Human L1CAM Carrying the Missense Mutations of the Fibronectin-Like Type III Domains Is Localized in the Endoplasmic Reticulum and Degraded by Polyubiquitylation

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Any mutations in the human neural cell adhesion molecule L1 (hL1CAM) gene might cause various types of serious neurological syndromes in humans, characterized by increased mortality, mental retardation, and various malformations of the nervous system. Such missense mutations often cause severe abnormalities or even fatalities, and the reason for this may be a disruption of the adhesive function of L1CAM resulting from a misdirection of the degradative pathway. Transfection studies using neuroblastoma N2a cells demonstrated that hL1CAM carrying the missense mutations in the fibronectin-like type III (FnIII) domains most likely is located within the endoplasmic reticulum (ER), but it is less well expressed on the cell surface. One mutant, L935P, in the fourth FnIII domain, was chosen from six mutants (K655 and G698 at Fn1, L935P and P941 at Fn4, W1036 and Y1070 at Fn5) in the FnIII domains to study in detail the functions of hL1CAM200kDa, such as the intracellular traffic and degradation, because only a single band at 200 kDa was detected in the hL1CAM200kDa-transfected cells. hL1CAM200kDa is expressed predominantly in the ER but not on the cell surface. In addition, this missense mutated hL1CAM200kDa is polyubiquitylated at some sites in the extracellular domain and thus becomes degraded by proteasomes via the ER-associated degradation pathway. These observations demonstrate that the missense mutations of hL1CAM in the FnIII domain may cause the resultant pathogenesis because of a loss of expression on the cell surface resulting from misrouting to the degradative pathway.

Key words: L1CAM; missense mutation; ERAD; human disease; ubiquitin

The neural cell adhesion molecule L1 (L1CAM) is critical for normal development of the nervous system, as demonstrated by multiple human X-linked disorders associated with L1CAM mutations (Fransen et al., 1997; Kamiguchi et al., 1998; Kenwick et al., 2000) and similar phenotypes seen in LICAM knockout mice (Dahme et al., 1997; Cohen et al., 1998). L1CAM has been implicated in multiple aspects of neural development such as migration of neuronal precursors (Lindner et al., 1983), neurite outgrowth (Lagenaur and Lemmon, 1987), axon fasciculation (Itoh et al., 1995; Kunz et al., 1998; Wiencken-Barger et al., 2004), synaptic plasticity (Itoh et al., 2005), and myelination (Haney et al., 1999; Itoh et al., 2000). L1CAM basically exhibits homophilic binding via extracellular interactions to play many important roles in the nervous system (Rathjen and Schachner, 1984; Lemmon et al., 1989; Fields and Itoh, 1996).

The human gene encoding L1CAM (hL1CAM) is located near the long arm of the X-chromosome in Xq28 (Djabali et al., 1990; Chapman et al., 1990). Different X-linked mental retardation syndromes, including HSAS syndrome (hydrocephalus due to stenosis of the aqueduct of Sylvius), were first attributed to mutations in the hL1CAM gene (Rosenthal et al., 1992); MASA

