The Intracellular Location and Function of Proteins of Neuronal Ceroid Lipofuscinoses

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Neuronal ceroid lipofuscinoses are a group of diseases characterized by accumulation of hydrophobic proteins in lysosomes of neurons and other types of cells. NCLs are caused by at least 8 mutant genes (CLN1-CLN8), though CLN4 and CLN7 have not yet been identified. Except for Cln1p, the protein encoded by CLN1, the defective proteins are associated with lysosomal accumulation of mitochondrial ATP synthase subunit c. Cln1p and Cln2p are soluble lysosomal enzymes, targeted to lysosomes in a mannose 6-phosphate dependent manner. Mutations in the lysosomal protease cathepsin D cause another NCL. Cln3p, Cln5p, Cln6p and Cln8p are thought to be transmembrane proteins. Cln3p and Cln5p are localized in the endosome-lysosomal compartment. Deficiency of endosomal membrane protein CLC-3, a member of the chloride channel family, causes NCL-like phenotype and lysosomal storage of subunit c. Herein, we review the features of NCL and NCL-related proteins and discuss the involvement of the proteins in lysosomal degradation of subunit c.

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INTRODUCTION

The neuronal ceroid lipofuscinoses (NCLs) comprise a group of fatal neurodegenerative disorders. NCLs are caused by mutations in at least 8 genes, resulting in accumulation of proteins in neurons and other cells. Based on age at onset of the disorders, clinical features, genetic linkage, mutation analysis and enzyme assay, they can be classified into eight forms (Table 1) (40, 55): infantile NCL (INCL; defective gene is CLN1; Santavuori-Haltia disease), classical late infantile NCL (LINCL; defective gene is CLN2; Jansky-Bielschowsky disease), juvenile NCL (JNCL; defective gene is CLN3; Spielmeyer-Vogt-Sjögren disease), adult NCL (defective gene is CLN4; Kufs disease;), three variant forms of LINCL (defective genes are CLN5, CLN6 and CLN7; Finnish, Costa Rican and Turkish variants) and a variant form of JNCL (defective gene is CLN8; progressive epilepsy with mental retardation (EPMR)/Northern epilepsy). In addition, 2 other JNCL variants are each caused by mutations in CLN1 or CLN2. It is thought that other types of NCLs which have been studied in animal models may also afflict humans (41, 91, 103).

SPECIFIC LYSOSOMAL STORAGE OF HYDROPHOBIC PROTEINS: SUBUNIT C OR SPHINGOLIPID ACTIVATOR PROTEINS

NCLs are characterized by accumulation of material, which has histochemical properties similar to lipofuscin/ceroid, in neurons and many other types of cells. Palmer and coworkers demonstrated that the material is two-thirds protein and that its major component is identical to the dicyclohexylcarbodiimide-reactive proteolipid which is also known as subunit c of the mitochondrial ATP synthase complex (F_0F_1-ATPase) (61, 62, 63). Subunit c is a very hydrophobic protein. It is composed of 75 amino acid residues with a theoretical molecular mass of 7 602 Da and is classified as a proteolipid because of its lipid-like solubility in chloroform-methanol mixtures. There is no evidence that the amino acid sequence of subunit c accumulating in the juvenile and late infantile forms is abnormal (18). Biochemical analysis has shown that subunit c accumulates in the lysosomal compartment of fibroblasts, except in INCL (26, 44).

In INCL, Saposins A and D accumulate in the cells. Saposins, also called sphingolipid activator proteins (SAPs), are small heat-stable glycoproteins needed for the hydrolysis of sphingolipids in lysosomes (90). They are derived from a common precursor, prosaposin, which is a 70-kDa glycoprotein (30). As described in more detail below, INCL is caused by deficiency of palmitoyl protein thioesterase 1 (PPT-1), but the relationship between the deficiency and the accumulation of SAPS is unclear.

DEFECT OF LYSOSOMAL ENZYME, PPT-1 IN INCL

INCL is caused by the mutation of the gene for palmitoyl protein thioesterase 1 (PPT-1) (93). PPT-1 is a long chain fatty-acid hydrolase that removes fatty acyl groups from various S-acylated proteins. Camp and Hoffmann have purified PPT-1 from bovine brain by utilizing its activity to remove palmitate from palmitoylated H-Ras. The enzyme had a molecular mass at 37 kDa and was sensitive to diethyl pyrocarbonate and insensitive to phenylmethylsulfonyl fluoride and N-ethylmaleimide (6). They have also cloned bovine and rat cDNA that encodes PPT-1. The deduced amino acid sequence of PPT-1 predicts a protein of 306 amino acids that contains amino acid motifs characteristic of thioesterase: Gly-X-Ser-X-Gly positioned near the NH2 terminus and Gly-Asp-His positioned near the COOH terminus of the protein (7). The optimal pH for Ha-Ras as a substrate was in the neutral range, but it was half-maximal in the range 4.5 to 5.5 in the model substrate palmitoyl CoA (92).

