Topological Analysis of Niemann-Pick C1 Protein Reveals That the Membrane Orientation of the Putative Sterol-sensing Domain Is Identical to Those of 3-Hydroxy-3-methylglutaryl-CoA Reductase and Sterol Regulatory Element Binding Protein Cleavage-activating Protein*

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The Niemann-Pick C1 (NPC1) protein is predicted to be a polytopic glycoprotein, and it contains a region with extensive homology to the sterol-sensing domains (SSD) of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-R) and sterol regulatory element binding protein cleavage-activating protein (SCAP). To aid the functional characterization of NPC1, a model of NPC1 topology was evaluated by expression of epitope-tagged NPC1 proteins and investigation of epitope accessibility in selectively permeabilized cells. These results were further confirmed by expression of NPC1 and identification of glycosylated domains that are located in the lumen of the endoplasmic reticulum. Our data indicate that this glycoprotein contains 13 transmembrane domains, 3 large and 4 small luminal loops, 6 small cytoplasmic loops, and a cytoplasmic tail. Furthermore, our data show that the putative SSD of NPC1 is oriented in the same manner as those of HMG-R and SCAP, providing strong evidence that this domain is functionally important.

Niemann-Pick type C (NPC) is a rare autosomal recessive disorder that results in progressive neurodegeneration and hepatosplenomegaly, leading to death during early childhood (1). The most prominent biochemical feature is the accumulation of low density lipoprotein-derived unesterified cholesterol in lysosomes (2, 3). In addition, cholesterol accumulates in the trans-Golgi network (TGN), and its relocation to and from the plasma membrane is delayed (1). In fibroblasts, the defect in cholesterol exit from lysosomes is accompanied by an attenuation in the down-regulation of two key components of cholesterol homeostasis, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-R) and the low density lipoprotein receptor (4, 5). Recently NPC1, the gene for the major linkage and complementation group, which maps to chromosome 18q11-12, was identified. It encodes an approximately 4.5-kilobase mRNA (6) that is predicted to produce a 1278-amino acid protein. At least 36 mutations have been described in NPC1 patients (6–10), and although most are found throughout the gene and do not reveal any functionally critical protein domains, a small cluster is located in a the carboxyl-terminal third of the protein in a region that possesses conserved cysteine residues (9). A minor group of NPC patients does not map to the NPC1 locus, and complementation studies (11, 12) have shown that these patients are defective for a second gene, NPC2, which is currently unknown.

The effects of reduced or absent NPC1 in both humans (4, 13) and animal models (1, 3) have been well studied, establishing this gene as an essential component of intracellular cholesterol trafficking. In addition, several Chinese hamster ovary cell mutants that display the NPC1 phenotype have been characterized (14) and most have been shown to be defective for the NPC1 gene (15). However, the precise function(s) of the NPC1 protein is still unknown. NPC1 has been shown to be a membrane glycoprotein that localizes to Lamp-positive organelles, presumably endosomes and lysosomes (16–18). Further studies have demonstrated that NPC1 resides primarily in late endosomes and only secondarily in lysosomes and the TGN (18). This is an important distinction in view of recent data suggesting that cholesterol accumulation in NPC cells occurs primarily in endosomes (19), which are sorting sites for various cellular components. In addition, NPC cells appear to be defective in their efflux of endocytosed sucrose and in the sorting of the mannose-6 phosphate receptor, suggesting that the retrograde movement of proteins and cargo from late endosomes to the TGN is perturbed (16, 19).

Interestingly, although NPC1 is not closely related to any known proteins, a cluster of five potential membrane-spanning sequences, from residues 616 to 797, is homologous to the sterol-sensing domain (SSD) found in two key regulators of cholesterol homeostasis, HMG-R and sterol regulatory element binding protein cleavage-activating protein (SCAP). In SCAP, a single point mutation in its SSD is sufficient to render the protein sterol-insensitive, indicating the importance of this region (20, 21), whereas in HMG-R the SSD mediates sterol-regulated degradation of the protein (22, 23). The transmembrane (TM) regions of NPC1, including the putative SSD, also share homology with regions of Patched (PTC), a receptor for
the cholesterol-activated protein sonic hedgehog (24, 25). Together these data support the view that the SSD is functionally significant.