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syndrome (mental retardation, aphasia, shuffling gait and adducted thumbs), X-linked complicated SP-1 (spastic paraplegia), or ACC (agenesis of the corpus callosum) have been located to Xq28, and the morphological abnormalities of these syndromes might result from deficiencies in cell migration or axonal pathfinding and fasciculation, so hL1CAM is a likely candidate gene causing these syndromes (Fransen et al., 1994; Jouet et al., 1994; Vits et al., 1994). HSAS, MASA, SP-1, and ACC are allelic disorders that represent overlapping clinical spectra of the same disease. Therefore, these disorders are classified as the L1CAM spectrum (Moya et al., 2002). The hL1CAM mutations account for 5% of all cases with hydrocephalus and are the most frequent genetic cause of this pathology. About 240 different pathogenic mutations have been identified in virtually all regions of the gene (Vos and Hofstra, 2010; the hL1CAM Mutation Database http://www.l1cammutationdatabase.info/). No key mutations have been observed. All kinds of mutations are found in human patients, including missense, nonsense, and frame shift mutations, deletions, insertions and splice site mutations. Most mutations in the hL1CAM are private point mutations that appear in only one family. Despite the wide range of symptoms, a certain correlation between the severity of the disease and the location of the mutation has been demonstrated (Bateman et al., 1996; Yamasaki et al., 1997; Fransen et al., 1998; Michaelis et al., 1998). Missense mutations within the extracellular domain may cause a severe phenotype. Substitutions involving the key residues that are responsible for maintaining the conformation of L1CAM produce the most severe forms of hydrocephalus and are also the most deleterious with regard to infant survival (Kamiguchi 1998; Michaelis et al., 1998; Fransen et al., 1998). However, the reasons for the high number of different hL1CAM missense mutations with severe consequences remain only partially understood. Potentially, these mutations might interfere with homophilic or heterophilic interactions of L1CAM or with the targeting of the protein to the cell surface (De Angelis et al., 1999, 2001; Moulding et al., 2000; Rünker et al., 2003; Schäfer et al., 2010a,b). The loss of function caused by hL1CAM mutations is caused by a failure of the above-described interactions on the cell surface, and this could be the result of changes in intracellular trafficking that reduce the cell-surface expression of L1CAM (Schäfer et al., 2010a). The current article offers a detailed characterization of a missense mutation in the extracellular domain of hL1CAM in the murine N2a neuroblastoma cells to obtain insight into the consequences of hL1CAM mutations associated with posttranslational processing or protein trafficking.

MATERIALS AND METHODS

Antibodies

Antibodies were obtained against Flag (Flag mAb, M2; Sigma-Aldrich, St. Louis, MO), ubiquitin (Ub pAb; Dako Cytomation, Glostrup, Denmark), L1CAM (goat polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA), polyubiquitin (FK1 mAb; Nippon Biotest Lab, Tokyo, Japan), the KDEL sequence of luminal proteins (KDEL mAb, 10C3, mouse IgG2a; Stressgen, Vancouver, British Columbia, Canada), and Golgi matrix protein of 130 kDa (GM130 mAb, mouse IgG1; BD Transduction Labs, San Jose, CA). The affinity-purified anti-L1CAM polyclonal antibody (L1 pAb) against the extracellular domain of L1CAM was raised using full-length rat L1CAM as the immunogen. Secondary antibodies (anti-rabbit IgG and anti-mouse IgG) conjugated to horseradish peroxidase were purchased from Thermo Pierce (Rockford, IL). Alexa 488- and Alexa 563-conjugated secondary antibodies were from Molecular Probes (Eugene, OR).

Mutant L1CAM cDNA Constructs

The human L1CAM (hL1CAM) mutations were created by using PCR-based site-directed mutagenesis (QuickChange Site-Directed Mutagenesis kit; Stratagene, La Jolla, CA) and were verified by DNA sequencing. Eleven different hL1CAM mutants were constructed (Weller and Gärtnert, 2001; De Angelis et al., 2001): hL1CAM with the pathogenic missense mutations R184Q (L1CAMR184Q), H210Q, I219T, C264Y, A265D, K655E, I698R, L935P (L1CAML935P), P941L, W1036L, and Y1070C (see Fig. 1A). These hL1CAM mutants, including wild-type L1CAM (hL1CAMWT) constructs, were cloned into the pCMV-Tag4 (Stratagene) and pEGFP-N1 (Clontech, Palo Alto, CA), respectively. The lysine (K) residues at 11 positions (1144, 1147, 1150, 1154, 1156, 1169, 1186, 1200, 1232, 1233, and 1235) in the cytoplasmic domain of hL1CAMWT (hL1CAMWT), hL1CAML935P, and hL1CAML935P-11KR were substituted with arginine (R) using PCR-based site-directed mutagenesis (QuickChange Site-Directed Mutagenesis kit; Stratagene).

Cell Culture and Transfection

Murine neuroblastoma N2a (N2a) cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a 5% CO2 incubator. Plasmid DNA was transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and maintained for an additional 48 hr in culture.

