A Nine-transmembrane Domain Topology for Presenilin 1*

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Presenilin (PS) provides the catalytic core of the β-secretase complex. β-Secretase activity leads to generation of the amyloid β-peptide, a key event implicated in the pathogenesis of Alzheimer disease. PS has ten hydrophobic regions, which can all theoretically form membrane-spanning domains. Various topology models have been proposed, and the prevalent view holds that PS has an eight-transmembrane (TM) domain organization; however, the precise topology has not been unequivocally determined. Previous topological studies are based on non-functional truncated variants of PS proteins fused to reporter domains, or immunocytotoxic staining. In this study, we used a more subtle N-linked glycosylation scanning approach, which allowed us to assess the topology of functional PS1 molecules. Glycosylation acceptor sequences were introduced into full-length human PS1, and the results showed that the first hydrophilic loop is oriented toward the lumen of the endoplasmic reticulum, whereas the N terminus and large hydrophilic loop are in the cytosol. Although this is in accordance with most current models, our data unexpectedly revealed that the C terminus localized to the luminal side of the endoplasmic reticulum. Additional studies on the glycosylation pattern after TM domain deletions, combined with computer-based TM protein topology predictions and biotinylation assays of different PS1 mutants, led us to conclude that PS1 has nine TM domains and that the C terminus locates to the lumen/extracellular space.

Most proteases operate in aqueous environments, but β-secretase, along with other intramembrane cleaving proteases, is unusual in the sense that it executes proteolysis in the lipid bilayer (1). β-Secretase is a high molecular weight protein complex composed of presenilin (PS)2 1 or 2, nicastrin, Aph-1, and Pen-2 (2–5). Growing evidence suggests that the polytopic PS1 protein is a TM aspartyl protease and that two of its active-site residues, Asp-257 in hydrophobic domain (HD) 6 and Asp-385 in HD 8, form the catalytic site of the β-secretase complex (6–8). An endoproteolytic cleavage within HD 7 of PS1 generates an N-terminal fragment (NTF) and a C-terminal fragment (CTF) (9). The NTF/CTF heterodimer is believed to be the biologically active form of PS. Nicastrin, Aph-1, and Pen-2 are all essential cofactors in the β-secretase complex, but their precise biochemical functions are not fully known. Nicastrin and Aph-1 have been proposed to form a subcomplex that can stabilize full-length PS, and the final addition of Pen-2 has been suggested to trigger PS endoproteolysis (10) and stabilize the NTF/CTF heterodimer (11). β-Secretase cleavage occurs within the lipid bilayer of the membrane through a mechanism referred to as “regulated intramembrane proteolysis” (12). Recently, not only APP, but a large number of other type-1 TM proteins, such as the Notch receptors, CD44 and E-cadherin, have been shown to undergo PS-dependent regulated intramembrane proteolysis (reviewed in Ref. 13). Given that target proteins of β-secretase are implicated in important processes like embryonic development and neurodegeneration, a molecular understanding of the β-secretase complex is warranted.

PS1 is a TM protein of ~50 kDa with 10 HDs that could all theoretically span the membrane. However, the proposed number of HDs that traverses the membrane varies between different studies (reviewed in Ref. 14) and is still under debate. The currently favored model suggests an eight-TM domain topology, positioning the N and C termini, as well as the large hydrophilic loop in the cytosol (15–17). Because PS is proposed to carry the catalytic site of the β-secretase complex, clarifying the topology of the protein is a crucial step in understanding the molecular mechanism of β-secretase activity: assessment of PS conformation and thereby the orientation of the catalytic Asp-257 and Asp-385 residues is critical for explaining how the protein functions as an enzyme.

Here we analyzed the topology of PS1 by introducing glycosylation acceptor sequences into human PS1. In agreement with the majority of previous studies on PS topology, we found that the first hydrophilic loop had a luminal orientation whereas the N terminus and large hydrophilic loop were in the cytosol. In contrast, our data suggested that the C terminus was located in the lumen of the endoplasmic reticulum (ER). These findings, corroborated by computer-based TM topology predictions and biotinylation assays of different PS1 mutants led us to propose a novel nine-TM domain model for PS1.

