Membrane Topology of γ-Secretase Component PEN-2*

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PEN-2 is an integral membrane protein that is a necessary component of the γ-secretase complex, which is central in the pathogenesis of Alzheimer’s disease and is also required for Notch signaling. In the absence of PEN-2, Notch signaling fails to guide normal development in Caenorhabditis elegans, and amyloid β peptide is not generated from the amyloid precursor protein. Human PEN-2 is a 101-amino acid protein containing two putative transmembrane domains. To understand its interaction with other γ-secretase components, it is important to know the membrane topology of each member of the complex. To characterize the membrane topology of PEN-2, we introduced single amino acid changes in each of the three hydrophilic regions of PEN-2 to generate N-linked glycosylation sites. We found that the N-linked glycosylation sites present in the N- and C-terminal domains of PEN-2 were utilized, whereas a site in the hydrophilic “loop” region connecting the two transmembrane domains was not. The addition of a carbohydrate structure in the N-terminal domain of PEN-2 prevented association with presenilin 1, whereas glycosylation in the C-terminal region of PEN-2 did not suggest, that the N-terminal domain is important for interactions with presenilin 1. Immunofluorescence microscopy with selective permeabilization of the plasma membrane of cells expressing epitope-tagged forms of PEN-2 confirmed the luminal location of both the N and C termini. A protease protection assay also demonstrated that the loop domain of PEN-2 is cytosolic. Thus, PEN-2 spans the membrane twice, with the N and C termini facing the lumen of the endoplasmic reticulum.

PEN-2 is a component of the high molecular weight γ-secretase complex that also contains, at a minimum, the integral membrane proteins APH-1, Nicastrin (Nct), and presenilin 1 (PS1) or presenilin 2 (PS2) (1–5). This complex is responsible for intramembranous proteolytic cleavage of both the amyloid precursor protein (APP) and the Notch receptor. APP, a type I integral membrane protein, is cleaved in its luminal domain by the membrane-anchored aspartyl protease BACE (for β-site APP cleaving enzyme), resulting in a C-terminal 99-amino acid-long membrane-anchored fragment termed C99 (6). C99 can be subsequently cleaved within its transmembrane domain by the γ-secretase complex. This cleavage results in both the production of the amyloid β peptide (7), which is most often 40–42 amino acids in length (8), as well as the C-terminal fragment of APP. The amyloid β peptide is prone to aggregation and is the main component of amyloid plaques, a pathological hallmark of Alzheimer’s disease (9). That the γ-secretase and APP are central to Alzheimer’s disease pathogenesis is clear as mutations in APP or the presenilins can result in autosomal dominant familial Alzheimer’s disease (10–12).

PS1 and its less abundant homolog PS2 are known to be necessary for γ-secretase activity and are likely the proteases that cleave APP and Notch (13, 14). After synthesis, PS1 is itself cleaved resulting in noncovalently associated N- and C-terminal fragments that are thought to be the active form of the enzyme. However, the presenilins do not function alone, being known to associate with other proteins that are required for γ-secretase function. Nicastrin, a type I integral membrane protein, was the first protein other than the presenilins to be identified as a member of the γ-secretase complex (5). Recently, two additional integral membrane proteins, PEN-2 and APH-1, both with uncertain membrane topologies and no other known functions, have been shown to be required components without which the γ-secretase cannot function normally (1, 2, 4, 15, 16). Furthermore, the various components of the complex appear to regulate each other and are required for normal maturation and trafficking of the other complex proteins. For example, in the absence of PS, Nct fails to become resistant to endoglycosidase H (endo H) and appears to be retained in the endoplasmic reticulum (ER) (17, 18) whereas PEN-2 levels are reduced significantly (4). Similarly, cleavage of PS1 into N- and C-terminal fragments does not occur following down-regulation of Nct or APH-1 and is reduced significantly following down-regulation of PEN-2 (15), which has been shown to be a limiting factor in PS1 cleavage (19). Furthermore, PEN-2 binds preferentially to the PS1 holoprotein (19).

