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Membrane topology of CLN3, the protein underlying Batten disease

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Abstract Juvenile neuronal ceroid lipofuscinosis, or Batten disease, is an autosomal recessive disorder characterized by progressive loss of motor and cognitive functions, loss of vision, progressively severe seizures, and death. The disease is associated with mutations in the gene CLN3, which encodes a novel 438 amino acid protein, the function of which is currently unknown. Protein secondary structure prediction programs suggest that the CLN3 protein has five to seven membrane-spanning domains (MSDs). To distinguish among a number of hypothetical models for the membrane topology of CLN3 we used in vitro translation of native, Flag epitope-labeled and glycosylation site-mutated CLN3 protein in the presence or absence of canine pancreatic microsomes. These were immunoprecipitated using antibodies specific for Flag or peptide sequences within CLN3 or left untreated. The results indicate that CLN3 contains five MSDs, an extracellular/intraluminal amino-terminus, and a cytoplasmic carboxy-terminus.

Key words: Batten disease; Membrane-spanning domain; Protein topology

1. Introduction

Juvenile neuronal ceroid lipofuscinosis (JNCL), or Batten disease, is one form of the neuronal ceroid lipofuscinoses (NCL), a collection of at least eight inherited neurodegenerative disorders with similar pathological features. JNCL is the most common inherited neurodegenerative disease in children, with an annual incidence from 1/25 000 to 1/40 000 live births [1,2]. The disease is inherited in an autosomal recessive pattern. Symptoms typically appear between ages 5 and 8, and include vision loss, gradual deterioration in motor and cognitive skills, changes in behavior, and progressively severe seizures. Over a period of 10–20 years this process inexorably culminates in persistent vegetative state and death. At this time, clinical interventions are limited to amelioration of symptoms and supportive care. No cure exists, and our understanding of the molecular pathogenesis of the disease remains immature despite a considerable amount of study.

JNCL is caused by defects in a recently discovered gene called CLN3, which is located on chromosome 16p12.1 [2]. The *CLN3* gene encodes a novel 438 amino acid protein (CLN3) the function of which is currently unknown. Homologs have been identified in dogs, mice, yeast, *Drosophila*, and nematodes, and the amino acid sequence of CLN3 appears to be highly conserved in evolution [3]. In most cases of JNCL the inherited defect is a 1.02 kb deletion of genomic DNA, resulting in a truncation of CLN3 such that the resultant protein contains the original 153 N-terminal residues followed by 28 novel amino acids [2,4].

In affected individuals, mutations of CLN3 result in pathologic intracellular inclusions in neurons and other cells of the body. Lysosomal accumulation of autofluorescent lipids (ceroid and lipofuscin) and high concentrations of a mitochondrial protein, ATP synthase subunit C, have been reported [5,6]. Recent immunofluorescence studies have shown that the predominant intracellular destination of nascent CLN3 is the lysosome [7–10].

As we begin to learn more about CLN3, the necessity of determining the structure of the protein becomes increasingly apparent. Clues to the function of CLN3 may be gained by knowing how CLN3 is distributed in the membrane, and which amino acid residues are accessible for interaction with cytoplasmic or non-cytoplasmic proteins. Computer-generated models of secondary structure suggest a transmembrane protein with between five and seven membrane-spanning domains (MSDs). Six of the membrane domains are scored as being highly likely by the PSORT prediction program [11], and some studies of the CLN3 gene have assumed a six-MSD model with the C-terminus and N-terminus both located in the extracellular milieu [12]. To date no empirical evidence to support the six-MSD model has been proffered.

To address the structure of CLN3, we have made use of the fact that incorporation of proteins into microsomes (endoplasmic reticulum (ER) membranes) protects regions of the protein from recognition by antibodies and that glycosylation activity only takes place on the luminal side of microsomes. We have utilized immunoprecipitation with membrane-bound CLN3 protein produced by transcription and translation in vitro and glycosylation scanning procedure to determine whether various epitopes lie within or outside of the ER membrane. Our data show that CLN3 is a type IIIB protein, with five MSDs, an intraluminal amino-terminus, and a cytoplasmic carboxy-terminus.