In these studies, we describe and validate a topological model for the structure of NPC1, showing that the orientation of the putative SSD is identical to those of HMG-R and SCAP. These data should support efforts in elucidating the precise function of this large membrane glycoprotein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium was purchased from Mediatech (Herndon, VA) and fetal bovine serum was from HyClone (Logan, UT). LipofectAMINE, L-glutamine, gentamicin, colcemid, and trypsin/EDTA were obtained from Life Technologies, Inc. Fluormount-G was purchased from Southern Biotechnology Associates (Birmingham, AL). All enzymes were obtained from New England Biolabs (Beverly, MA) except for N-glycosidase F, which was from Roche Molecular Biochemicals (Indianapolis, IN). The goat anti-mouse IgG-fluorescein isothiocyanate (FITC) and goat anti-rabbit IgG-FITC were also from Roche Molecular Biochemicals (Indianapolis, IN), except for M2 anti-FLAG, which was from Sigma-Aldrich.

**Creation of FLAG-tagged NPC1 Plasmids**—All plasmids were constructed using the wild-type (wt) human NPC1 cDNA. For analysis of the full-length NPC1 protein, hydrophilic loops A to E were tagged with the FLAG peptide sequence DYKDDDDK by inserting an adapter oligonucleotide at a unique restriction endonuclease site in the wt NPC1 cDNA. For loops A to E, these sites were NdeI, ClaI, BstI, BclIWI, and Bsa36I and resulted in the insertion of the following sequences at the indicated amino acid positions: 36-FLAG-Y, 306-FLAG-I, 519-FLAG-M, 2436-FLAG-HA, respectively (Fig. 1A). A pictorial representation of the predicted topology of NPC1 is shown with cylinders denoting TM domains, including the putative SSD, in boldface. Heavy lines depict the large hydrophilic portions (loops A–E) above and below the cylinders to indicate their luminal or cytosolic location, respectively. The letter F within the ovals denotes the locations of the FLAG tags.

![Topography of NPC1](image)

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For complete permeabilization, cells were fixed in methanol for 6 min at 4 °C and blocked with 1% BSA in PBS. Proteins were detected by incubation with 0.2 μg/ml M2 anti-FLAG antibody and 1.4 μg/ml rabbit anti-α-galactosidase A (α-Gal A) polyclonal antibody (27) in blocking solution for 45 min, followed by a wash with PBS, and then incubation with 2 μg/ml anti-mouse IgG-FTC and 2 μg/ml anti-rabbit IgG-rhodamine-X secondary antibodies in blocking solution. After further washing, the cells were mounted in Fluoromount G and visualized using a Nikon Eclipse fluorescence microscope equipped with a charge-coupled device (CCD) camera (Nikon, Melville, NY).

To selectively permeabilize the plasma membrane, cells were incubated at 4 °C for 45 min in sucrose buffer (1% BSA, 0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl2, 1 mM EDTA, 10 mM HEPES, pH 7.4) containing 0, 1, 1.5, or 2 μg/ml digitonin and with the same concentrations of M2 FLAG and α-Gal A antibodies used for complete permeabilization. The cells were then fixed with methanol and incubated in blocking solution for 30 min before addition of secondary antibodies and visualization as above.

**Analysis of Truncated Glycosylated Proteins**—For expression of various NPC1 constructs, COS-7 cells were grown in 10-cm diameter dishes and transfected using 4 μg of DNA, 20 μl of LipofectAMINE, and 30 μl of Plus reagent, according to the manufacturer’s recommendations. The transiently expressed proteins were analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis (PAGE), essentially as described previously (27). Briefly, at 36 h post-transfection, the cells were metabolically labeled with [35S]methionine for 16 h. Cells were harvested using PBS, 2 mM EDTA and centrifuged at 800 × g for 10 min. The cell pellet was resuspended in buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 2 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 25 μg/ml N-acetyl-leucinal-leucinal-norleucinal, 1 μM/ml phenylmethylsulfonyl fluoride, and 0.1 mM Pefabloc), disrupted by sonication using a Branson sonifier (Smith-Klein, Philadelphia, PA), and centrifuged at 22,000 × g for 10 min. The resultant cell membrane pellet was resuspended in 0.5 ml of 0.5 M NaCl, 50 mM Tris, 2 mM EDTA, 1% Igepal, supplemented with the above proteinase inhibitors, using 10 passages through a 22-gauge needle, prior to rocking for 15 min and centrifugation at 22,000 × g for 10 min. The solubilized membranes were incubated for 1 h with 2.8 μg/ml of anti-FLAG M2, followed by a 45-min incubation with protein G-agarose beads (Roche Molecular Biochemicals). The immunoprecipitates were washed with buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Igepal, 1 mM EDTA, 0.25% gelatin, 0.2% NaN3), resuspended in SDS-gel loading buffer, heated for 10 min at 65 °C, and subjected to SDS-PAGE analysis (28). N-Glycosidase F digestions were carried out as described previously (27).