How the components of the γ-secretase complex interact and

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‡ The abbreviations used are: Nct, Nicastrin; PS1, presenilin 1; PS2, presenilin 2; APP, amyloid precursor protein; endo H, endoglycosidase H; ER, endoplasmic reticulum; TX-100, Triton X-100; SLO, streptolysin O; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; HA, hemagglutinin; HEK, human embryonic kidney; PBS, phosphate-buffered saline; Tricine, N,N,N'-tris(2-hydroxyethyl)glycine; mAb, monoclonal antibody; PNGase F, peptide N-glycosidase F.

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regulate the expression and the activity of other members of the functional complex is unknown. The membrane topology of PEN-2, a 101-amino-acid-long protein with two potential transmembrane domains, using three independent and complementary approaches. Glycosylation-scanning mutagenesis, which takes advantage of the fact that secretory and membrane glycoproteins are glycosylated only on the luminal side of the ER (25), was employed to biochemically determine the regions of PEN-2 that are inserted into the lumen of the ER. This strategy has been used successfully to determine the topologies of other proteins (26–28). In addition, we used immunofluorescence to demonstrate that the N- and C-terminal domains of epitope-tagged PEN-2 were detectable only when the ER and Golgi membranes were permeabilized and not when the plasma membrane was selectively permeabilized. That the tagged and mutated PEN-2 proteins retained their original structure, and therefore topology, was confirmed by demonstrating that all PEN-2-tagged and mutant proteins co-immunoprecipitated with PS1 as judged by co-immunoprecipitation analysis. To confirm the results from the glycosylation scanning and immunofluorescence approaches, a protease protection assay using selectively permeabilized cells was utilized to demonstrate that not only were the N- and C-terminal domains of PEN-2 luminal but that the hydrophilic domain connecting the two hydrophobic, putative transmembrane domains was cytosolic. Finally, using the glycosylated PEN-2 mutant proteins as tools to study γ-secretase assembly, we found that glycosylation near the N terminus of PEN-2 disrupted the physical association between PEN-2 and PS1 and that PEN-2 likely associates with other γ-secretase complex components in the ER very shortly after its synthesis. Thus, the γ-secretase component PEN-2 spans the membrane two times and is oriented such that its N- and C-terminal domains are inserted into the lumen of the ER.

EXPERIMENTAL PROCEDURES

Plasmids and Site-directed Mutagenesis—APHA-1 and PEN-2 were cloned from a liver cDNA library (Clontech, Palo Alto, CA) using standard PCR methods (Expand high fidelity polymerase; Roche Molecular Biochemicals). Primers were synthesized based on published sequences (forward, 5′-CACCATGAGCACGAGCTGTC-3′; reverse, 5′-GGGGGCCAGGGGT-3′), and the resulting PCR products were cloned into pcDNA3.1D-5′/HisTOPO (Invitrogen) using the manufacturer’s protocol. Construction of the BACE-HA (29), BACE-KK HA (29), BACE-46N reverse, 5′-TCAGGGTGCCAGGGGTTA-3′, and the resulting PCR products were cloned into pcDNA3.1D-5′/HisTOPO (Invitrogen) using the manufacturer’s protocol. Construction of the BACE-HA (29), BACE-KK HA (29), and PS1 (30) plasmids were described previously.

Two methods were used to introduce site-directed nucleotide substitutions. PEN-2 E105S and PEN-2 A46N were created using QuikChange site-directed mutagenesis using mismatched, overlapping PCR primers (E105S forward, 5′-CGAGTGTCCGCAAGAGAACCTAC-3′; reverse, 5′-AGGCCTCTGGGTTGCAAAACAGAAGGCGGAAC-3′; A46N forward, 5′-AGGCCTCTGGGTTGCAAAACAGAAGGCGGAAC-3′; reverse, 5′-TTGCCCTTCTGCTGTAAGTTGGAAGGAAAGCT-3′). The PEN-2 S93N was created by introducing the glycosylation site using standard PCR in which the entire PEN-2 coding sequence is included the sequence for the HA epitope tag as an overhang at the 5′ end (5′-CACCATGAGCACGAGCTGTC-3′) with a reverse primer that added a stop codon after the PEN-2 coding sequence (5′-TCAGGGTGCCAGGGGTTA-3′). The 3′ HA tag was similarly introduced using the forward primer described above and a reverse primer (5′-TCAGGGTGAGTGTGTGGGAC-3′). The resulting PCR products were cloned as above. Plasmid DNA was purified using Qiagen plasmid kits (Qiagen, Valencia, CA). The identity of all plasmids was confirmed by sequencing.