MATERIALS AND METHODS

Expression Constructs—The reporter constructs that were used in the luciferase-based reporter system to monitor β-secretase activity have previously been described (18, 19). The PS1 CTF construct was in pcDNA3.1Zeo(-) (Invitrogen), and the Notch ΔE expression construct has been described previously (20). Mutations were introduced into the PS1 encoding sequence by site-directed mutagenesis according to the QuikChange protocol ( Stratagene) and verified by sequencing. Mutagenesis to generate the constructs PS1_N, PS1_HL1, PS1_large HL, PS1_HD10, PS1_C, PS1_optC, PS1_CAHD8, PS1_CAHD8–9, PS1_K395E, and PS1_K395E,Q464K were performed on wild type PS1 in the pcDNA3 vector (Invitrogen). The oligonucleotide pairs used

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2 The abbreviations used are: PS, presenilin; ER, endoplasmic reticulum; APP, amyloid β precursor protein; TM, transmembrane; NTF, N-terminal fragment; CTF, C-terminal fragment; HD, hydrophobic domain.
were: N:forward, 5′-CGACCCCAAGTTAACTCCAGCAGGTGTTGGAGCAAGATG-3′; N:reverse, 5′-CATCTGGCCTCCCAACCCCTTGAGTTGTTGACAGGAGCTCTGGATCTTCTGTCGATCTGCTTTGTTGAGGATAATTTAAAC-3′; opt:forward, 5′-C-GGAGAGGTCACTATGATATAAAATTGAGGTAAGAGGCTGTTTTCTACAATTCCACAGATTATCTTGTACAGCC-3′; opt:reverse, 5′-CCATGGGATTCTAACCCTAAGTACTGTTGATATAAAATTGAGGTAAGAGGCTGTTTTCTACAATTCCACAGATTATCTTGTACAGCC-3′; K395E:forward, 5′-CTACAGTGTTCGGTGTTGAGCAGCACAGGCTGTTTTGTTGAGGATAATTTAAAC-3′; K395E:reverse, 5′-CCATGCCTGGTTCGGAGGCTGTTTTGTTGAGGATAATTTAAAC-3′; Q464K:forward, 5′-GCCTGAGTTCGGTGTTGAGGCTGTTTTGTTGAGGATAATTTAAAC-3′; Q464K:reverse, 5′-CCGAAAA-TATGCTGATATAAATATGTTGAGGTAAGAGGCTGTTTTCTACAATTCCACAGATTATCTTGTACAGCC-3′.

The PS1_K395E and PS1_K395E,Q464K mutations were also introduced in the previously described pENTR2B-PS1 (21). pCAG-PS1_K395E and pCAG-PS1_K395E,Q464K were generated by Gateway cloning technology (Invitrogen).

**Cell Culture and Transfection**—Cells derived from PS1 mice (21) or PS1_K395E and PS1_K395E,Q464K mice were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2.4 mM l-glutamine, 0.1 mM non-essential amino acids. The medium was used at a 1:2 dilution ratio and supplemented with puromycin (1 μg/ml) for 1 week. Single puromycin-resistant colonies were expanded and analyzed for PS expression.

**Endoglycosidase H Treatment**—Cells transiently transfected in 6-well tissue culture plates were harvested 24 h post-transfection and resuspended in 0.4 ml of 10 mM HEPES (pH 7.4) containing 10 mM KCl, 5 mM EDTA, 5 mM EGTA, 250 mM sucrose, 100 mM NaCl, 1.5 mM MgCl2, and protease inhibitor mixture (Roche Applied Science). Cells were passed through a 20-gauge needle 20 times and centrifuged at 800 × g for 5 min. Postnuclear supernatants were collected and centrifuged at 25,000 × g for 15 min. Pellets were resuspended in a buffer composed of 5 mM HEPES (pH 7.4), 125 mM sucrose, 100 mM sodium citrate, 100 mM NaCl, 5 mM KCl, 2.5 mM EDTA, and 2.5 mM EGTA, 0.75 mM MgCl2, 0.3% SDS, 0.6% β-mercaptoethanol, and protease inhibitors mixture (Roche Applied Science). Endoglycosidase H (Roche Applied Science) was added when indicated (1 μl per 25-μl sample), and deglycosylations were performed in 37 °C for 2 h.