The expressed enzyme contained an asparagine-linked oligosaccharide chain (29), and was recovered in the bottom fractions with lysosomal enzyme markers by Percoll density gradient centrifugation (92). In COS-1 cells, PPT- 1 was transported to lysosomes via a mannose-6-phosphate receptor-mediated pathway, and its localization was disturbed when the enzyme had the

Type of NCL	Gene	Locus	Gene product	Localization	Storage protein
Congenital	?	?	(Cathepsin D?)	Lysosome	SAPs****
Infantile	CLN1	1p32	PPT*	Lysosome	SAPs****
Classical LINCL	CLN2	11p15	TPP-I**	Lysosome	Subunit c
LINCL (Finnish Variant)	CLN5	13q22	Cln5p (membrane protein)	Lysosome	Subunit c
LINCL (Costa Rican Variant)	CLN6	15q21-23	Cln6p (membrane protein)	?	Subunit c
LINCL (Turkish Variant)	CLN7	?	?	?	Subunit c
Classical JNCL	CLN3	16p12	Cln3p (membrane protein)	Lysosome	Subunit c
EPMR/NG***	CLN8	8p23	Cln8p (membrane protein)	ER	Subunit c
JNCL (Variant)	CLN1	1p32	PPT*	Lysosome	SAPs****
JNCL (Variant)	CLN2	11p15	TPP-I**	Lysosome	Subunit c
Kufs' Disease	CLN4	?	?	?	Subunit c

*Palmitoyl Protein Thioesterase, **Tripeptidyl Peptidase I, ***Progressive epilepsy with mental retardation or Northern epilepsy, ****Saposins

Table 1. The major forms of neuronal ceroid lipofuscinoses (NCLs).

commonest INCL mutation, Arg122Trp (29, 76, 92). This means that a novel lysosomal enzyme deficiency is behind INCL. In situ hybridization analysis showed that PPT-1 transcripts are found widely, but not homogeneously, in brain (33). In rodent brain, PPT-1 staining has shown a typical punctate pattern in neurons, especially in hippocampal neurons and Purkinje cells (32, 81). The expression of PPT-1 in rat brain was developmentally regulated; its expression increased with maturation of the CNS, and reached a maximum in young adulthood (32, 81). This may indicate that PPT-1 had a significant role in the early developmental stages of neuronal cells.

Non-lysosomal localization of PPT-1 was suggested by a study using cells expressing PPT-1. In mouse primary neurons and brain tissues, PPT-1 was localized in synaptosomes and synaptic vesicles but did not co-localize with lysosomal marker protein LAMP1. Furthermore, PPT-1 was targeted specifically to axons in mature neurons, as indicated by its colocalization with growth-associated protein 43 and synaptophysin. Polarized axonal targeting of PPT-1 may perhaps indicate a role of PPT-1 in the exocytotic pathway of neurons and may show that it is associated with synaptic function (1, 47).

PPT-1 knockout mice were viable and fertile but showed NCL-like pathology with prominent accumulation of autofluorescent lipopigment throughout the CNS, together with pronounced cerebral atrophy. They developed spasticity at a mean age of 21 weeks and died by 10 months of age. The PPT-1 deficient mouse is an excellent model and may be useful for testing a therapy (24).

PPT-2, the homologue of PPT-1, had comparable palmitoyl-CoA thioesterase activities (78). Its optimal pH is neutral and it is localized in the lysosomes. However, it had a different substrate specificity from that of PPT-1, and is suggested to play a unique role in the hydrolysis of lipid thioesterase. The PPT-2 knockout mice were also viable and fertile, but showed milder CLN symptoms than the PPT-1 knockout mice. The PPT-2 deficient mice may indicate a phenotype for possible human disorder (24, 25).