2. Materials and methods

2.1. Construction of Flag-labeled proteins

The plasmids used to examine CLN3 structure were created by altering portions of the CLN3 coding sequence from the plasmids pRSV-CLN3, pAdRSV-CLN3-Flag and pGEX-CLN3 [9]. The plas-

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mid pCDNA3.1(+)Zeo was obtained from Invitrogen (Carlsbad, CA, USA). The plasmid pAdRSV-CLN3-Flag contains the CLN3 gene with an exogenous Flag epitope (encoding the amino acid sequence DYKDDDDK) inserted just distal to the carboxy-terminus; henceforth it will be referred to as CLN3-F_{438}. CLN3 and CLN3-F_{438} DNA sequences were subcloned into pCDNA3.1Zeo from pRSV-CLN3 and pAdRSV-CLN3-Flag, respectively, by restriction endonuclease digestion using *Xba*I and *Not*I, followed by ligation with T4 ligase, and transformation of DH5α *Escherichia coli*. The plasmids pCDNA-CLN3-F_{260} and pCDNA-CLN3-F_{321} were prepared using polymerase chain reaction (PCR) to introduce Flag epitopes into the CLN3 gene at amino acids 260 and 321, respectively. Oligonucleotide sequences are available upon request. These fragments were ligated into appropriately restricted pCDNA, checked by DNA sequencing, and subcloned into pCDNA3.1Zeo.

2.2. Construction of glycosylation mutants

The CLN3 segment encoding the amino acids 87–438 was generated by PCR, using as a template the whole-length CLN3 sequence, and was ligated into *Eco*RI–*Xho*I-digested pCDNA3.1Zeo. The resultant plasmids pCDNA-CLN3(87–483) and pCDNA-CLN3 were used as the template for oligonucleotide-directed mutagenesis which converts the Asn codon in the glycosylation site Asn-310 to a Gln codon (N310Q). Further mutagenesis on amino acid 199 converting Ala to Asn (A199N) was performed on pCDNA-CLN3(87–483)N310Q. The mutagenesis was done with a kit from Stratagene (La Jolla, CA, USA).

2.3. In vitro transcription and translation

Different plasmids were transcribed and translated in vitro in the presence or absence of canine pancreatic microsomes using a T7-coupled reticulocyte lysate system (TNT-T7; Promega, Madison, WI, USA). Translated proteins were labeled by metabolic incorporation of [^{35}S]methionine (1000 Ci/mmol) purchased from NEN (Boston, MA, USA).

2.4. Immunoprecipitation of CLN3 from intact microsomes

The antibodies Q516 and Q438 were generated by DNA immunization, as described in [10], and recognize peptides corresponding, respectively, to amino acids 2–18 and 250–264 of CLN3. The reaction mixture (40 μl) from in vitro transcription and translation was diluted with 500 μl of dilution buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, and 0.2 μM aprotinin) and incubated with 2 μg anti-CLN3 antibody (Q516 or Q438) for 4 h at 4°C. Membranes were isolated by centrifugation at 70 000 rpm for 30 min and resuspended in 500 μl dilution buffer containing 1% NP-40. Reactions without microsomal membranes did not undergo this initial ultracentrifugation step. The samples were then mixed with 100 μl of immobilized protein A (Pierce, Rockford, IL, USA) for 1 h at 4°C and the beads were collected by centrifugation at 14 000 rpm for 2 min. The beads were washed twice with dilution buffer containing 1% NP-40 and once with dilution buffer alone. After the last wash, the pelleted beads were resuspended in 50 μl of 6×Laemmli buffer. The samples were resolved on a 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gradient gel, fixed, dried at 80°C under vacuum, and analyzed by autoradiography.

2.5. Immunoprecipitation of Flag constructs from intact microsomes

The plasmids pCDNA-CLN3, pCDNA-CLN3-F_{260}, pCDNA-CLN3-F_{321} and pCDNA-CLN3-F_{438} were transcribed and translated in vitro as described above. Control plasmids were αRENaC-F_{Cterm} and αRENaC-F_{EC} (generously provided by Peter Snyder, University of Iowa, Iowa City, IA, USA), which encode Flag-labeled membrane-bound polypeptides in which the Flag epitope is located, respectively, on the cytosolic side or intraluminal side of the membrane [13]. The reaction mixture (40 μl) was diluted with 500 μl of dilution buffer and incubated with 2 μg anti-Flag M2 antibody for 4 h at 4°C. Isolation of microsomal membranes and immunoprecipitation were performed as described above.