Immunoprecipitation (IP) and Immunoblot (IB) Analyses

N2a cells were lysed using Dounce glass homogenizer ( Kontes) in RIPA buffer (20 mM Tris-HCl, pH 7.4, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P40, 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, 10 mM N-ethyl-maleimide) and containing protease inhibitors [0.4 mM Pefabloc SC (Roche, Indianapolis, IN), 1 μg/ml aprotinin (Roche), 1 mM benzamidine (Sigma), 1 μM leupeptin (Roche), 10 μg/ml oμ2-macroglobulin (Roche), 1 μM pepstatin A (Calbiochem, La Jolla, CA), 10 μg/ml egg white trypsin inhibitor (Calbiochem), 10 μg/ml soybean trypsin inhibitor (Sigma), and 1 mM iodoacetamide (Sigma)] and incubated for 30 min at 4°C. Lysates were cleared by centrifugation at 15,000g for 20 min at 4°C. The protein concentrations of lysates and homogenates were

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Fig. 1. Expression patterns of missense mutations in relation to the domain structure of hL1CAM in N2a cells. A: Schematic representation of the hL1CAM showing domain structure: Ig-like domains (IgC2) I–VI, fibronectin-type III repeats 1–5 (FnIII), transmembrane region (TM), and cytoplasmic domain (CD). These constructs were cloned into the pCMV-Tag4 to conjugate with Flag-tag at the C-terminal. The locations of the mutations (underscored) are indicated (circles). B: Immunoblot of lysates from vector-transfected N2a cells. Wild type (WT), five mutations of IgC2 domains (R184Q, H210Q, I219T, C264Y, and A426D), or six mutations of the FnIII domains (K655E, G698R, L935P, P941L, W1036L, and Y1070C) are transiently transfected in N2a cells. The upper molecular weight (MW) band is approximately 220 kDa, and the lower MW band at approximately 200 kDa would be visible. Mock-transfected N2a cells served as a negative control. C: These typical photographs of immunocytochemistry show the hL1CAM expression in five hL1CAM mutations of IgC2 domains (R184Q, H210Q, I219T, C264Y, and A426D) of the transiently transfected N2a cells. D: These typical photographs of immunocytochemistry demonstrated hL1CAM expression in six hL1CAM mutations of the FnIII domains (K655E, G698R, L935P, P941L, W1036L, and Y1070C) of the transiently transfected N2a cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

determined using the BCA protein assay kit (Thermo Pierce). Supernatants were incubated with L1 pAb, anti-Flag monoclonal antibody (Flag mAb) for 5 hr, followed by protein G Sepharose beads (GE Healthcare, Uppsala, Sweden) for 1 hr. Immunoprecipitates were washed six times with lysis buffer, and bound proteins were eluted with SDS-sample buffer, resolved by NuPage 3–8% Tris-acetate gel (Invitrogen) and transferred electrophoretically to PVDF membranes. All non-specific reactivity was blocked with 5% skim milk in 20 mM Tris-HCl (pH 7.4), 136.7 mM NaCl, 0.1% Tween 20 (TBS-T) for 1 hr at RT and PVDF membranes were probed with specific antibodies, L1 pAb, Flag mAb, Ub pAb, and FK1 mAb, diluted in TBS-T overnight at 4°C. After washing the membranes with TBS-T three times for 5 min each, the membranes were then incubated with secondary antibody solution containing HRP-conjugated anti-rabbit IgG and anti-mouse IgG for 1 hr at room temperature (RT). The membranes were washed extensively with TBS-T four times for 8 min each, and immunoreactivity was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Pierce, Rockford, IL). Band intensities were quantitated and analyzed in ImageQuant software (Bio-Rad, Hercules, CA).

Deglycosylation of L1CAM

Cell lysates of N2a cells at 48 hr were prepared after transient transfection for deglycosylation of L1CAM as described above. The denatured lysates (20 μg protein of cell lysates) were incubated overnight at 37°C in 50 mM sodium phosphate (pH7.5), 1% NP-40, and protease inhibitor mixture with 750 U peptide-N-glycosidase F (PNGase F)/20 μg protein (BioLabs) and 50 mM sodium citrate (pH 5.5) and protease inhibitor mixture with 750 U endoglycosidase H (EndoH)/20 μg protein (BioLabs).