**Luciferase-based Reporter Assays**—Transfections for the luciferase-based reporter assays were carried out in 24-well tissue culture plates essentially as described (23). Where indicated, the γ-secretase inhibitor DAPT was added (1 μM) to the transfected cells. Cells were lysed in 100 μl of lysis buffer per well (10 mM Tris, pH 8, 1 mM EDTA, 150 mM NaCl, and 0.65% Nonidet P-40), and luciferase activity was monitored luminometrically after addition of luciferin (BioThema) and ATP (BioThema). The β-galactosidase activities of the cell lysates were determined to equalize for differences in transfection efficiencies. Arbitrary β-galactosidase normalized luminescence units are presented as percent activity compared with wild type PS1 response.

**Co-immunoprecipitations**—Transfections were carried out in a 6-well format on cells cultured to near confluence. Co-immunoprecipitations were performed on lysates 48 h post transfection. Cells were lysed in 0.5 ml co-immunoprecipitation buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, Pro tease Inhibitor Mixture (Roche Applied Science) and 1% CHAPS) and briefly sonicated. Lysates were subjected to ultracentrifugation (100,000 × g, 20 min). The CHAPSO-soluble supernatant was pre-cleared with a mixture of protein A- and G-Sepharose (Amersham Biosciences). The pre-cleared supernatants were incubated with primary antibody (dilution 1:300) overnight, and proteins were pulled down with a mixture of protein A- and G-Sepharose. Pre-immune serum was used as negative control.

**Biotinylation and Streptavidin Precipitation of Cell Surface Proteins**—Cultures of BD8 cells and BD8 cells stably expressing PS1, PS1_K395E, or PS1_K395E,Q464K were biotinylated as described previously (24). Cells were lysed in whole cell extract buffer (20 mM HEPES, pH 7.8, 0.42 M NaCl, 0.5% Nonidet P-40, 25% glycerol, 0.2 mM EDTA, 1.5 mM MgCl2, 1 mM dithiothreitol) supplemented with protease inhibitors mixture (Roche Applied Science). To break protein-protein interactions, SDS was added to a final concentration of 2%, and the samples were heated at 55 °C for 5 min. Radioimmune precipitation assay buffer was subsequently added to dilute the SDS concentration to 0.4%, and biotinylated proteins were precipitated using streptavidin–agarose beads (Pierce).

**Immunoblotting**—Immunoprecipitated proteins and proteins from cell lysates were resolved on 10–20% Tricine gels (Invitrogen), transferred to nitrocellulose membranes, and detected by appropriate antibodies. The immunoblots were developed using horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) and ECL substrate (Pierce).

**Antibodies**—The antibodies used for co-immunoprecipitations of PS1 were polyclonal antibody Ab14, raised against the N terminus of PS1 (25), and monoclonal antibody MAB5232 (Chemicon) detecting PS1 CTF. For immunoblotting of PS1, monoclonal antibody NT1 directed toward the N terminus (26), and MAB5232 were used. Nicastrin was immunoprecipitated and detected using polyclonal antibody N1660 (Sigma). Polyclonal antibody UDI was used for immunoblotting of Pen-2 (27). Monoclonal anti-β-actin antibody (Sigma) was used for immunoblotting.