TPP-I, A LYSOSOMAL SERINE CARBOXYL PEPTIDASE, IS DEFICIENT IN LINCL

In 1997, Sleat and colleagues demonstrated the absence of a single lysosomal protein in brain with LINCL by proteomic analysis, and identified mutations in the CLN2 gene (77). The CLN2 gene product,Cln2p, was identified to be a 46-kDa glycoprotein and was predicted as a lysosomal protein by its modified carbohydrate, mannose 6-phosphate (man-6-P) (77). This protein showed a significant amino acid sequence similarity to bacterial pepstatin-insensitive carboxyl proteases (BPICP) (58, 59). Furthermore, corresponding enzymatic activity, that is pepstatin-insensitive endopeptidase (Pepinase) activity, was deficient in LINCL autopsy specimens. (77). Lysosomal localization was confirmed by immunofluorescence and subsequently by subfractionation analysis. Pulse-chase analysis showed that Cln2p was synthesized as a 67 kDa precursor and processed into a 46 kDa mature protein ($T_{1/2}$ = 1~2 h) (13, 22). Immunoblotting analysis showed reactivity in brain, liver, kidney, heart, stomach, colon, adrenal gland and testis (42, 46). Cln2p was immunohistochemically detected in the cytoplasmic vesicles of all these tissues, whereas it was completely absent in these organs in the LINCL cells (13, 79).

Before long, sequence analysis showed that Cln2p is identical to tripeptidyl peptidase I (TPP-I) (68, 96). LINCL fibroblasts were found to lack TPP-I activity (14, 96). This was unexpected because the Cln2p was considered to be the endopeptidase pepinase, but TPP-I turned out to be

an exopeptidase that cleaves tripeptides from the N-terminus of oligopeptides. The controversy was solved by a study using purified TPP-I (14); the enzyme had both TPP-I and pepinase activities, with a maximal activity at pH 3.0 as pepinase and at pH 4.5 as TPP-I. Both activities were strongly inhibited by Ala-Ala-Phe-CH_2Cl (AAF-CMK) (14). From these results, pepinase was identified as TPP-I itself. Tyrostatin, an inhibitor of BPICP, had no effect on the activity of pepinase/TPP-I. The mature enzyme was unstable at neutral pH and lost almost all activity within 30 minutes at 37°C (14). The native form of Cln2p/TPP-I was examined by gel filtration and shown to be a dimer or a larger form in rat spleen, brain or kidney (9, 38, 95). Because pepinase/TPP-I activity was lost in the cells with late infantile NCL, its activity should be measured as a marker of LINCL. Immunochemical analysis did not reveal Cln2p/TPP-I protein in cells from patients with LINCL with several different mutations, but the expression level of CLN2 was not significantly different from that in control cells. This means that mutated Cln2p/TPP-I may be degraded soon after being synthesized (13).

Lin and colleagues purified and characterized C-terminal hexahistidine-tagged human Cln2p/TPP-I produced from insect cells transfected with a baculovirus vector. They showed that Cln2p/TPP-I was synthesized as a catalytically inactive protein with an apparent size of 66 kDa and was converted into mature 46 kDa autocatalytically at acidic pH. They suggested that the Cln2p/TPP-I was inhibited by DFP, though it required a long time and a high concentration of DFP (48). It has recently been proposed that Cln2p/TPP-I be classified as a member of a new group of peptidases, that is, as a serine-carboxyl peptidase (60, 101, 102).

That specific accumulation of subunit c of ATP synthase is caused by deficiency of TPP-I (10, 11, 12) has been proven by immunodepletion of TPP-I and a study using AAF-CMK, the specific inhibitor of TPP-I (12,15). In the initial degradation of subunit c, TPP-I cleaves off tripeptides and then some of the aspartic protease. Furthermore, subunit c degradation in cells cultured from patients with LINCL was made possible by the addition of purified TPP-I (15).

What is the real substrate responsible for the neurodegenerative disease? Although it is reasonable to consider subunit c as a natural substrate, TPP-I may be responsible for degradation of neurotoxic peptides or maturation of certain neuropeptides. It has been reported that TPP-I effectively cleaves angiotensin II, substance P, amyloid β, glucagon, cholecystokinin-(29-33)-amide, and growth hormone (3, 38, 97). Bernardini and Warburton suggested that cathepsin C may substitute TPP-I. However, cathepsin C was scarcely detected in the brain (3). If TPP-I worked on these substrates in the brain, lack of TPP-I would contribute directly to pathogenesis.