3. Results

3.1. Sequence analysis of CLN3

The Batten disease protein, CLN3, is extremely hydrophobic. The hydrophilicity plot in Fig. 1 [14] demonstrates several large hydrophobic regions of greater than 15 amino acids, indicating that CLN3 is likely be an integral membrane protein with multiple MSDs. The PSORT database [11] identified up to seven potential MSDs in CLN3. Of these seven domains, six have very strong probability scores of −3 to −7. One of the putative MSDs (MSD 3) has a score of +0.11, and is therefore unlikely to be a true transmembrane domain.

Several secondary protein-structure prediction programs were used to analyze the amino acid sequence of CLN3, including PSORT [11], nnPredict [15,16], and SSP [17] databases. The program nnPredict uses a neural network to analyze an amino acid sequence for helix elements, turns, and β-strands. The accuracy of the computational neural network may approach 79% [15,16]. SSP has a probability of correct prediction ranging from 0.78 to 0.82 for helices longer than six residues and strands longer than eight residues [17]. The previously published six-MSD model of CLN3 structure proposed by Janes et al., in which both the COOH- and NH_2-termini are intraluminal (in the lysosome) or cytoplasmic (if on the plasma membrane), corresponds to the structure predicted by PSORT if the one unlikely MSD is excluded [12].

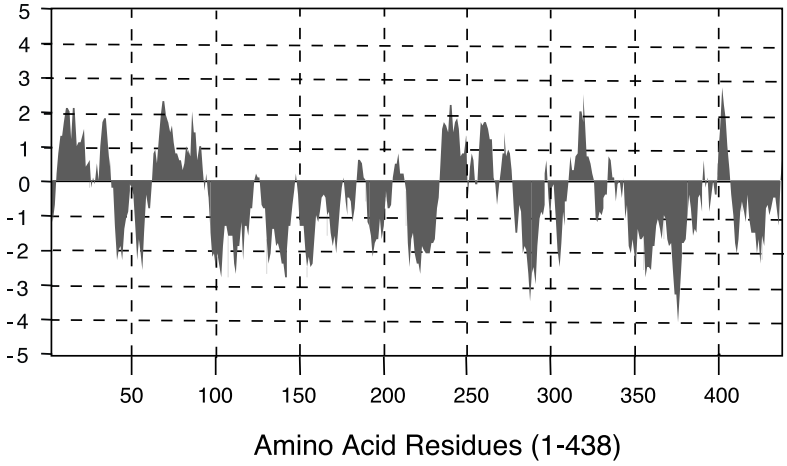

Fig. 1. Secondary structure analyses of CLN3. Kyte–Doolittle plot of the primary sequence of CLN3, showing domains of the protein which are hydrophobic.