**RESULTS**

**Glycosylation Acceptor Site Assay Defines a Luminal Localization for the C Terminus of PS1**—To determine the topology of PS1, glycosylation acceptor site assay (Asn-Xaa-Ser/Thr) was used. Where Xaa can be any amino acid except proline) were introduced into PS1 in different positions as shown in Fig. 1A. The glycosylation acceptor site assay for topology studies is based on the Asn residue of the sequence Asn-Xaa-Ser/Thr being glycosylated in the luminal compartment of ER, by transfer of high man-
FIGURE 1. A, schematic depiction of wild type PS1 and PS1 mutants generated for assessment of protein topology using a glycosylation acceptor site assay. Numbered boxes represent hydrophobic domains (HDs), and the letters Y indicate incorporated glycosylation acceptor sequences (Asn-Xaa-Ser/Thr). B, immunoblots of lysates from transfected BD8 cells using an antibody against the N terminus of PS1 revealed shifts in migration (open circles) of PS1 full-length (FL) (lanes 5 and 11) and PS1 NTF (lane 5). Treatment with endoglycosidase H (EndoH) resulted in a species that migrated with wild type PS1 (lanes 6 and 12). C, studies on the ability of the mutants to restore γ-secretase activity in PS-deficient cells using a luciferase-based reporter system for intramembrane cleavage of C99 (upper panel). Reconstitution of γ-secretase activity is presented as percent activity compared with wild type PS1. Error bars represent the standard error of the mean. Expression of the different PS1 variants was confirmed by immunoblotting using an antibody toward PS1 NTF (middle panel). Open circles indicate glycosylated PS1 molecules. γ-Secretase activity was also assessed by examining production of Notch ICD from Notch ΔE (lower panel). Notch ICD produced by PS1_HL1 appeared after a longer exposure. D, intramembrane cleavage of C99 in cells transfected with PS1_HL1 was inhibited by the γ-secretase inhibitor DAPT. Reconstitution of γ-secretase activity is presented as percent activity compared with wild type PS1. Error bars represent the standard error of the mean. E, studies of γ-secretase complex assembly for PS1 mutants illustrated in A. Co-immunoprecipitations were performed using an antibody toward nicastrin. Lysates (upper panel) and co-precipitations (lower panel) were analyzed by immunoblotting using antibodies toward PS1 NTF and CTF. Open circles show glycosylated PS1 mutants. Asterisks indicate unspecific bands.
nose oligosaccharides in an enzymatic reaction catalyzed by oligosaccharyltransferase (28). Hence, glycosylation acceptor sites in TM proteins become glycosylated if they face the lumen but not if they are located in the membrane or the cytosol.

To avoid interference from endogenous PS, we introduced the different constructs into BD8 cells, in which the PS1 and PS2 genes have been ablated (22). Glycosylation patterns for the transiently expressed constructs were assessed by immunoblotting (Fig. 1B). The introduction of a glycosylation acceptor site in the first hydrophilic loop situated between HD 1 and HD 2 (construct PS1_HL1) resulted in a shift in migration for both the full-length protein and the NTF (Fig. 1B, lane 5). Endoglycosidase H treatment reduced the size of the protein, confirming that the shift was caused by glycosylation (Fig. 1B, lane 6), and indicating that the first loop had a luminal/extracellular orientation. In contrast, the constructs with a glycosylation acceptor site in the N-terminal tail of PS, preceding HD1 (construct PS1_N), or in the large hydrophilic loop between HD 7 and HD 8 (PS1_large HL) were not glycosylated in the ER lumen (Fig. 1B, lanes 3–4 and 7–8, respectively). This indicates that the N terminus and the large hydrophilic loop face the cytosol, which is in agreement with most current topological models (15–17, 29–31). Similarly, no glycosylation was detected when a glycosylation acceptor site was introduced into HD 10 of PS1, which is generally believed to be in the cytosol (construct PS1_HD10, Fig. 1B, lanes 9 and 10). Interestingly, the construct with the glycosylation acceptor site in the absolute C terminus (PS1_C) was partially glycosylated, indicating that the C-terminal was located in the lumen (Fig. 1B, lane 11). Deglycosylation by treatment with endoglycosidase H resulted in disappearance of the upper band, confirming that the shift was caused by a glycosylation (Fig. 1B, lane 12). Taken together, the glycosylation patterns for PS1_HD10 and PS1_C suggest that HD 10 spans the membrane and positions the C terminus in the lumen, at least in a subset of PS1 molecules.