Biotinylation Assay for Detection of Cell Surface L1CAM

Surface biotinylation was carried out as described by Schmidt et al. (1997). In brief, cells were washed twice with ice-cold PBS-CM 48 hr after transfection. Surface proteins were biotinylated by incubating cells with 0.3 mg/ml Sulfo-NHS-SS-biotin (Pierce, Rockford, IL) in PBS-CM for 30 min at 4°C. Biotinylation was terminated by incubation with 20 mM glycine in PBS-CM at 4°C for 10 min, followed by extensive washing with PBS-CM. Excess biotin was quenched by washing three times with DMEM containing 0.1% BSA, followed by an additional wash with ice-cold TBS (10 mM Tris-HCl pH 7.4, 154 mM NaCl). Biotinylated cells were then lysed directly in RIPA buffer. The biotinylated proteins were precipitated with streptavidin-immobilized on agarose beads (Thermo Pierce) at 4°C for 2 hr. Agarose beads were pelleted by centrifugation and washed five times in RIPA buffer. Precipitated proteins were solubilized by addition of SDS-sample buffer to the agarose beads. The eluted proteins were separated by SDS-PAGE, and proteins were quantified by IB analysis using L1 pAb.

Indirect Immunofluorescence Staining of Fixed and Living Cells

For fixed cells, the medium was removed from the coverslips, and cells were fixed with 4% PA in PBS for 15 min at 4°C and washed twice with PBS. Coverslips were placed on parafilm in a humid chamber and incubated with 1% BSA in PBS for 1 hr at RT. The blocking buffer was removed by aspiration, and the coverslips were covered and incubated with L1 pAb and KDEL mAb or GM130 mAb overnight at 4°C in a humid chamber. The coverslips were washed three times with 0.1% BSA in PBS and incubated with secondary antibody solution containing Alexa 488-conjugated anti-rabbit IgG, Alexa 563-conjugated anti-mouse IgG for 1 hr at RT in the dark. Finally, the cells were washed three times with PBS and mounted on slides with Vectashield (Vector, Burlingame, CA). For living cells, L1 pAb were added to the coverslips and incubated at 4°C
for 30 min. The coverslips were placed into 12-well dishes and washed three times with PBS-5% FCS at RT. Next, the coverslips were placed on parafilm again, covered with PBS-5% FCS containing the secondary Alexa 488-conjugated anti-rabbit IgG antibody in a 1:200 dilution, and incubated at 4°C for 20 min in the dark. Finally, the coverslips were washed twice with PBS-5% FCS, fixed with 4% PA in PBS, washed with PBS, and mounted on slides with Vectashield.

**Imaging and Quantification**

The cells were rinsed with PBS 48 hr after transfection, fixed in 4% formaldehyde for 30 min, and permeabilized with 0.3% Triton X in PBS for 30 min. Primary antibodies were diluted in PBS containing 10% FBS. The cells were examined using a FV-1000 laser scanning confocal microscope (Olympus, Tokyo, Japan) with a ×63 oil immersion objective lens. Images were analyzed using an arbitrary threshold for the determination of fluorescence intensity of L1CAM in Meta-morph imaging software (Universal Imaging Corp., Downingtown, PA). Fifty cells were photographed and analyzed for each construct in each of at least three independent experiments.

**Measuring Molecular Weight With IB**

The mobility (Rf) of a molecule in gel electrophoresis is determined by its free solution mobility (mobility in a gel of 0%) and the sieving action of the gel matrix. The relative mobilities are determined solely by the sieving action of the gel. This sieving action is proportional to the molecular weight of the particular protein. An empirical plot of log(molecular weight) vs. Rf for several standards of known molecular weight was used to determine the molecular weight of unknowns (Dunkeri and Rueckert, 1969).