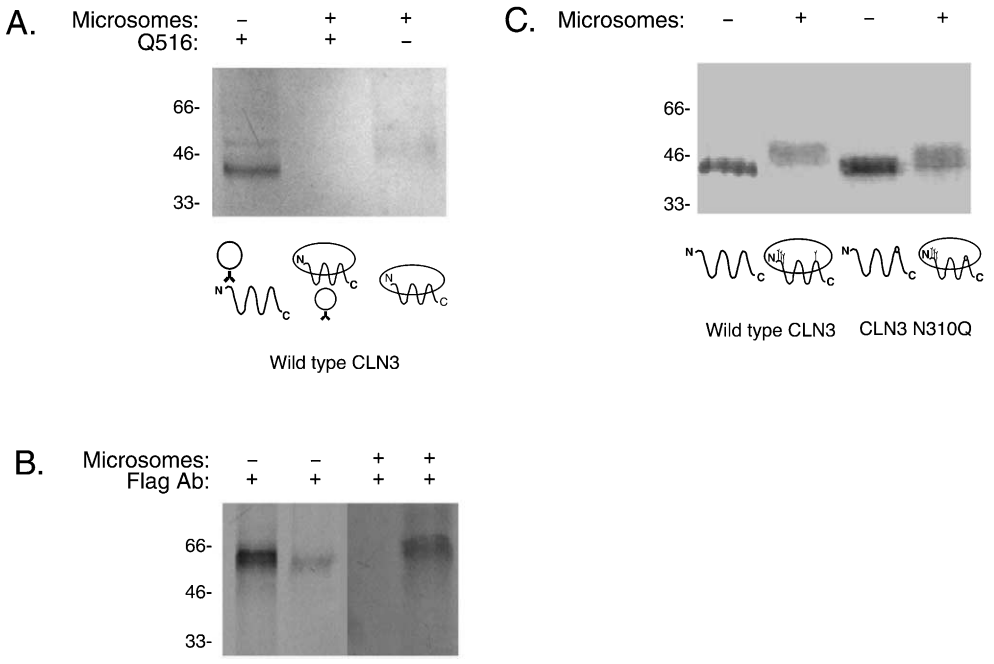

Fig. 2. The amino-terminus of CLN3 is intraluminal. A: CLN3 was translated in vitro in a cocktail containing [^{35}S]Met. Translation occurred in the absence (lane 1) or presence (lanes 2 and 3) of microsomes. After translation, Q516 antibody (lanes 1 and 2) was added and precipitates isolated (see Section 2). Residual microsomal membranes were collected by centrifugation to confirm incorporation of CLN3 into membranes (lane 3). Immunoprecipitates and non-precipitated microsomes were resolved by SDS–PAGE and visualized by autoradiography. B: Control study demonstrating the validity of the approach as originally described by Snyder et al. [13]. Proteins were translated in a [^{35}S]Met-containing cocktail, in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of microsomes. Translated products were immunoprecipitated with anti-Flag antibodies, and immunocomplexes dissociated and fractionated on SDS–PAGE. Lanes 1 and 3 are representative of translated ENaC subunits with Flag epitopes located in extracellular domains. Lanes 2 and 4 are representative of translated ENaC subunits with Flag placed in a cytoplasmic region. C: pCDNA-CLN3 (lanes 1 and 2) and pCDNA-CLN3N310Q (lanes 3 and 4) were translated in vitro in a cocktail containing [^{35}S]Met in the absence (lane 1and 3) or presence (lanes 2 and 4) of microsomes.

3.2. *Localization of the amino-terminus by immunoprecipitation and glycosylation mutagenesis*

CLN3 protein was prepared by in vitro transcription and translation and immunoprecipitated with Q516, an antibody which recognizes an epitope within the amino-terminal 20 amino acids of CLN3. Electrophoresis of the translated product revealed a peptide with a molecular weight of 43–45 kDa (Fig. 2A, lane 1). When translated in the presence of microsomal membranes, intraluminal epitopes become inaccessible to antibodies; this space is equivalent to the lumen of the ER, and as such is equivalent to the lysosomal lumen or the extracellular space. Fig. 2A, lane 2 is representative of three independent experiments and shows that Q516 did not precipitate CLN3 from intact microsomes. Subsequent purification of the supernatant-containing unprecipitated microsomes indicated that the protein was properly translated and incorporated into the membrane (Fig. 2A, lane 3). The molecular weight of translated CLN3 increases slightly, reflecting the addition of N-linked oligosaccharide moieties during post-translational processing in the microsomes.

Additional control experiments confirmed that the lack of immunoprecipitation by Q516 was due to inaccessibility of the antigen rather than a technical difficulty with the experiments. Two plasmids encoding Flag-epitope-containing proteins were tested. These plasmids contain the coding sequence for the α subunit of the amiloride-sensitive sodium channel from rats (αRENaC). The plasmid αRENaC-F_{EC} contains a Flag epitope at the amino-terminus, which is located in the extracellular milieu and thus protected from precipitation by Flag antibody. The plasmid αRENaC-F_{Cterm} contains Flag at the carboxy-terminus, which is cytoplasmic and therefore accessible to the antibody [13]. The representative data shown in Fig. 2B replicate the published data and show that Flag M2 antibody precipitates both proteins. However, only αRENaC-

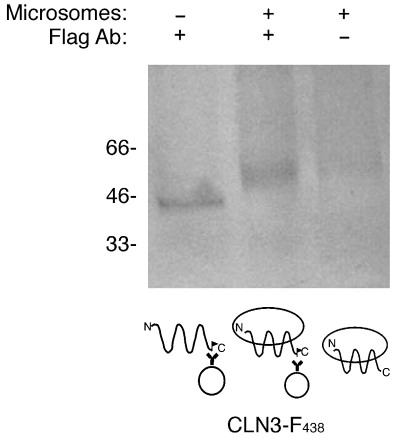

Fig. 3. The carboxy-terminus of CLN3 is cytoplasmic. CLN3 was translated in vitro in a cocktail containing [^{35}S]Met. Translation occurred in the absence (lane 1) or presence (lanes 2 and 3) of microsomes. After translation, anti-Flag antibody (lanes 1 and 2) was added and precipitates isolated. Residual microsomes were isolated by centrifugation. Translated products were fractionated by SDS–PAGE and then visualized by autoradiography.