Antibodies—HA-tagged PEN-2 and BACE proteins were labeled with mAb HA11 (Covance, Princeton, NJ). Calnexin was recognized using Calnexin H-70, a rabbit polyclonal antibody to the luminal N terminus (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). PS1 was immunoprecipitated using affinity-purified PSI “M” antibody, a rabbit polyclonal sera raised against the loop domain of PS1. This peptide was expressed as a His-tagged recombinant protein BL 21 E. coli and then injected into rabbits to generate the crude antisera before affinity purification. BACE-KK HA was blotted using “85,” a rabbit polyclonal rabbit reactive against BACE homology domain within the ER lumen.

Cell Culture and Western Blotting—Six-well plates of HEK293T, BHK-21, CHO, HeLa, or QT6 cells were transfected using Gene Porter lipid transfection reagent (Gene Therapy Systems, Inc., San Diego, CA). 24 h after transfection, the cells were lysed in radioimmuno precipitation assay buffer (0.1% SDS, 1% Triton X-100 (TX-100), 5 mM EDTA, 50 mM Tris, pH 8, 150 mM NaCl) with Complete protease inhibitor (Roche Molecular Biochemicals) and then sonicated and cleared by centrifugation (10,000 × g, 10 min, 4°C). Cell lysates from 200,000 cells were deglycosylated using peptide N-glycosidase F (PNGase F) or endoglycosidase H (endo H) (New England Biolabs, Inc.) following the manufacturer’s instructions. Control samples were treated identically with H2O used in place of enzyme. The samples were incubated for 3 h at 37°C for SDS-PAGE. All samples were run on precast 10–20% Tris-HCl Chemicon gels (Bio-Rad Laboratories, Hercules, CA) and transferred to PVDF membranes. The antigens were detected by Western blot with the appropriate antibodies followed by imaging with an ECL® plus Western blotting detection system (Amersham Biosciences) or SuperSignal West Femto maximum sensitivity substrate (Pierce) on a Fujifilm (Stanford, CA) LAS-1000 camera.

Immunofluorescence Microscopy—HeLa cells grown on glass coverslips were transfected with the BACE-HA, the PEN-2 N-terminal HA, the PEN-2 C-terminal HA, or the PEN-2 S93N plasmids using the Gene Porter transfection reagent. Twenty-four h after transfection, the cells were fixed with PBS and permeabilized with PBS. To positively permeabilize the permeabilize of appropriate cells, 300 μl of activated streptolysin O (SLO) was added to the plate at 200 units/ml for 5 min at 37°C preceding fixation and then washed with PBS. The cells were fixed for 5 min in 2% formaldehyde and then rinsed in PBS. Complete permeabilization was obtained by incubating fixed cells with 0.1% Triton-X100 for 10 min at room temperature. All cells were blocked for 10 min in PBS supplemented with 4% fetal calf serum and then incubated with primary antibodies to HA (1:500) and calnexin (1:100) in 4% fetal calf serum for 1 h at room temperature. In the selective permeabilization experiments, HA-tagged PEN-2 and BACE constructs were detected with a mouse monoclonal antibody to HA11 (Covance). The luminal N terminus of expressed calnexin was assayed using calnexin H-70. In the Golgi co-localization studies, the PEN-2 HA constructs were detected with a rabbit polyclonal antibody to HA11 (Abcam, Cambridge, United Kingdom), and GM130 was detected with a mouse monoclonal antibody to GM130 (Transduction Laboratories, Lexington, KY). Cells were then incubated with fluorescence-conjugated secondary antibody (1:500 each) and 4′,6-diamidino-2-phenylindole dihydrochloride (1:1000) in 4% fetal calf serum. Secondary antibodies Alexa Fluor 488 (goat anti-rabbit) and Alexa Fluor 594 (goat anti-mouse) were obtained from Molecular Probes (Eugene, OR). Following a final wash, the coverslips were mounted using Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL) and sealed with nail polish. Fluorescence was examined at 60 × magnification with a Nikon E600 microscope utilizing UV illumination.