**RESULTS**

**hL1CAM<sup>L935P</sup> Is Expressed as a Protein Variant With a Reduced Molecular Weight**

The N2a cells endogenously expressed mouse L1CAM with two bands of 220 kDa (mL1CAM<sup>220kDa</sup>) and 200 kDa (mL1CAM<sup>200kDa</sup>), and the mL1CAM<sup>220kDa</sup> was predominantly expressed (Fig. 1B). A similar result was obtained for N2a cells transiently transfected with hL1CAM<sup>WT</sup>. Both the 220- and the 200-kDa bands were detected by IB analysis with L1 pAb in the immunoprecipitate with Flag mAb of Flag-tagged hL1CAM<sup>WT</sup>-transfected cells. Eleven different hL1CAM pathogenic missense mutants were constructed (Fig. 1A), cloned into the pCMV-Tag4, and transfected into N2a cells to analyze the expression levels of the different hL1CAM missense mutations in the immunoglobulin C2 (IgC2) and fibronectin-like type III (FnIII) domains (Weller and Gärtner, 2001; De Angelis et al., 2001). Flag-tagged hL1CAM mutants-transfected N2a cells were subjected to immunoblot analysis with Flag mAb (Fig. 1B). For the hL1CAM<sup>WT</sup>, the more prominent band demonstrated a molecular weight of approximately 220 kDa. Five missense mutations in the IgC2 domain expressed both high- and low-molecular-weight bands (about 220 and 200 kDa), and the 220-kDa band was the more highly expressed, except for hL1CAM<sup>R184Q</sup>. The signal intensity of 220 kDa had the same intensity as the 200-kDa band in cultures transfected with hL1CAM<sup>R184Q</sup>. The upper band corresponds to the full-length and fully glycosylated form of L1CAM located at the cell surface (Zisch et al., 1997). Five hL1CAM mutations of the IgC2 domains (R184Q, H210Q, I219T, C264Y, and A426D) and one mutation of the FnIII domain (K655E) were found to be strongly expressed on the cell surface in the transiently transfected N2a cells, as shown in Figure 1C. In contrast, the signal intensity of 220 kDa was much weaker than that of the 200-kDa band in four missense mutations in the FnIII domains in transfected cultures, except for K655E. Immunocytochemical studies showed the three hL1CAM mutations of FnIII domains (G698R, L935P, and P941L) not to be expressed on the cell surface; instead, they are intracellularly detected in transiently transfected N2a cells, whereas W1036 and Y1070 mutants at Fn5 are detected on both the cell surface and the intracellular organelles (Fig. 1D). Only a single band at approximately 200 kDa was detected in lysates of hL1CAM<sup>L935P</sup>-transfected cells regardless of longer time periods of posttransfection or exposure. The L935P mutant was chosen to study the functions of hL1CAM<sup>200kDa</sup> such as the intracellular traffic and the degradation. In these transfection experiments, the cell numbers of these transiently transfected mutants did not decrease after 48 or 72 hr in comparison with mock-transfectants (data not shown). This evidence indicates the overexpression of hL1CAM mutants in N2a cells did not cause cell death.

**Glycosylation Form of hL1CAM With L935P Mutation**

The most likely explanation for the reduced molecular weight of the L1CAM is a change in the glycosylation pattern. N2a cells were lysed 48 hr after transfection, and the protein was digested with PNGase F and EndoH. PNGase F treatment caused the 220- and 200-kDa bands of hL1CAM<sup>WT</sup> and hL1CAM<sup>L935P</sup> to shift to an approximately 160-kDa band, the estimated molecular weight of deglycosylated hL1CAM protein (Fig. 2A). On the other hand, the 220 kDa of hL1CAM<sup>WT</sup> was not cleaved by treatment with EndoH, although the 200 kDa of hL1CAM<sup>200kDa</sup> shifted to a 160-kDa band (Fig. 2A). This indicates the 220 kDa of hL1CAM in the transfected N2a cells has asparagine (N)-linked complex oligosaccharides, whereas the 200-kDa bands of hL1CAM<sup>WT</sup> and hL1CAM<sup>L935P</sup> have either high-mannose or hybrid oligosaccharides (Fig. 2B). This suggests that the lower molecular weight form of hL1CAM is located within the ER or the Golgi network.