CATHEPSIN D DEFICIENCY ALSO CAUSES NEURONAL CEROID LIPOFUSCINOSIS

Cathepsin D knockout ($CD^{-/-}$) mice manifest seizures with trembling and stiff tails and become blind near the terminal stage of the disease. They usually grow normally for up to 2 weeks of age, after which they cease to grow, and die at around P26 as a result of intestinal necrosis and lack of eating. The mice begin to manifest repetitive seizures from about P20 and some of them have severe tonic seizures and die from respiratory arrest. In electrophysiological analysis of hippocampal slices, spontaneous burst discharges consisting of 4 to 15 population spikes superimposed on a prolonged positive deflection (60-150 msec) were recorded from the stratum pyramidale of both the CA1 and the CA3 regions (41).

In the terminal stage (P23-26), the neurons were completely filled with granular osmiophilic deposits (GRODs), some of which were surrounded by double-layered membranes, like those of autophagosomes and autolysosomes. Fingerprint profiles also appeared in neuronal cell bodies and retinal pigment epithelial cells; surprisingly, the neuronal cell bodies were immunopositive for subunit c of mitochondrial F_1F_0-ATP synthase, however the activity and protein levels of TPP-I were elevated. The accumulation of subunit c was also detected in peripheral tissues such as the liver, kidney, and heart (43).

Microglia of $CD^{-/-}$ mice with large round cell bodies but few processes appeared in cerebral cortex and thalamus after P16. At P24, the microglia often encircled neurons that contained autolysosomes, indicating phagocytic activity. These morphologically transformed microglia markedly expressed inducible NO synthase (iNOS) which was also detected in the murine intestines. Although NO is a biological messenger molecule, high levels are toxic. L-N^G-nitro-arginine methylester (L-NAME), a competitive NOS inhibitor, or S-methylisothiourea hemisulfate (SMT), an iNOS inhibitor partially but significantly reduced the total number of TUNEL-positive cells in the thalamus of $CD^{-/-}$ mice. Furthermore, these inhibitors could partially lessen the decrease in body weight and the intestinal atrophy of $CD^{-/-}$ mice and prolong the survival time. These results suggest that NO production through iNOS activity in microglia and peripheral macrophages contribute to secondary tissue damages such as neuronal apoptosis and intestinal necrosis, respectively (42, 56).

An ovine model showed different symptoms to those of the cathepsin D knockout mouse model. Cathepsin D activities were also undetectable in congenital ovine NCL (CONCL). The mutation associated with CONCL results from asparagines substituting the aspartate that corresponds to Asp295 in human cathepsin D. This residue is essential for the catalytic function of cathepsin D, and its mutation leads to production of an enzymatically inactive but stable protein. Newborn CONCL lambs were very weak; they trembled and were unable to stand. Surprisingly, Western blot analysis revealed that the steady-state level of cathepsin D protein was markedly higher in CONCL brains than in control brains. Electron microscopic examination of CONCL neuronal storage bodies revealed GRODs. The storage material contained high level of SAPs A and D, and there was no accumulation of mitochondrial ATP synthase subunit c (91). The difference in phenotypes between $CD^{-/-}$ mice and CONCL lambs is probably due to the presence of enzymatically-inactive cathepsin D in CONCL lambs. These results suggest a link between lysosomal degradation of subunit c and SAPs.

CLN3P IS A LYSOSOMAL MEMBRANE PROTEIN

The CLN3 gene was positionally cloned in 1995, and has been shown to be responsible for JNCL (89). The accumulating material is mainly composed of subunit c.

Cln3p, encoded by CLN3, is a lysosomal membrane protein rather than a lysosomal enzyme (16). The predicted product of the cDNA is a novel protein of 438 amino acids. A search for protein motifs in Cln3p revealed pattern matches for 4 N-glycosylation sites, 2 O-glycosylation sites, 2 glycosaminoglycan attachment sites, 2 cAMP- and cGMP-dependent protein kinase phosphorylation sites, 6 protein kinase C phosphorylation sites, 8 casein kinase II phosphorylation sites, and 12 N-myristoylation sites. Hydropathy calculation predicted 5 hydrophobic regions that might be potential membrane-spanning regions at amino acids 38-61, 93-233, 278-310, 345-399 and 408-438 (34, 89). However, the membrane topology of Cln3p remains controversial (16, 51). Lysosomal localization of this protein was suggested by studies using cultured cells which over-expressed Cln3p (20, 35). Järvelä and colleagues showed that the mutation behind JNCL causes mislocalization of Cln3p (36).