Co-immunoprecipitation—HEK293T cells overexpressing PS1 and the appropriate PEN-2 plasmid (or green fluorescent protein) were grown to 80% confluency in 10-cm² plates. Cells were lysed with 0.5 ml of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% CHAPS, 1 mM EDTA, 1 mM EGTA) with Complete protease inhibitor (Roche Molecular Biochemicals) followed by centrifugation at 10,000 × g for 10 min at 4°C. All immunoprecipitation steps were performed at 4°C. After pre-clearing with protein A/G plus agarose (Santa Cruz Biotechnology, Inc.) for 20 min, the cell lysates were incubated with PS1 “M” antibody previously coupled to protein A/G plus agarose (Santa Cruz Biotechnology, Inc.) for 20 min. All immunoblots were eluted at 100°C for 5 min with 20 μl of sample buffer (0.08 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.005% bromophenol blue). Samples were analyzed by immunoblotting as described above.
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Protease Protection—HEK293T cells in six-well plates were transfected with calcium phosphate to overexpress the PEN-2 N-terminal HA, the PEN-2 C-terminal HA, or BACE-KK HA constructs. The next day, the cells were washed three times in PBS and then treated with 300 µl of trypsin-EDTA and incubated for 10 min at 37 °C. Upon resuspension, the cells were then selectively permeabilized by the addition 200 µl of PBS, 200 µl of SLO in PBS (1000 units/ml), or 200 µl of 0.2% TX-100 in PBS and incubated for 5 min at 37 °C. The cells were then treated with proteinase K (100 µg/ml) and incubated for 30 min at 37 °C after which time proteinase K was inhibited by the addition of phenylmethylsulfonyl fluoride (1.0 mM). The cells were lysed by the addition of 120 µl of 5% radiolabeled precipitation assay buffer with Complete protease inhibitor (Roche Molecular Biochemicals) and then sonicated and cleared by centrifugation (10,000 × g, 10 min, 4 °C). PEN-2 samples were analyzed on 16.5% Tris-Tricine Chemicon gels (Bio-Rad) while BACE samples were run on 4–15% Tris-HCl gels (Bio-Rad). Immunoblotting was carried out as described above using anti-HA for PEN-2 and “85” for BACE.

RESULTS

Glycosylation Scanning Mutagenesis—PEN-2 associates with PS1 and Nct, both of which are integral membrane proteins. It is effectively solubilized from cells with nonionic detergents, and it possesses two domains of the appropriate length and hydrophobicity to constitute transmembrane domain regions. However, it is also possible that only one of these putative domains actually spans the membrane. Therefore, there are four possible topological models for PEN-2 (Fig. 1A).

To address the membrane topology of PEN-2 we took advantage of the absolute asymmetry of the N-linked glycosylation machinery, which adds core high mannose structures only on protein domains within the lumen of the ER. Because PEN-2 lacks N-linked glycosylation sites, we introduced single amino acid substitutions in each of the three hydrophilic domains of PEN-2 to generate Asn-X-(Ser/Thr) sequons (Fig. 1B). The three mutants were expressed using either N-terminal or C-terminal HA epitope tags, resulting in a total of six constructs. Each of the four possible membrane topologies of PEN-2 would result in a unique glycosylation pattern if the inserted sequons in the luminal domains are utilized. Therefore, by examining the utilization of sequons in these PEN-2 mutant proteins, it would be possible to deduce its membrane topology.