**hL1CAM With L935P Mutation Is Not Expressed on the Cell Surface**

Immunocytochemistry and biotinylation-cell surface assay were performed using L1 pAb and precipitation of biotinylated proteins, respectively, in hL1CAM<sup>WT</sup>- and
hL1CAM<sup>L935P</sup>-transfected cells to determine whether the hL1CAM<sup>L935P</sup> was expressed on the cell surface. The hL1CAM<sup>L935P</sup> mutated protein did not immunocytochemically appear to be on the cell surface (Figs. 1D, 3C,F). To quantify the cell surface expression levels of hL1CAM<sup>L935P</sup> in N2a cells, a biotinylation assay for the detection of cell surface hL1CAM<sup>L935P</sup> was performed. The biotinylated protein was precipitated by streptavidin-agarose beads and analyzed by immunoblotting with Flag mAb (Fig. 3G). Although the 220-kDa band was detected in both the lysate and membrane fractions of hL1CAM<sup>WT</sup> transfectants, cells transfected with hL1CAM<sup>L935P</sup> did not show the 200-kDa band associated with the plasma membrane (Fig. 3G). Therefore, no L1CAM expression was observed on the cell surface in the L935P mutant, and this finding was consistent with the loss of hL1CAM<sup>L935P</sup>-immunopositive live cells after transfection (data not shown). The 200-kDa protein form, present in lysates of N2a cells transfected with hL1CAM<sup>L935P</sup>, was not labeled by biotin on the cell surface, indicating that it is not transported to the plasma membrane but was located inside the cells. Double immunocytochemistry was performed using L1 pAb and KDEL mAb or GM130 mAb in N2a cells transfected with hL1CAM<sup>L935P</sup> to investigate the intracellular localization of the hL1CAM<sup>L935P</sup> protein. The hL1CAM<sup>L935P</sup> was detected in cytoplasm, but not at the cell surface.

hL1CAM<sup>L935P</sup> Localized in the ER Is Conjugated With High-Mannose Oligosaccharides

Lysates of hL1CAM<sup>WT</sup> transfectant were treated with two exoglycosidase inhibitors to be compared with the molecular size of hL1CAM<sup>L935P</sup> to determine whether hL1CAM<sup>L935P</sup> is identical to hL1CAM<sup>200kDa</sup> in...
The neurological disorders caused by mutations in all parts of the \textit{hL1CAM} gene are characterized by increased mortality, mental retardation, and various malformations of the nervous system (Fransen et al., 1995). Missense mutations in the \textit{hL1CAM} gene affecting the extracellular domain often result in a severe phenotype, whereas mutations in the highly conserved cytoplasmic domain generally cause more moderate phenotypes. This study focused on the functional consequences of extracellular \textit{hL1CAM} mutations \textit{in vitro} in an attempt to understand the reasons for the frequent occurrence of severe missense mutations in the extracellular domain of \textit{hL1CAM}. The current study was a detailed characterization of a missense mutation in the extracellular domain of \textit{hL1CAM} in the widely used N2a cells, which was performed to obtain further insight into the consequences of \textit{hL1CAM} mutations associated with either post-translational processing or protein trafficking.