Recently, we detected Cln3p endogenous in brain and liver. It has a molecular mass of around 55 kDa and also around 60 kDa (16), depending on the complex-type oligosaccharides attached. Liver Cln3p was specifically localized in the lysosomal membranes and was not detected in the endoplasmic reticulum or the Golgi apparatus. Unexpectedly, the concentration of Cln3p was much lower in brain than in liver (16); it had been assumed that brain contains large amounts of Cln3p, because NCL is a neurodegenerative disease. However we detected only small quantities of Cln3p in cerebrum after immunopurification. Of course, this does not mean that it does not have an important role in the central nervous system.

Cln3p knockout mice were created by targeted disruption of the CLN3 gene (39, 45, 53). Their neurons and other type of cells had accumulated material that was autofluorescent. The material was situated in membrane-bound inclusions with multilamellar rectilinear/fingerprint appearance, which is typical of ceroid lipofuscinosis. The inclusions contained subunit c of mitochondrial ATP synthase. In addition, there was increased activity in the brain of the lysosomal protease Cln2p/TPP-I, as seen in Batten disease (chronic juvenile form) (37, 53). Knockout animals showed neuropathological abnormalities with loss of certain cortical interneurons and hypertrophy of many interneurons in the hippocampus (53). Fifty percent of Cln3 knockout mice died after flurothyl-induced seizures (45). Apoptotic cells were observed in the photoreceptor layer of CLN3 knockout mice, but the degree of retinal degeneration was not extensive (72). $Cln3^{\Delta ex7/8}$ knock-in mice that have ~1 kb deletion in the CLN3 gene displayed recessively inherited degenerative changes in retina, cerebral cortex and cerebellum, as well as neurological defects and premature death (8).

The function of Cln3p has also not yet been identified, but BTN1 (yeast homologue of human CLN3) might give some clues (64). A BTN1-deletion yeast strain (Δbtn1) was resistant to D-(-)-threo-2-amino-1-[p-nitrophenyl]-1,3-propanediol (denoted ANP), a breakdown product of chloramphenicol. Normal BTN1+ strains could not grow in the presence of ANP at pH6.8 or higher, but could when the pH of the growth medium was artificially decreased. Δbtn's resistance to ANP was due to an elevated ability to acidify the growth medium and therefore neutralize the toxicity of ANP. The pH of vacuoles in (btn strains was also decreased temporarily during the course of growth (65). If lack of Cln3p also causes a decrease in lysosomal pH, proteins which are normally degraded in the lysosomes may either accumulate or aggregate due to decreased pH. Furthermore, the activity of protease involved in degradation of such proteins might be reduced by low pH. Thus, altered pH homeostasis seems to be the underlying cause of juvenile NCL.

Golabek and colleagues suggested that the lysosomal pH in cultured human embryonic kidney cells was increased by the expression of GFP or FLAG labeled Cln3p. The effect, however, was not detected by expression of mutant Cln3p (R334C) (21). Contrary, Holopainen et al claimed that the lysosomal pH was elevated in the fibroblasts with juvenile NCL (31). The discrepancy may be caused by differences in the techniques used to assess the function of Cln3p, however the measurement of pH in the intracellular organelles seems to be more important.

OTHER CLN TRANSMEMBRANE PROTEINS

Cln5p. The Finnish variant of late-infantile NCL (CLN5) was recently distinguished from classical NCLs. Santavuori et al examined 22 patients in Finland with an onset age of 4.5 to 7 years and found that the spectrum of LINCL is wider than had previously been thought, and they actually preferred to denote the disease as a variant form of Jansky-Bielschowsky type(69). Their observations imply that there are two variant forms of NCLs, CLN5 and a progressive epilepsy with mental retardation (EPMR) which is also called Northern epilepsy)(CLN8) (70, 83). CLN5 has later onsetting clinical signs, progresses more slowly and leads to less severe brain atrophy. They assigned one of the NCL variants to chromosome 13 (70).

The gene that encodes the protein associated with CLN5 has been identified by positional cloning. It is approximately 4.1 kb and consists of four exons with an open reading frame of 1380 bp. Hybridization of an RNA dot blot suggested high levels of expression in aorta, kidney, lung and pancreas of adults. The levels of expression in brain, heart, kidney, liver, spleen and lung of fetuses were fairly uniform, but in fetal thymus it was about twice as much (71). The CLN5 gene product, Cln5p, was predicted to be a novel protein of 46 kDa and to have a calculated pI of 8.41. There are several potential sites at which it can be modified: protein kinase C phosphorylation sites, casein kinase II phosphorylation sites, tyrosine kinase phosphorylation sites, N-myristoylation sites and N-glycosylation sites. CLN5 has 4 in-frame AUG codons at the 5′ end which are used to produce polypeptides with molecular masses of 47, 44, 42, and 40 kDa in in vitro translation (71).