Each construct was expressed in HEK293T cells and analyzed by SDS-PAGE and Western blot with a monoclonal antibody to the HA epitope tag. The E10S and S93N PEN-2 mutants were glycosylated with variable efficiency as demonstrated by the slower migration of the glycosylated mutant proteins in comparison to the wild type PEN-2 (Fig. 2). These constructs were also glycosylated when they were transiently expressed in HeLa, BHK-21, Chinese hamster ovary, and QT6 cells (data not shown). The A46N mutant was not glycosylated. The results were similar whether the epitope tag was at the N (Fig. 2A) or C (Fig. 2B) terminus, though the E10S mutant was glycosylated more efficiently in the context of the N-terminal epitope tag. These results make it unlikely that the epitope tags disrupt PEN-2 membrane topology.

To confirm that the change in mobility was caused by N-linked glycosylation, cell lysates expressing PEN-2 or the E10S, A46N, or S93N mutants were treated with PNGase F, which removes N-linked oligosaccharides. After digestion, the E10S and S93N mutant proteins quantitatively migrated with wild type PEN-2 indicating the shift in migration was in fact because of glycosylation. Because N-linked oligosaccharides are added only on protein domains within the ER, these results provide clear biochemical evidence that the N-terminal and C-terminal domains of PEN-2 are inserted within the ER lumen (Fig. 3).

Immunofluorescence with Selective Membrane Permeabilization—The biochemical data provided substantial evidence supporting a PEN-2 topology in which both the N- and C-terminal domains are inserted in the ER lumen. However it is possible, though unlikely, that the single amino acid substitutions introduced to generate N-linked glycosylation sites disrupted the normal membrane topology of wild type PEN-2. Therefore, it was necessary to confirm the proposed membrane topology of PEN-2 using an independent approach. To do so, we expressed both N- and C-terminally tagged PEN-2 in HeLa cells and performed immunofluorescence microscopy using a variety of membrane-permeabilization conditions. If the N- and C-terminal domains of PEN-2 are within the ER lumen, then N- and C-terminally tagged PEN-2 proteins should only be detected if both the cell membrane and the ER/Golgi membranes are permeabilized using TX-100. In contrast, if the plasma membrane was selectively permeabilized using SLO, leaving the ER/Golgi membranes intact, the epitope-tagged proteins would not be detected by immunofluorescence microscopy.

To clearly demonstrate that the examined cells were appropriately permeabilized, several controls were used. We used an antibody to the luminal domain of calnexin, a molecular chaperone present at high levels in the ER (31), to control for the integrity of the ER membrane. We also used an antibody directed against an epitope tag in the cytoplasmic domain of BACE-HA, a type I transmembrane protein. Therefore, selective permeabilization of the plasma membrane should result in the recognition of BACE by exogenously added antibody,
whereas permeabilization of all cellular membranes would result in the detection of both BACE and calnexin.

HeLa cells were transfected with BACE-HA, N-terminally tagged PEN-2, or C-terminally tagged PEN-2 and processed for immunofluorescence microscopy. HeLa cells were treated with SLO to selectively permeabilize the plasma membrane only or were treated with TX-100 to permeabilize all cell membranes. In SLO-permeabilized HeLa cells, only BACE could be detected; staining of calnexin or PEN-2 was not observed (Fig. 4). However, N- and C-terminally tagged versions of PEN-2, as well as calnexin, were readily detected following permeabilization with TX-100. Thus, both N- and C-terminally tagged PEN-2 were detected only when the ER/Golgi membrane and the plasma membrane were permeabilized and not when the plasma membrane was permeabilized alone (Fig. 4). These results are consistent with the biochemical studies and demonstrate that PEN-2 has a membrane topology in which the N- and C-terminal domains are inserted into the ER lumen.

**Protease Protection**—The above results, using two independent methods, demonstrated that the N- and C-terminal domains of PEN-2 were lumenal. However, the possibility remained that PEN-2 was entirely lumenal and attached to the membrane by some means other than the putative transmembrane domains. For this reason, we used a protease protection assay to determine whether that the hydrophobic domain connecting the two putative transmembrane domains could be proteolytically cleaved by proteinase K in SLO-treated cells, whereas the luminal domains would be protected. We reasoned that if the loop domain was indeed cytosolic, an N-terminal and C-terminal fragment would be released. These fragments could be visualized by overexpressing either the N-terminal or C-terminally epitope-tagged PEN-2 proteins. Treatment of intact cells with proteinase K should have no effect on PEN-2 holoprotein whereas full permeabilization of cells with TX-100 should result in complete degradation of PEN-2.