**RESULTS**

**Predicted Membrane Topology of NPC1**—The predicted NPC1 protein sequence was analyzed to identify potential transmembrane (TM) or hydrophilic domains. Using the TMpred algorithm (29) at EMBnet-CH, the TM domains and topology of the hydrophilic loops of NPC1 were evaluated. The NPC1 protein is predicted to have 15 TM regions (Fig. 1A). The assignment of predicted TM regions 4–8 is based on their relative hydrophobicity and their high degree of similarity to the sterol-sensing domains of SCAP and HMG-R (30, 31) (Fig. 1A, SSD). Based on these observations and computer analyses, the predicted model for NPC1 topology is shown in Fig. 1B and consists of 15 TM domains, 5 hydrophilic loops ranging in size from ~50 amino acids (loop B) to ~265 amino acids (loop A), and a 27-amino acid cytoplasmic tail containing a potential endosomal/lysosomal dileucine targeting motif. In addition, the model predicts that the amino terminus of NPC1 (loop A) remains in the lumen upon entering the endoplasmic reticulum (ER).

**Characterization of Full-length Epitope-tagged NPC1 Pro-
To validate the locations of the major hydrophilic loops A to E in the predicted model (Fig. 1B), a series of epitope-tagged, full-length NPC1 proteins was constructed and analyzed. These epitope-tagged NPC1 proteins were expressed in human NPC1 fibroblasts to determine whether the addition of the tags interfered with their correct processing, transport, and/or function. Transfected cells were costained with filipin and the anti-FLAG antibody to determine whether cells expressing the FLAG-tagged NPC1 were corrected for the NPC1 phenotype, thus producing filipin-negative cells. 50–100 NPC1-positive cells were scored for correction. For all constructs ~60–90% of positive cells appeared to be corrected, with the exception of the loop C construct for which about 22% of cells were corrected. Representative fields for each construct are shown in Fig. 3; human NPC1 cells positive for NPC1 expression (left panels; arrows) show a dramatic decrease in filipin staining (right panels; arrows) compared with neighboring cells, which are negative for NPC1 expression. Only one construct, which contained the tag in loop C (Fig. 3C), did not appear to complement the NPC1 phenotype. Upon careful inspection it is clear that NPC1 containing the loop C tag does not colocalize with the stored cholesterol and thus is unable to mobilize cholesterol, similar to results obtained by mutagenesis of the NPC1 tail (32). These results indicate that the FLAG-tagged NPC1 proteins are functional and, with the exception of the loop C construct, were correctly targeted to the endosomal/lysosomal system, the site of cholesterol accumulation in NPC1 cells. Thus, these epitope-tagged NPC1 proteins remained functionally active and could be used to determine the membrane topology of NPC1.

Epitope Mapping by Selective Permeabilization and Immunofluorescence—The full-length FLAG-tagged NPC1 constructs were transiently expressed in COS-7 cells following electroporation. Because COS-7 cells are easy to transfect and maintain, the majority of experiments were carried out using these cells. Initial studies showed no differences in the processing, distribution, and functional integrity of the expressed proteins, indicating that NPC1 is topologically identical whether expressed by human or simian cells (data not shown). To determine the positions of the hydrophilic domains, selective membrane permeabilization and immunofluorescence microscopy were used. At 24 h post-transfection, the cells were subjected to immunofluorescence analysis under one of three conditions: no permeabilization (absence of detergent); selective permeabilization of the plasma membrane using low concentrations of digitonin; or full permeabilization by methanol fixation. All NPC1 proteins were detected with the M2 FLAG antibody and a fluorescein-conjugated secondary antibody. As a control for permeabilization, the intralysosomal protein α-Gal A was detected using a specific polyclonal antibody and a rhodamine-conjugated secondary antibody. Pilot studies indicate that NPC1 proteins tagged at loops A, C, and D (panels A, C, and D, respectively) were undetected in selectively permeabilized cells, indicating a luminal location, whereas the loop B and tail protein was detected (panels B and G, respectively), showing that they were cytosolic. For loop E two different tagged NPC1 proteins were expressed (panels E and F), and both showed a luminal location. The first was observed at the plasma membrane surface of nonpermeabilized cells, whereas the second protein was undetectable in permeabilized cells.
cated that concentrations of digitonin below 3 μg/ml specifically permeabilized the plasma membrane but not intracellular membranes, as demonstrated by the inability of the α-Gal A antibody to detect its target protein within lysosomes (not shown). Under these conditions, cytosolic protein epitopes were detected while luminal ones, including the α-Gal A control protein, were negative. Nonpermeabilized cells were used as a negative control while transfection efficiency and locations of expressed proteins were analyzed in fully permeabilized cells.