**The Lower Molecular Weight of hL1CAM in N2a Cells**

A high-molecular-weight (220 kDa) band was expressed predominantly on the cell surface in the transfectants of missense-mutated IgC2 domains (R184Q, H210Q, I219T, C264Y, and A426D) to a similar extent in N2a cells transfected with \textit{hL1CAM} WT, whereas the FnIII domains of the \textit{hL1CAM} missense mutants were expressed predominantly in the 196-kDa form and at
very low levels in the 220-kDa form on the cell surface, except for K655E (Figs. 1, 4). The transiently transfected N2a cells with hL1CAML935P eliminated the 220-kDa form of the protein from the cell surface, although a dominant 220-kDa form was observed in endogenous mL1CAM, and hL1CAMWT and IgC2 domain mutants-transfected cells. Instead, the 196-kDa form of hL1CAM was detected as the prominent L1CAM-immunoreactive band in these FnIII domain mutants (Figs. 1, 4). However, this 196-kDa form was not labeled in cell surface biotinylation experiments, indicating an intracellular localization of hL1CAML935P. Therefore, the hL1CAML935P was primarily retained inside the cells. These results suggest that the fourth FnIII domain mutation, hL1CAML935P, results in impaired protein trafficking in N2a cells. Although N2a cells originated from peripheral neurons, the hL1CAM missense mutants are expressed in the neurons of CNS and PNS. The 220-kDa and 200 (196)-kDa forms in Na2 cells corresponded to 200 kDa and 180 (176) kDa in CNS neurons respectively, although the molecular size of the each core protein is 160 kDa (data not shown). This 20-kDa difference is dependent on glycosylation processes in the Golgi network between CNS neurons and N2a cells. However, the trimming of oligosaccharide chains of L1CAM during the N-linked oligosaccharide biosynthetic pathway in the ER is not different between CNS neurons and N2a cells. Therefore, the current experiments using N2a cells are appropriate to study hL1CAM dynamics in the ER.

Retention of Misfolded hL1CAM Protein in the ER

How might the hL1CAML935P protein be retained intracellularly? The hL1CAML935P mutant accumulated intracellularly, especially in the ER, but not the Golgi apparatus, as shown by double immunocytochemistry (Fig. 2). These in vitro observations demonstrate that a mutation in the extracellular fourth FnIII domain of hL1CAM may interfere with the targeting of the protein to the Golgi apparatus, suggesting that other mutations in the extracellular domain of hL1CAM might lead to the retention of misfolded hL1CAM mutants in the ER. Two possible explanations for the retention in the ER are misfolding of the protein and aberrant glycosylation of hL1CAM. The ER is the subcellular site where glycoproteins such as L1CAM are synthesized, acquire their proper folding, and become de novo N-glycosylated. The precursor oligosaccharyl moiety (Glu3Man9GlcNAc2) at Asn-X-Ser/Thr sites of L1CAM is trimmed to an Man8-GlcNAc2 during the N-linked oligosaccharide biosynthetic pathway, by sequential exoglycosidase reactions that are catalyzed by α-glucosidase I, α-glucosidase II, and ER α-mannosidase I in the ER (Kornfeld and Kornfeld, 1985; Moremen et al., 1994). Two exoglycosidase inhibitors, CAST and DMJ, which inhibit α-glucosidase I and II, and ER α-mannosidase I, respectively, were used to treat to N2a cells in order to explore the relationship between oligosaccharide structure and molecular size of hL1CAMWT in the ER (Elbein, 1991). The hL1CAMWT bearing Man5GlcNAc2 was 200 kDa in DMJ-treated N2a cells. These results show that the difference in molecular weight between hL1CAMWT bearing Man5GlcNAc2 and hL1CAML935P was approximately 4 kDa, corresponding to the molecular weight of 22 mannoses (180.16 g/mol/Man × 22 putative N-glycosylation sites; Kobayashi et al., 1991), suggesting that hL1CAML935P contains...
Man₈-GlcNAc₂. Although the folded L1CAM with Man₈-GlcNAc₂ theoretically interacts with L-type lectins that transport L1CAM from the ER to the Golgi apparatus, hL1CAML⁹₃₅P was retained in the ER regardless of the presence of Man₈-GlcNAc₂. The evidence suggests that hL1CAML⁹₃₅P is misfolded and that L₉₃₅P in the fourth FnIII domain might be important for folding of the protein structure. Other sites in FnIII domains (G698 at Fn1 and P941 at Fn4) also might be involved in the mechanisms of hL1CAM folding, because these missense mutants expressed predominantly the lower molecular weight (196–200 kDa) form in the ER (Figs. 1 and 3, Supp. Info. Fig. 1). This monitoring of misfolded glycoproteins in the ER has been called an ER quality control, a mechanism that prevents export of misfolded proteins from the ER to the Golgi apparatus (Hammond and Helenius, 1995; Kopito, 1997). The misfolding of the L₉₃₅P mutant bearing Man₈-GlcNAc₂ may be caused by the destabilization of the domain that interrupts the hydrogen bonds in the secondary structure, such as β-sheets. Leucine 935 is involved in hydrogen bonding with cysteine 928 in the helix region of the far β-sheet of the hL1CAM (Bateman et al., 1996). The missense L₉₃₅P mutation substituted by proline for leucine is expected to affect both the structure and the function of the hL1CAM protein. Proline has a fixed angle because of its imino group, which acts as a structural disruptor in the secondary structural errors between β-sheets, thus causing abnormal protein folding and conformation. Therefore, the mutant predicted affects the structure of a domain resulting in reduced cell-surface expression and shows intra-ER retention of hL1CAM in the cells. Indeed, missense mutations in nearby sites (L₉₃₅P, R₉₃₇P, and P₉₄₁L) have been identified in association with X-linked hydrocephalus (Jouet et al., 1995; Du et al., 1998; Wilson et al., 2009). We speculate that the fourth FnIII domain in hL1CAM may play an important role in protein folding and trafficking in the ER.