To demonstrate that the proteinase K had appropriately localized activity in this assay, we used BACE-KK HA as a control. This type I transmembrane protein contains a dilysine ER retention motif in the C terminus and is therefore found predominantly in the ER (29). Because the orientation and localization of this protein is known, we could predict that protease treatment under selective permeabilization would result in a slight increase of mobility on SDS-PAGE because of degradation of the 32-amino acid cytosolic C terminus of epitope-tagged BACE. As expected, we found that proteinase K digestion of SLO-permeabilized cells resulted in a slight increase in the mobility of BACE-KK HA, whereas proteinase K digestion of TX-100-treated cells degraded BACE-KK HA more completely (Fig. 5A). This demonstrated that the protease protection assay with selective permeabilization was an appropriate system with which we could analyze PEN-2.

HEK293T cells overexpressing C-terminally tagged PEN-2 subjected to the protease protection assay revealed a shift of ~5 kDa in the migration of PEN-2 when cells selectively permeabilized with SLO were digested with proteinase K (Fig. 5B). The tagged holoprotein contains 110 amino acids and consistently migrates at ~14 kDa. The C-terminal fragment would contain between 50 and 71 amino acids depending on the cleavage site or sites of proteinase K. Thus, the observed shift in migration of ~5 kDa is consistent with cleavage of the holoprotein in the loop domain. As expected, proteinase K digestion of intact cells did not alter PEN-2 migration, whereas treatment of TX-100-permeabilized cells with proteinase K re-
resulted in the complete digestion of PEN-2. In addition, because the amount of protein detected in untreated cells was similar to that in intact, proteinase K-treated cells, this experiment provides additional evidence that overexpressed PEN-2 is not found in significant levels on the cell surface. Similar results were obtained when N-terminally epitope-tagged PEN-2 was used (data not shown). Together, these results support the model of PEN-2 as a protein with two transmembrane domains. The N- and C-terminal domains are luminal, and the loop domain is cytosolic.

**Membrane Topology of PEN-2 Mutants Is Intact**—Having shown, using three independent methods, a luminal orientation for the N- and C-terminal domains of PEN-2, we sought to determine whether the alterations we introduced had perturbed the structure or membrane topology of PEN-2. We reasoned that if PEN-2 is able to associate with any of its γ-secretase partners, then it can be inferred that the structural integrity and membrane topology of the tagged protein must be undisturbed. To examine this, we co-transfected HEK293T cells with PS1 and with each of the PEN-2 constructs. Subsequently, the cells were lysed and subjected to immunoprecipitation with an antisera to PS1. The immunoprecipitates were analyzed by SDS-PAGE and Western blot with an antibody to the HA epitope tag present in the PEN-2 constructs. We found that in each case the overexpressed PEN-2 protein co-immunoprecipitated with PS1 indicating that the tags and single amino acid substitutions introduced did not disrupt membrane topology of PEN-2 (Fig. 6A). Notably, PEN-2 S93N, which is efficiently glycosylated, was co-immunoprecipitated with PS1. This indicates that neither the S93N amino acid change nor the carbohydrate structure disrupted the interaction of PEN-2 with PS1. However, whereas the non-glycosylated E10S species was readily co-immunoprecipitated with PS1, the glycosylated species of E10S was undetectable, even after prolonged exposure of the blot (Fig. 6B). This finding indicated that whereas the E10S mutation itself did not disrupt association of PEN-2 with PS1, the addition of a carbohydrate structure in the N-terminal domain did, suggesting that the N-terminal domain of PEN-2 is important for interactions with PS1. Whether this disruption resulted from a change in how PEN-2 folds when it is glycosylated at this position, or because the carbohydrate structure directly interfered with the physical interaction between PEN-2 and PS1, remains to be determined. Taken together, we conclude that both PEN-2 E10S and PEN-2 S93N exhibited a membrane topology that was unperturbed by the amino acid substitution. This result, when considered together with the fact that both sites are glycosylated, adds support to our model that the N- and C termini reside in the ER lumen.