Fig. 4 shows the results of immunofluorescence detection of proteins from the FLAG-tagged constructs. Representative cells are shown for each condition. In fully permeabilized cells, a bright, vesicular staining pattern for the intraorganelle marker α-Gal A was observed, indicating complete antibody access. However, in digitonin-permeabilized cells there was no α-Gal A staining, confirming that permeabilization was selective for the plasma membrane. The six full-length NPC1 proteins, containing FLAG tags in domains A–E and the cytoplasmic tail, were analyzed (Fig. 4). Detection of the loop A-tagged NPC1 protein in the fully but not the partially permeabilized cells indicated that loop A was luminal. Similarly, detection of the loop B-tagged protein in digitonin-permeabilized cells, accompanied by a negative α-Gal A signal, indicated that this epitope, and thus loop B, was cytosolic (Fig. 4B). In contrast, the FLAG tags in loops C and D were both undetectable in digitonin-treated cells, suggesting their luminal location (Fig. 4, C and D). These results indicated that the orientation of loops D and E was reversed with respect to our predicted model, implying that the predicted TM3 domain does not span the membrane. This suggests that loops C and D comprise a single luminal loop and loop E is shifted from a cytoplasmic position to the luminal one. The results for loop E confirmed its luminal location, assuming elimination of TM3. However, its distribution was surprising. This protein was detected in large quantities at the plasma membrane of nonpermeabilized cells (Fig. 4E). Interestingly, the membrane staining appeared speckled and uneven, perhaps indicating that some form of aggregation occurred at the cell surface. However, this phenomenon does not apparently interfere with NPC1 function, because the protein successfully reversed the cholesterol storage observed in filipin-stained human fibroblasts (Fig. 3E). The mechanism by which a FLAG tag in this luminal loop causes mislocalization of NPC1 to the plasma membrane is currently being investigated.

To further confirm the location of loop E, two different approaches were used. First, an additional construct containing the FLAG tag at a different position within loop E was expressed and analyzed as above. The expressed protein did not mislocalize to the plasma membrane. As shown in Fig. 4F, failure to detect the FLAG-tagged protein in digitonin-treated cells confirmed the previous conclusion that loop E is luminal. Second, an antibody directed against loop E was used to detect its epitope in permeabilized cells that expressed wt NPC1, as above, lending further support to the luminal location of loop E (data not shown).

Notably, the above results indicate that elimination of TM3 from our topological model reverses the orientation of the SSD. In this orientation, the amino terminus of the SSD is luminal, in complete agreement with the topological models for the HMG-R and SCAP SSDs, further supporting the notion that the SSD plays an important role in cholesterol homeostasis in these proteins. Also, in the absence of TM3, the position of the NPC1 tail shifts from the cytoplasm to the lumen. Because the carboxyl-terminal dileucine (LLNF) motif normally functions in the cytoplasm to target proteins to the endosomal/lysosomal system (33), the precise number of TM domains and, therefore,