Polyubiquitylation of L1CAM Via the ERAD Pathway

Misfolded glycoproteins with Man₈-GlcNAc₂ interact with M-type lectins that translocate the glycoproteins from the ER to the cytoplasm, and misfolded cytoplasmic glycoproteins are degraded by the ubiquitin-proteasome pathway, the so-called ERAD. The irreversibly misfolded hL1CAML⁹₃₅P might have been targeted for degradation via the ERAD pathway. The current experiments demonstrate that the hL1CAM was ubiquitylated by two different processes. The hL1CAML¹¹KR did not detect Ub signals and in addition did not show FK-1-positive bands (Fig. 5), suggesting that hL1CAMWt could be mono- or multiubiquitylated in the cytoplasmic domain. Recently, one report indicated that L1CAM is ubiquitylated in the cells and that this ubiquitylated L1CAM is degraded by lysosome (Schäfer et al., 2010b). On the other hand, hL1CAML⁹₃₅P was polyubiquitylated in the extracellular domain of L1CAM, because hL1CAML⁹₃₅P-¹¹KR was also polyubiquitylated (Fig. 5C). These findings suggest that the misfolded hL1CAML⁹₃₅P might be moved from the ER to the cytoplasm and then polyubiquitylated. It is likely that the ubiquitylated hL1CAML⁹₃₅P, as an ERAD substrate, is degraded by proteasomes, because degradation of the hL1CAML⁹₃₅P was inhibited by the proteasome inhibitor lactacycin, but not by a lysosome inhibitor, bafilomycin (data not shown). Therefore, the misfolded hL1CAML⁹₃₅P with Man₈-GlcNAc₂ translocates from the ER to the cytoplasm and, then, is polyubiquitylated, and the polyubiquitylated hL1CAML⁹₃₅P is finally degraded by the proteasomes.

In conclusion, we found that a low-molecular-weight (196 kDa) missense mutated hL1CAM of the FnIII domains localized predominantly in the ER, and it did not move to the cell surface via the Golgi apparatus. In addition, the missense mutated hL1CAMs of the FnIII domains were found to be ubiquitylated in the extracellular domain, thus leading to destruction via ERAD. These data are consistent with a model in which some missense mutations in hL1CAM lead to a loss of function, such as cell–cell interactions by mistargeting and degradation. Although this general mechanism has been suggested in the past, these new data provide a specific molecular mechanism by which this can occur.

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Supplementary Figure 1. Intracellular localization and cell-surface expression of hL1CAMWT and hL1CAMG698R and hL1CAMP941L in N2a cells. Double immunofluorescence labeling of exogenous hL1CAM (Flag mAb, green) with the ER marker (KDEL mAb, red) or Golgi marker (GM130 mAb, red) is shown for hL1CAMWT hL1CAMG698R and hL1CAMP941L and they are representative of the patterns of expression observed in this study. The colocalization of hL1CAMWT, hL1CAMG698R and hL1CAMP941L with the ER marker is visualized by yellow staining.