**PEN-2 Glycoforms Are Endoglycosidase H-sensitive**—Because PEN-2 S93N associated with PS1, and because modification of its N-linked oligosaccharide to a complex, endo H-resistant form would serve as a convenient marker to monitor transport of the γ-secretase complex to at least the medial-Golgi, we subjected lysates of cells expressing PEN-2 S93N to digestion with endo H. PEN-2 S93N was sensitive to endo H when expressed in QT6 cells (Fig. 6), as well as in HEK293T, BHK-21 and Chinese hamster ovary cells (data not shown). This was true whether PEN-2 S93N was expressed by itself or co-expressed with PS1, Nct, and APH-1 (Fig. 7). Interestingly, when PEN-2 S93N was overexpressed with the other known γ-secretase components, a portion of the PEN-2 S93N protein failed to be glycosylated. This was observed in both QT6 cells (Fig. 7) and HEK293T cells (data not shown) and occurred only if all known γ-secretase components were expressed and not when PEN S93N was transfected alone. This suggests that in the presence of the complete γ-secretase complex PEN-2 S93N rapidly associates with another member, or members, of the complex, thus disrupting accessibility of the glycosylation machinery to the introduced sequon. Because N-linked glycosylation is a co-translational process, the association of PEN-2 to the γ-secretase component or components is likely to be rapid.

The failure of the S93N mutant to acquire resistance to endo H could mean that the glycosylated protein is not transported to the medial-Golgi or that it is transported, but the carbohydrate structure is not modified by Golgi-associated enzymes. To distinguish between these possibilities, we transiently expressed N- and C-terminally tagged PEN-2 S93N in HeLa cells and processed them for immunofluorescence microscopy. Double labeling of completely permeabilized cells demonstrated...
that both wild type PEN-2 and PEN-2 S93N co-localized with GM130 (Fig. 8), a cis-Golgi matrix protein (32). This indicates that the PEN-2 S93N mutant was transported to the Golgi complex. It is therefore reasonable to conclude that PEN-2 S93N failed to become endo H-resistant, because the carbohydrate was not modified by Golgi-associated enzymes and was not due to a failure to leave the ER.

DISCUSSION

Both PEN-2 and APH-1 were identified as members of the γ-secretase complex using mutagenesis genetic screens in C. elegans (15, 16), and a number of recent studies have confirmed these results in both Drosophila and mammalian systems (1, 2, 4). Together, PEN-2, APH-1, Nct, and PS may comprise the entirety of the γ-secretase complex as their combined molecular weight approximates at least some estimates of the mass of the purified γ-secretase activity (3, 5, 33, 34), and saturating mutagenesis in C. elegans has failed to identify other putative components of this complex (15). With three additional proteins now known to associate with PS, it will be important to address the roles that each component plays in transport of the complex and in regulating its activity, both with regards to substrate specificity and access, as well as in the precise site of enzymatic cleavage of substrates like APP, which can be cleaved by γ-secretase at several positions.

Whereas APH-1 is a multi-pass transmembrane protein, PEN-2 is predicted to traverse the membrane two times (15). Both proteins are essential for Notch-mediated activities in C. elegans, and both are required for γ-secretase activity (1, 2, 4, 15). That the four identified γ-secretase components physically associate is clear. Antibodies to PS1 or PS2 co-immunoprecipitate PEN-2, Nct, and APH-1 (2, 4). Similarly, antibody to Nct co-immunoprecipitates PS1, PS2, PEN-2, and APH-1 (2, 4), whereas antisera to APH-1 co-immunoprecipitates the PSI terminal fragments, PSI holoprotein, and Nct (1). In addition to physically associating with each other, changing the expression of any single component may impact the expression of the others. For example, Steiner et al. (4) found that ablation of PSI expression caused a strong reduction in PEN-2 levels. Likewise, when Nct expression was down-regulated by small interfering RNA treatment, PEN-2 levels were also reduced (4). When PEN-2 expression was reduced by small interfering RNA treatment, levels of both PSI terminal fragment and mature Nct were reduced (4). In contrast, APH-1 levels are not greatly affected in cells lacking PS1 and PS2 (1). Thus, expression of at least these three components of the γ-secretase complex is coordinately regulated, helping to explain why overexpression of any single component typically fails to enhance γ-secretase activity. Our results with the S93N mutant, which is glycosylated less efficiently when the other known γ-secretase components are overexpressed, suggests that PEN-2 enters the γ-secretase complex rapidly after or during its synthesis.