**Fig. 5. Localization of FLAG epitopes in truncated NPC1 proteins in semipermeabilized cells.** The FLAG-tagged truncated NPC1 proteins were transiently expressed in COS-7 cells and detected by immunofluorescence, as described in Fig. 4. In A–F, expression of proteins from the pLaTM1, pLbTM2, pLeTM3, pLdTM4, pLdTM6, and pLeTM13 constructs (see Fig. 2), respectively, in nonpermeabilized (Non), completely permeabilized (Complete), and selectively permeabilized (Digitonin) cells is shown. The pLaTM1, pLdTM4, and pLeTM6 proteins were detected in selectively permeabilized cells indicating their cytosolic location. Proteins from pLbTM2 and pLeTM3 were detected in nonpermeabilized cells at the plasma membrane, and that of pLeTM13 was undetected in selectively permeabilized cells, confirming that their FLAG-tagged epitopes were luminal.
the correct orientation of the tail needed to be established. Thus, a construct in which the tail was removed and replaced by a FLAG tag was used. As anticipated, the epitope tag was undetected in nonpermeabilized cells but was accessible following treatment with digitonin, indicating a cytosolic location for the tail (Fig. 4G). This is consistent with other proteins that contain the dileucine lysosomal targeting motif at their carboxyl terminus. However, for the model to correctly reflect the location of the tail, one of the predicted TM domains after TM13, most probably TM14, which has a lower hydropathy score than TM15 (Fig. 1A), would have to be eliminated as a potential transmembrane domain.

Expression and N-Glycosylation Analysis of Truncated NPC1 Proteins—To further confirm the NPC1 topology suggested by the above results, a number of truncated NPC1 constructs, whose sequences terminated after TM1, 2, 3, 4, 6, and 13 (Fig. 2), were expressed and analyzed as above. Based on the predicted model, constructs terminating after TM1, 4, and 6 were expected to have a FLAG tag on the cytosolic side of the membrane. pLaTM1 protein was undetected in nonpermeabilized cells but showed distinct plasma membrane staining in digitonin-treated cells, suggesting that its FLAG tag was cytosolic (Fig. 5A). pLbTM2 and pLeTM3 were also detected at the plasma membrane and because nonpermeabilized cells were stained, this indicated a luminal position for both, as expected in the absence of TM3 (Fig. 5, B and C). Interestingly, the pLdTM4 protein did not localize to the plasma membrane but showed a predominantly vesicular appearance, suggesting the presence of a targeting signal that either facilitated retrieval from the plasma membrane or caused intracellular sequestration (Fig. 5D). Because the tag was detected in digitonin-treated cells, its cytosolic location was confirmed, further supporting the absence of TM3. pLeTM6 gave similar results, although its location was less vesicular and displayed a Golgi and/or ER pattern of distribution (Fig. 5E). pLeTM13 protein had a distribution similar to that of TM6 but was not detected in digitonin-treated cells, indicating a luminal tag position (Fig. 5F). These results confirmed previous observations that TM3 is not present and that loops A, C, D, and E are luminal.

Because the results obtained using the truncated NPC1 proteins were in complete agreement with the results obtained using the full-length FLAG-tagged NPC1 proteins, we extended our studies to investigate the level of glycosylation, if any, of each truncated protein. Loops that are glycosylated would have to enter the ER lumen, lending further support to their luminal location. The truncated proteins were expressed, immunoprecipitated, and then deglycosylated using N-glycosidase F, prior to SDS-PAGE analysis (Fig. 6). Comparison of the estimated molecular weights of the deglycosylated and untreated proteins allowed the extent of N-linked glycosylation to be determined (Table I). For pLaTM1 protein, there was a clear band shift after N-glycosidase F treatment (Fig. 6A), indicating that the amino terminus (loop A) was heavily glycosylated and therefore located in the ER lumen. Because the average molecular mass of one high mannose-type oligosaccharide is about 2 kDa, the shift of approximately 11 kDa probably represents glycosylation at all five potential sites. The protein expressed from pLbTM2 also showed an approximate shift of 11 kDa, and because there was no apparent increase in the extent of deglycosylation in comparison to the shift seen for pLaTM1, it was presumed that the glycosylation site in loop B was not utilized and that this loop was cytoplasmic, as predicted. The results for pLcTM3 expression are also in agreement with the proposed model. A band shift of about 16 kDa indicated that loop C was glycosylated, and it was therefore located in the lumen (Fig. 6A).

| Construct  | Total number of N-glycosylation consensus sites | Predicted molecular mass, without glycosylation (kDa) | Observed molecular mass, without glycosylation (kDa) |
|------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| pLaTM1     | 5                                             | 31.7                                         | 40                                            |
| pLbTM2     | 6                                             | 40.3                                         | 45                                            |
| pLcTM3     | 10                                            | 59.8                                         | 65                                            |
| pLdTM4     | 13                                            | 70.8                                         | 81                                            |
| pLeTM6     | 13                                            | 78.8                                         | 82                                            |
| pLaTM1     | 19                                            | 127.9                                        | 144                                           |

* Accurate determination of the molecular mass shifts for this construct was not possible (see text).