F_{Cterm} could be precipitated from microsomes (compare Fig. 2B, lanes 3 and 4).

We next asked if the glycosylation pattern of CLN3 in the presence of microsomes was retained when the residual glycosylation site at Asn310 was removed, but the three potential glycosylation sites at the amino-terminus of CLN3, Asn-46, Asn-49, and Asn-71 were retained. If the amino-terminus was cytoplasmic, the N310Q mutation would ablate glycosylation-induced shifts. On the contrary, if the amino-terminus was intraluminal, there should be no or little alteration in the band shift compared to wild-type CLN3. As seen in Fig. 2C, there was no change in the glycosylation pattern between wild-type CLN3 and N310Q, confirming that the amino-terminus is intraluminal.

3.3. Localization of the carboxy-terminus by immunoprecipitation

The plasmid pCDNA-CLN3-F_{438} contains the CLN3 gene with a Flag epitope inserted between the last amino acid (residue 438) and the stop codon. The six-MSD model of CLN3 predicts this region to be intraluminal. As shown in Fig. 3, Flag M2 antibody precipitated the pCDNA-CLN3-F_{438} protein, thus indicating that the C-terminus is cytoplasmic. There was no cross-reactivity between the Flag epitope and CLN3. That the C-terminus and the N-terminus reside on opposite sides of the membrane indicates that CLN3 has an odd number of MSDs, and therefore rules out the proposed six-MSD model.

3.4. Localization of other regions of CLN3 by immunoprecipitation and glycosylation mutagenesis

To differentiate between the most likely remaining models (the five-MSD and seven-MSD models) we focused upon three other regions of CLN3. In the seven-MSD model, amino acid residue 199 would be on the cytoplasmic side of the membrane, amino acid residues 250–260 would be intraluminal and amino acid residue 310 or 321 would be on the cytoplasmic side of the membrane. The reverse of this arrangement would rule out the seven-MSD model.

A CLN3 construct lacking the three amino-terminal glycosylation sites, CLN3(Δ1–87), was modified to contain a new glycosylation site at residue 199. As shown in Fig. 4A, A199N