Primary amino acid sequencing has shown that Cln5p contains 2 hydrophobic regions, at positions 76 to 91 and 353 to 373. This suggests that the polypeptide has 2 transmembrane domains. Membrane fractionation analysis using Triton X-114 has shown that the longest 47 kDa form of Cln5p is a transmembrane protein. Actually, the construct encoding the first 107 amino acids produces a polypeptide associated with the membrane fraction. Recently, soluble Cln5p has been obtained in transiently transfected BHK-21 cells. It

is possible that usage of the third or fourth methionine results in a soluble protein, implying that the second hydrophobic region is not sufficient for membrane association of the polypeptide (94).

Coimmunoprecipitation and in vitro binding assays revealed that Cln5p specifically interacts with TPP-I and Cln3p. All of the Cln5p mutants studied were able to interact with Cln3p, suggesting that the interacting domain of Cln5p is located in the amino terminal region of the protein. In vitro binding further confirmed the interaction between Cln3 and Cln5p, and also showed that their interaction occurs directly, ie, without assisting proteins. In contrast, TPP-I was able to bind with wild type Cln5p only. Importantly, both the FIN_M polypeptide which lacks only 16 C-terminal amino acids, and EUR polypeptide which has a substituted amino acid at position 279, cannot bind with TPP-I. This implies that the TPP-I-interacting domain of Cln5p most likely resides in the C-terminal part of the protein. TPP-I initiates the degradation of ATP synthase subunit c, and Cln3p is reportedly involved in pH homeostasis of lysosomes in which subunit c is degraded. Therefore, Cln5p may be involved in the accumulation of subunit c through its interaction with TPP-I and Cln3p (94).

The subcellular localization of Cln5p was studied in transiently transfected culture cells using confocal microscopy. The staining pattern of wild type Cln5p showed almost complete colocalization with the lysosomal membrane protein lgp120. FIN_M and EUR mutants are also targeted to lysosomes in COS-1 cells, however FIN_M mutant showed predominantly Golgi staining in BHK-21 cells (94).

Cln6p and Cln7p. Costa Rican variant LINCL (CLN6) has been reported as having clinically mixed features of LINCL and JNCL (Pakistan type, Indian type, Greece type and Portugal type also have a mutations in the same gene) (98, 73). Its clinical onset is delayed until 5 to 7 years of age and it follows a mild course. Ultrastructural examination of these accumulating bodies has revealed predominantly curvilinear bodies, with some fingerprint profiles. The gene for CLN6 was mapped to chromosome 15q21-23 and was predicted to be orthologous to the genes underlying NCL in nclf mice and in South Hampshire and Merino sheep (4, 5, 99). Nclf is a spontaneous mouse mutation which causes a recessively inherited NCL-like disease with a phenotype almost the same as that observed in mnd/mnd mice. Homozygous nclf mice developed progressive retinal atrophy early in life and became paralyzed at around 9 months of age (4).

The CLN6 gene product, Cln6p, is predicted to be a novel 311-amino acid transmembrane protein with unknown function and localization. CLN6 has no homology with known proteins or functional domains, although the sequence is highly conserved across vertebrate species, having 90% identity between the human and mouse amino acid sequences. The majority of mutations result in a frame shift or nonsense change, with the introduction of a premature stop codon. However, as some patients had a 3-base pair deletion or missense mutation within the protein's hydrophilic loop (E72Q, I154del, Y171del, Y221S, E241T and W300R) (2, 19, 74, 75, 87), the loop probably has an important role in the normal function of the protein.

Recently, manganese-dependent superoxide dismutase (Mn^{2+}-SOD) was reported as being significantly and specifically increased in the fibroblasts and brain extracts of both human and ovine models affected with CLN6. Confocal fluorescence microscopy and immunohistochemical studies revealed the presence of Mn^{2+}-SOD in mitochondria of CLN6 fibroblasts and in both neurons and hypertrophic astrocytes in CLN6 brain sections. These results may reflect the oxidative stress and/or the production of proinflammatory cytokines characteristic of CLN6 (28).

CLN7, the mutant gene that causes Turkish variant LINCL, has not yet been mapped. It was not found in any of the known NCL loci, despite having an earlier onset and being a more severe phenotype (98). However, it may be an allelic variant of ERMR, because the region of shared homozygosity has been identified on chromosome 8p23, which is where CLN8 is located (52).