The results from the larger truncated proteins were less conclusive than those for loops A to C. The pLdTM6 construct was substituted for the pLdTM4 construct because of inefficient immunoprecipitation of the pLdTM4 protein (Fig. 6B),
even though immunofluorescent staining indicated that this protein was efficiently expressed in COS-7 cells. Following immunoprecipitation of the pLdTM6 protein, SDS-PAGE analysis revealed multiple bands, thus complicating estimations of the band shift (Fig. 6B). A similar problem was encountered with the pLeTM13 and full-length NPC1 proteins (not shown). However, following deglycosylation, both the pLeTM6 and pLeTM13 proteins showed a qualitatively greater shift than pLeTM3, indicating that loops D and E were glycosylated and thus located in the lumen (Fig. 6B and Table I). The additional bands may represent protein aggregation, or they may reflect additional forms of post-translational modification. Interestingly, there is a 5- to 8-kDa discrepancy between the predicted and observed molecular masses for the deglycosylated pLaTM1, pLbTM2, and pLcTM3 proteins (Table I). This may reflect additional post-translational modification of loop A, which contains six consensus sites for myristoylation. Further studies are necessary to determine whether NPC1 is subject to other types of post-translational processing.

Together, these data independently verify the positions of hydrophilic domains A–C and confirm the absence of TM3. Removal of TM14 to correctly orient the tail results in the topological model of NPC1 shown in Fig. 7. This model consists of 13 transmembrane domains, 3 large luminal and 4 small hydrophilic loops, 6 small cytoplasmic loops, and a cytoplasmic tail. Elimination of the proposed TM3 (Fig. 1) results in a reversal of the location of domains D and E, thus placing the putative SSD in the same orientation as those of the HMG-R and SCAP proteins. Because the carboxyl terminus tail is cytosolic, an additional membrane domain has been removed between TM13 and 15 in our modified model.

**DISCUSSION**

The data obtained in these studies are consistent with a topological model in which NPC1 has 13 TM domains, a luminal amino terminus and a cytosolic carboxyl-terminal tail. The results from the glycosylation analyses of truncated NPC1 proteins and the results obtained from investigating the antibody accessibility of FLAG-tagged NPC1 proteins in selectively permeabilized cells are also in agreement with this model. The prediction for TM3 has been determined to be incorrect. These studies have shown that loop D is luminal, not cytosolic as predicted, thus indicating that loops C and D are fused and that the proposed TM3 does not span the membrane. In addition, loop E, which is predicted to be cytosolic, is in fact luminal, suggesting that the orientation of protein domain from loop D to at least loop E is reversed. Thus, the orientations of loops D–E as well as transmembrane domains 4, 6, and 13, and presumably all intervening sequences, are reversed with respect to the predicted model (Fig. 1B). The loss of TM3 from the topological model is not surprising in view of the hydrophobicity profile in which TM3 has the lowest score compared with the other predicted membrane-spanning regions of NPC1 (Fig. 1A). Finally, immunolocalization of the NPC1 tail in cells with selectively permeabilized plasma membranes has confirmed its cytoplasmic location, which is expected, because the tail contains a dileucine targeting motif (33, 34) that normally functions in the cytosol to target proteins to an endosomal/lysosomal compartment.

We therefore propose a new model for the topology of NPC1 (Fig. 7), which is in agreement with our extensive experimental data. In this model, the original TM3 does not span the mem-
brane and the proposed loops C and D form one luminal do-
main. In addition, because our amended model reverses
the orientation of the middle portion of the protein but main-
tains the cytosolic tail, the 14th predicted TM domain, which is
very close to TM15, is presumed to be absent. Therefore, our data
for NPC1 is consistent with a membrane glycoprotein that con-
tains 13 transmembrane domains oriented as in Fig. 7, with a
luminal amino terminus and cytosolic carboxyl-terminal tail.
The three largest hydrophilic domains are luminal, and the
majority of the remaining portions of the NPC1 protein is
embedded within the membranes. Of interest, all three luminal
hydrophilic domains are approximately the same length (~250
amino acids), although the importance of this observation is
currently unclear.

The results from our topological analyses have added to
current speculation regarding the possible functional role of
the domain from amino acids 615 to 797 (6, 35). This region of
NPC1 is homologous to the SSD of HMG-R and SCAP, two
cholesterol-regulated proteins (22, 31). In HMG-R, the putative
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