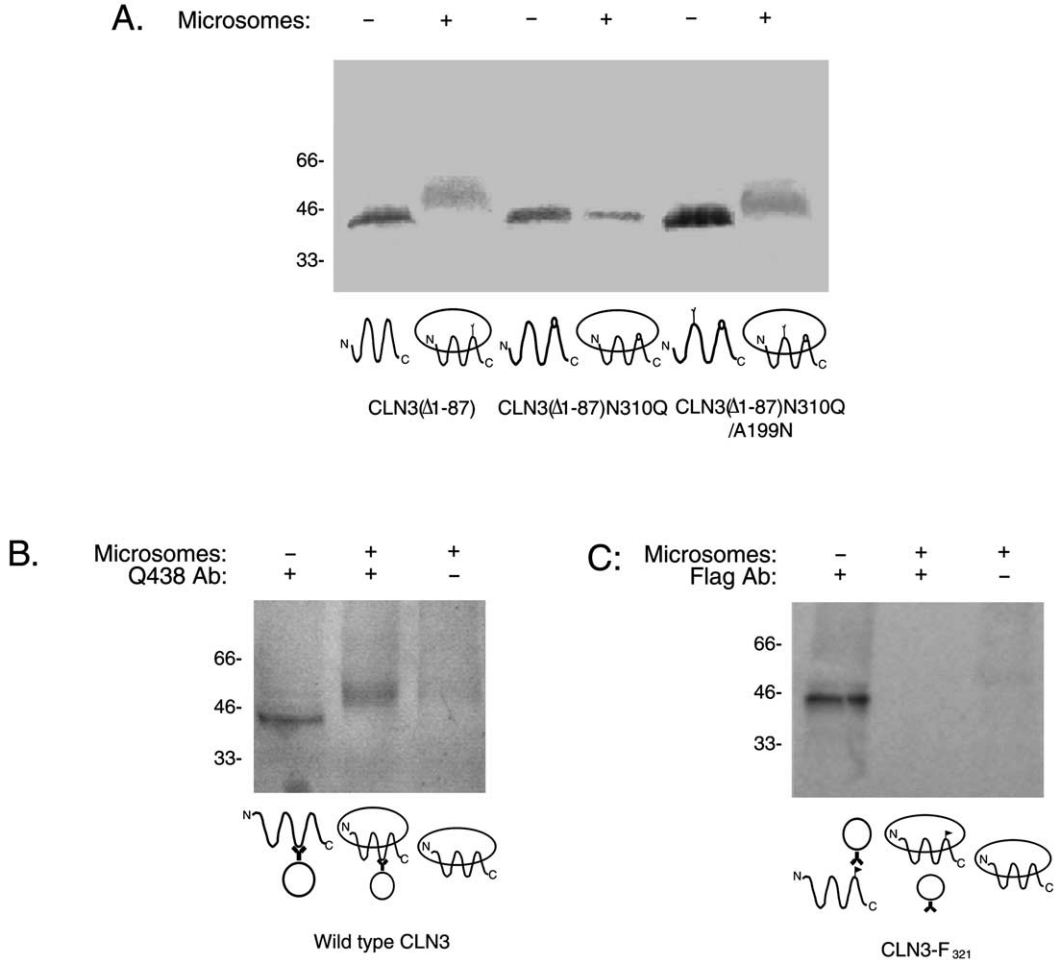

Fig. 4. A: Glycosylation site insertion and disruption localize residues 199 and 310 to the lumen. pCDNA-CLN3(Δ1–87) (lanes 1 and 2), pCDNA-CLN3(Δ1–87)N310Q (lanes 3 and 4) and pCDNA-CLN3(Δ1–87)N310Q/A199N were translated in vitro in a cocktail containing [^{35}S]Met in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of microsomes. B: The epitope spanning aa 250–264 of CLN3 is located in the cytoplasm. CLN3 was translated in vitro as described in the legend to Figs. 2 and 3. Translation occurred in the absence (lane 1) or presence (lanes 2 and 3) of microsomes. Precipitation of translated products (lanes 1 and 2) was done using anti-CLN3 antibody Q438. C: Residue 321 of CLN3 is not cytoplasmic. CLN3-$Flag_{321}$ was translated in vitro as described in the legend to Figs. 2 and 3. Translation occurred in the absence (lane 1) or presence (lanes 2 and 3) of microsomes. Precipitation of translated products (lanes 1 and 2) was done using anti-Flag antibody.