Cln8p. Another type of variant LINCL, which occurs predominantly in northern Finland, was initially described as an EPMR that was also called Northern epilepsy. There is neuropathological evidence for classifying EPMR as an NCL (83): in most of the neuronal cells, accumulating autofluorescent material was positive for Luxol fast blue, PAS and Sudan black B (27, 67). The accumulation of mitochondrial ATP synthase subunit c and small amounts of SAPs in the neurons of brains were detected by immunocytochemical staining. The EPMR gene, CLN8, is located on the short arm of chromosome 8, and was identified in 1999 (66). It encodes a putative transmembrane protein composed of 286 amino acid polypeptide (Cln8p). A missense mutation, 70C→G, whereby arginine is substituted for glycine (R24G) was found to be homozygous in EPMR patients and heterozygous in carrier parents (66). Cln8p is 33 kDa in COS-cells that transiently expressed the polypeptide. Cln8p was not processed during its maturation and was not modified by N-glycosylation. Immunostaining of recombinant Cln8p in transiently transfected BHK, Hela and CHO cells revealed a reticular labeling pattern typical of endoplasmic reticulum (49).

The CLN8 defect was detected in the naturally occurring mouse model, the motor neuron degeneration (mnd) mouse. Histopathologically, the mnd mouse showed accumulation of autofluorescent material that was immunoreactive with antibodies against subunit c of F_0F_1-ATP synthase. From these results, the mnd mouse has been proposed as a model for NCL (17). The coding sequence of the human and mouse genes were found to be 82% and 85% identical at the nucleotide and the polypeptide levels, respectively. A homozygous mutation (267-268insC, codon90) results in a frame shift and a truncated polypeptide of 116 amino acids (17).

Recently, CLN8 was suggested to be a member of a large eukaryotic family of homologues that includes 2 proteins that are resident on the endoplasmic reticulum: Lag1p and human TRAM (translocating chai-associating membrane protein). The homology domain of these proteins named TLC (TRAM, LAG1, and CLN8 homology domains) usually extends over approximately 200 amino acids and contains at least 5 predicted membrane-spanning domains (100). Lag1p and its homologue Lac1p are thought to be relevant to acyl-CoA-dependent ceramide synthase. Hu-

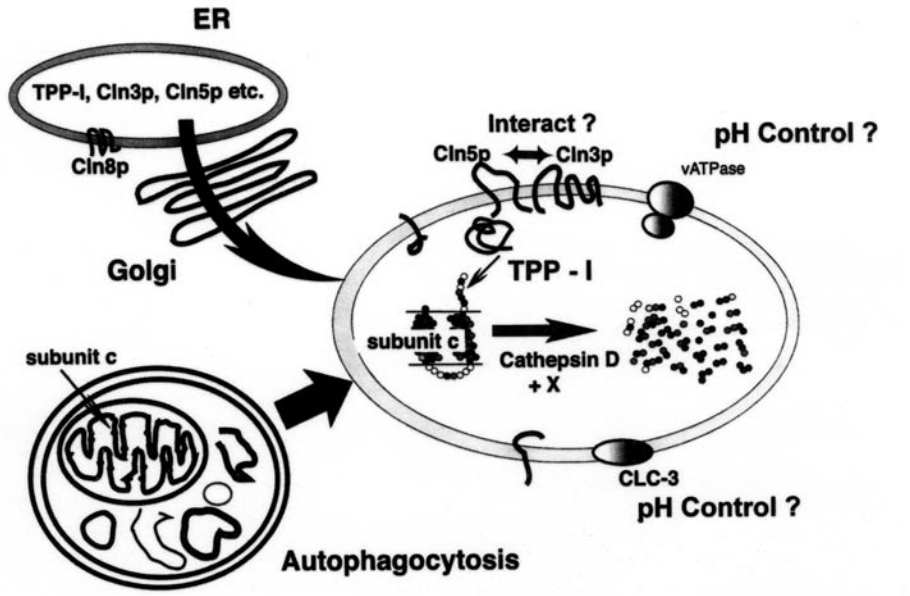

Figure 1. The lysosomal/endosomal system.

man LAG1 homologues could rescue the viability of lag1Δ lac1Δ yeast cells and restore acyl-CoA-dependent ceramide and sphingolipid biosynthesis, whereas CLN8 and TRAM could not restore viability to lag1Δ lac1Δ yeast mutant. It may be that these proteins need a factor not available in yeast (23).