Our results show that the membrane topology of PEN-2 is such that both N- and C termini mediate interactions with the luminal components of the other members of the γ-secretase complex, including the large ectodomain of Nct and the extracellular loops of both PS and APH-1. Our results with the E10S mutant are consistent with this hypothesis. Whereas E10S could be co-immunoprecipitated with antibodies to PS1, the glycosylated fraction of E10S could not, suggesting that the presence of a large, hydrophilic carbohydrate structure in the N-terminal domain of PEN-2 precludes or at least destabilizes interactions with PS1. In contrast, the presence of an identical carbohydrate structure in the C-terminal domain of PEN-2 had no apparent effect on PS1 interactions. Whether these domains interact with other components of the γ-secretase complex remains to be determined, but the results here will help guide mutagenesis efforts designed to uncover regions and specific residues that are important for these noncovalent interactions.

The presence of one or more N-linked carbohydrate structures in a protein affords a way to indirectly monitor the efficiency and rate of transport through the secretory pathway by following carbohydrate processing events that occur at specific sites within the ER and Golgi complex. Thus, the fact that addition of an N-linked carbohydrate structure in the C-terminal domain of PEN-2 did not obviously affect its association with PS1 makes this construct a potentially useful tool for monitoring not just the transport of PEN-2 but of the γ-secretase complex, as well. However, PEN-2 S93N did not acquire endo H resistance, even when co-expressed with all other known members of the γ-secretase complex, suggesting that it is either not transported or transported but not processed. We favor the latter alternative, because PEN-2 S93N co-localized in part with GM130 (Fig. 7), a cis-Golgi matrix protein (32). This indicates that the PEN-2 S93N mutants is transported to the Golgi complex but fails to become endo H-resistant, because the carbohydrate structure is not modified by Golgi-associated enzymes. In this regard, PEN-2 S93N is similar to certain other glycoproteins, including Nct, which contain one or more N-linked carbohydrate chains that are not processed by Golgi-associated enzymes even though they transit this organelle (24, 35). It may be of interest to introduce carbohydrate addition sites in other regions of the PEN-2 C-terminal domain in the hopes of obtaining a glycosylated version of PEN-2 that is fully processed and so can be used as a marker for intracellular transport events.

Identifying the components of the γ-secretase complex is an important step in ultimately understanding its structure, function, and regulatory components. Obvious questions to address include the roles complex members play in PS cleavage and activity, regulation of transport and activity, and whether they play a role in substrate presentation. Differences in how these components interact with each other in different cellular compartments could affect the way in which APP is cleaved, impacting the amount and type of amyloid β peptide that is produced. With the topology of PEN-2 now known, structure-function studies, as well as attempts to reconstitute the γ-secretase complex, can benefit from this information.

![Fig. 8. PEN-2 S93N is transported to the Golgi apparatus. HeLa cells transfected with C-terminally tagged PEN-2 (A) or N-terminally tagged PEN-2 S93N (B) were immunolabeled with anti-HA rabbit polyclonal antibody and anti-GM130 mouse monoclonal antibody and detected with fluorescently conjugated secondary antibodies. The columns, from left to right show the following: green (PEN-2), red (GM130), Merge (green (PEN-2), red (GM130), blue (DNA)). Both PEN-2 proteins partially co-localized with the Golgi marker GM130](http://www.jbc.org/Downloaded from http://www.jbc.org/ by guest on July 24, 2018)
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