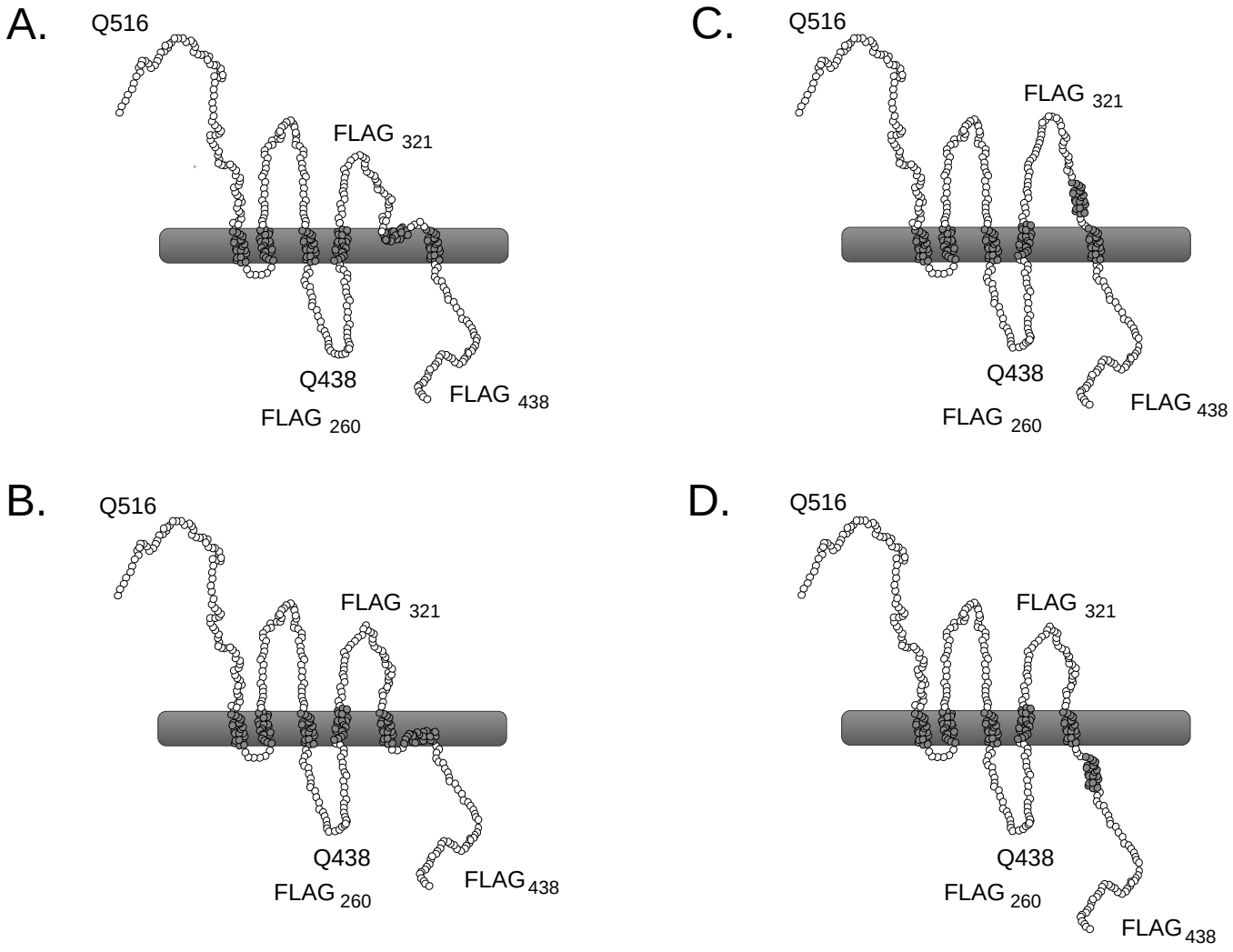

Fig. 5. Models for the orientation of CLN3 in the membrane. The data presented are consistent with CLN3 containing five domains which span the entire width of the membrane. CLN3 has an intraluminal, or extracellular, amino-terminus, and a cytoplasmic carboxy-terminus. It is possible that MSD 6 or MSD 7 could orientate within, rather than through, the membrane (panels A and B, respectively). Alternatively, MSD 6 may reside in the vesicle lumen (panel C). A fourth model can also be proposed, with MSD 7 located in the cytoplasm. This is the least favored of the four models.

resulted in a shift consistent with increased glycosylation, relative to protein translated in the absence of microsomes. These data support that residue 199 is intraluminal.

The region corresponding to amino acid residues 250–260 was evaluated both by insertion of a Flag epitope and by a recently developed antibody recognizing this region of the native CLN3 protein. The antibody Q438, generated by DNA immunization, recognizes a peptide corresponding to amino acid residues 250–264 of CLN3. This antibody precipitated microsomal membrane-bound CLN3 (Fig. 4B, lane 2). The plasmid pCDNA-CLN3-F_{260}, containing the Flag epitope inserted between amino acids 260 and 261 of CLN3, was likewise immunoprecipitated by Flag M2 antibody, suggesting that this region of the protein is cytoplasmic (data not shown).

Finally, we confirmed the position of the carboxy-terminal residues of CLN3; residue 310 by removing the endogenous glycosylation site in CLN3(Δ1–87), and residue 321 by Flag epitope insertion. As seen in Fig. 4C, CLN3-321_{Flag} was protected by the microsomal membrane from recognition by anti-Flag antibody. Glycosylation-scanning experiments corroborated this observation. Glycosylation was detected when CLN3(Δ1–87) was translated in the presence of microsomes (Fig. 4A, lanes 1 and 2), while no molecular weight shift was observed in the double mutant CLN3(Δ1–87)N310Q (Fig. 4A, lanes 3 and 4). Together, these experiments localize MSD 5 within the membrane, and argue against the seven-MSD model.

4. Discussion

Our studies of CLN3 indicate that it is a transmembrane protein with an odd number of MSDs. Our findings are most consistent with a structural model in which the protein has five domains that fully span the membrane, a cytoplasmic carboxy-terminus, and an amino-terminus which is located in the lumen of the ER.