CLC-3 DEFICIENCY LEADS TO PHENOTYPES SIMILAR TO NCL

Chloride channels are thought to regulate electrical excitability, the ionic composition of intra- and extracellular compartments, and cell volume. In mammals, the CLC family consists of nine genes belonging to three different branches. Clcn3p, the product of CLC3, has been detected in endosomal compartments and in synaptic vesicles of neurons. While swelling-activated currents were unchanged in mice with disrupted CLC-3 ($Clcn3^{-/-}$ mice), acidification of synaptic vesicles was impaired, and there was severe postnatal degradation of the retina and the hippocampus. Together with developmental retardation and higher mortality, $Clcn3^{-/-}$ mice showed neurological manifestations such as blindness, motor coordination deficit, and spontaneous hyperlocomotion. In histological analysis, the $Clcn3^{-/-}$ mice showed a pattern of progressive degeneration of the retina, hippocampus and ileal mucosa, which resembled the phenotype observed in $CD^{-/-}$ mice (80, 103). By immunohistochemistry and Western blot analysis, accumulation of subunit c was also detected in the lysosomes of $Clcn3^{-/-}$ mice. In addition, an elevation of endosomal pH was detected in the $Clcn3^{-/-}$ mice (103). These results suggest that the neurodegeneration in the $Clcn3^{-/-}$ mice might be caused by an abnormality in the environment that leads to degradation of cellular proteins. This process has been associated with the phenotype of NCL.

MECHANISM OF THE SPECIFIC ACCUMULATION OF SUBUNIT C

Cytoplasmic components, including mitochondria, are engulfed by autophagy and are degraded in lysosomes (Figure 1). Autophagy is a process of regulated turnover of organelles and proteins that occurs during development and under conditions of stress such as starvation (54). This process, which exists in all eukaryotic cells, is tightly controlled, but in extreme cases results in cell death. Major insights into the molecular and biochemical pathways have come from genetic studies in yeast, and recently a wealth of information about autophagic pathways in mammalian cells has emerged (57, 84, 85, 86). In NCLs, autophagic processes initially proceed normally, but the subsequent process, namely, lysosomal degradation of sequestered proteins, is impaired. There are several reports on vacuolar processing machinery in yeast. Inside the vacuolar lumen (lysosomal lumen in mammals), before proteases can access their substrates, Atg15 (Cvt17) (88), a presumptive lipase activity and Atg22 (Aut4) of unknown function are required to degrade the inner boundary of autophagic bodies (82). The final stage of substrate degradation requires protease activities. However, little is known about how the engulfed organelles and hydrophobic proteins are degraded in the lysosomes in mammalian cells.

At least 4 elements are required for lysosomal degradation of subunit c: 1) TPP-I, 2) cathepsin D, 3) lysosomal acidic pH and 4) lysosomal membrane proteins. Normal function of 2 lysosomal proteinases is the minimum requirement for degradation of the subunit c. Lysosomal pH homeostasis is also important for degradation of sequestered substrate proteins, including subunit c. Lysosomal accumulation of subunit c in CLC3-deficient mice supports this idea (103); CLC3-deficiency led to an elevation of pH within endosomes. The molecular mechanism in which deficiency of CLN membrane proteins leads to specific accumulation of subunit c has not yet been explained. What is the role(s) of lysosomal membrane proteins (Cln3p, Cln5p, Cln6p) in the degradation of subunit c? Based on co-immunoprecipitation and in vitro binding assays, Vesa and colleagues have suggested that Cln5p interacts with TPP-I and Cln3p (94). Further systemic studies are necessary to elucidate the interaction of all CLN membrane proteins, pH control machinery (v-ATPase and CLCs) and TPP-I. CLN membrane proteins may regulate the activity of TPP-I by modulating the substrate binding to the enzyme or may control lysosomal trafficking and localization of other CLN proteins.

THE MECHANISMS RELATING TO NEURODEGENERATION

Specific accumulation of subunit c is a characteristic feature of NCLs except INCL. However, the relationship between subunit c accumulation and neurodegenerative disease remains unexplained. How can accumulation of subunit c lead to neurodegenerative disorders? Perhaps it is toxic to the membranes of neurons. Specific accumulation of proteins or other material has also been detected in many kinds of lysosomal diseases and sometimes it accompanies neurodegeneration. McGeoch and Guidotti claim that accumulation of too much subunit c may function as a cation channel (50). However, as yet there is no clear proof of a relationship between the ac-

cumulation of subunit c and neurodegeneration in NCL. It is necessary to examine the relationship between protein deposition and cell degeneration to understand at the molecular level the pathogenesis of NCLs.

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