteins do not interact with each other. *Neurogenetics* *3*, 41–44.