The fact that the carboxy-terminus and amino-terminus lie on opposite sides of the ER membrane rules out models of protein structure in which there exist even numbers of MSDs.

This includes the six-MSD model proposed by Janes and colleagues [4,12]. The five-MSD model illustrated in Fig. 5 shows the relative locations of the six most favored MSDs (1, 2, 4–7). We conclude that MSD 3, which has a score of +0.10 and is predicted to form a relatively small β structure, is unlikely to be a true MSD. The cumulative data on epitope precipitation with antibodies targeted to the aa 260 (cytoplasmic) and aa 321 regions (intraluminal) and glycosylation scanning on residues 199 (intraluminal) and 310 (intraluminal) ruled out a seven-MSD structure and confirmed that MSD 5 does in fact span the membrane. Of MSDs 6 and 7, the latter has a higher probability of being membrane-bound than does the former. However, our data are consistent with either one of these putative MSDs spanning the entire width of the membrane, and the other embedded within the membrane (Fig. 5A,B).

Data using viral-mediated or plasmid-based transfections to over-express CLN3 supports our findings. Järvelä et al. [8] showed that a small amount of CLN3 is found at the plasma membrane. In vitro trafficking work in our laboratory showed that the amino-terminus of native CLN3 is accessible to the Q516 antibody but not to the Q438 antibody (Davidson and Mao, unpublished observations). Furthermore, unpublished data from phage display studies suggest the possibility of an interaction between two regions of the CLN3. A peptide corresponding to amino acid residues 67–91 was used as bait and was found to interact with peptides corresponding to amino acids 163–215 of CLN3 (Davidson, unpublished observation). Binding between these two regions, if confirmed, would require that they be located in the same compartment as is predicted by our five-MSD model (Fig. 5).

In contrast to the previously published model of CLN3, we have demonstrated that the C-terminal domain resides on the cytoplasmic side of the membrane. Analysis of the amino acid sequence of the carboxy-terminal region of CLN3 indicates several motifs that could potentially interact with cytoplasmic adapter complexes involved in sorting of integral membrane proteins [18,19]. The C-terminal tail of CLN3 contains three tyrosine-based motifs and several LL- and LI-pairs. These motifs are found in other lysosomal membrane proteins such as LAMP-1, LIMP-II and LAMP-2 [18–20]. Although the LL- and LI-motifs are unlikely to be targets for adapter protein complex binding, two of the tyrosine-based motifs, aa 378–382 and 387–391, show high sequence similarities to other lysosomal targeting or internalization motifs [18]. The third tyrosine-based motif, aa 370–374, lies embedded within putative MSD 7.

In addition to the 1.02 kb deletion which is present in a majority of patients, there exist disease-causing point mutations in other regions of the protein [4,21,22]. Patients who are compound heterozygotes and have the 1.02 kb deletion coupled with a transition mutation resulting in substitution of phenylalanine for valine at the 330th amino acid residue (V330F) are afflicted with JNCL, as are compound heterozygotes who have the mutation R334H. Compound heterozygous patients with L101P, L170P, or E295K substitutions may have a more protracted and less severe clinical course, featuring visual loss but modest impairments of cognition and motor function [4]. Although the truncation mutant created by the 1.02 kb genomic deletion shows defective intracellular trafficking and accumulates in the ER [8], studies to date indicate that disease-causing point mutations do not grossly disrupt trafficking to the lysosome but rather CLN3 function [10]. Interestingly, our model of CLN3 places all but one of these point mutations within the lumen of the ER. Because some CLN3 traffics to the cell surface (Davidson and Mao, unpublished observations), there exists the possibility that the CLN3 mutations may affect appropriate protein–protein interactions with substances in the extracellular milieu, or with other membrane proteins. Alternatively, if CLN3 is found to be a transport protein, presence on the cell membrane may allow for transport of its target molecule into the cell cytoplasm as well as in the lysosome. Testing of these hypotheses requires further experimentation.

In summary, the juvenile form of Batten disease, or JNCL, is a devastating and presently incurable neurologic disease resulting from mutations in a lysosomal integral membrane protein with five MSDs. Our efforts to determine the membrane structure of the protein provide a number of clues that assist our ongoing efforts to delineate CLN3 function and its role in the molecular pathogenesis of JNCL.

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