Glycosylation Acceptor Site Mutants Form Functional γ-Secretase Complexes—Compared with the use of other reporter domains, glycosylation acceptor site constructs represent a less invasive topology tracing technique, but we wanted to assure that the introduction of the glycosylation acceptor sites did not affect the functionality of the engineered PS1 molecules. We therefore analyzed the mutants for γ-secretase activity using a previously described luciferase-based γ-secretase reporter assay (18, 19). Intramembrane cleavage of C99-GVP, a protein mimicking β-secretase-processed APP with an intracellular Gal4/VP16 domain (18), was monitored by UAS-luciferase activation in BD8 cells transiently transfected with the different glycosylation acceptor site mutants. The mutants were all functional in this assay, although PS1_HL1 and to some extent also PS1_HD10 and PS1_C had reduced γ-secretase activity compared with wild type PS1 (Fig. 1C, upper panel). Expression of the different PS1 mutants was verified by immunoblotting using an antibody directed toward PS1 NTF (Fig. 1C, middle panel). Interestingly, the full-length form of PS1_HD10 appeared to have reduced stability. However, the amount and stability of NTF generated from PS1_HD10 seemed to be unaffected, which indicated that the mutant had a native conformation.

To corroborate the results from the luciferase-based APP reporter assay, we examined the ability of the PS glycosylation acceptor site constructs to mediate γ-secretase cleavage of another target protein: the Notch receptor. We transiently transfected BD8 cells with Notch ΔE, a membrane-tethered, N-terminally truncated Notch 1 receptor that is constitutively cleaved by γ-secretase (20), together with the different PS constructs. Western blotting revealed that all PS constructs were able to generate a fragment corresponding to the Notch intracellular domain (Notch ICD) through γ-secretase-mediated cleavage of Notch ΔE (Fig. 1C, lower panels). However, the PS1_HL1 construct exhibited considerably lower activity, such that Notch ICD could only be detected after a long exposure of the film (Fig. 1C). To confirm that the results obtained with PS1_HL1 in the luciferase assay was due to γ-secretase activity, we treated transfected cells with the specific γ-secretase inhibitor DAPT (Fig. 1D). DAPT blocked activation of the reporter system, lending support to the notion that PS1_HL1 can form functional γ-secretase complexes.

To verify that the introduction of the glycosylation sites did not interfere with correct complex assembly, co-immunoprecipitations using an antibody toward nicastrin were performed under conditions shown to keep the complex intact (32) (Fig. 1E). All glycosylation acceptor site mutants were co-immunoprecipitated with nicastrin, as shown by immunoblotting with antibodies toward PS1 NTF and CTF (Fig. 1E). Importantly, PS1_HL1, PS1_HD10, and PS1_C, which had reduced γ-secretase activity co-precipitated with nicastrin (Fig. 1E, lanes 4, 6, and 7). Moreover, an antibody directed toward PS1 CTF co-precipitated PS1 NTF, and an antibody specific for the N terminus of PS1 co-precipitated Pen-2 for all constructs (data not shown). Taken together, this suggests that the constructs adopted a native conformation. However, the CTF of PS1_C that was immunoprecipitated with nicastrin was apparently not glycosylated (Fig. 1E, lane 7). In fact, although the full-length PS1_C construct was glycosylated we could not detect any glycosylated CTF generated from PS1_C in either cell lysates or upon immunoprecipitation (Fig. 1E, lane 7).

To be efficiently glycosylated a glycosylation acceptor site must not be located too close to the protein’s C terminus (33). Thus, the most parsimonious explanation for the partial glycosylation of PS1_C is a suboptimal position of the glycosylation acceptor site. An alternative explanation for the appearance of a double band could be the presence of a mixture of PS1 molecules, with different topological orientations. To resolve this issue, we optimized the C-terminal glycosylation acceptor site to achieve a more complete glycosylation of PS1, and to permit detection of glycosylated species of CTF. The best result was obtained when the glycosylation acceptor site was inserted between the glutamic acid 442 and the phenylalanine in position 465 (construct PS1_optC, Fig. 2A). Upon expression in PS-deficient cells, PS1_optC appeared to be fully glycosylated, and glycosylation of both full-length PS1 and PS1 CTF was confirmed by endoglycosidase H treatment (Fig. 2B, lanes 3 and 4). The functionality of the mutant was analyzed with the γ-secretase reporter assay as well as by analyzing the conversion of Notch ΔE to Notch ICD (Fig. 2C). PS1_optC restored γ-secretase activity to almost the same extent as wild type PS1. Importantly, Western blot analysis showed that all CTF derived by endoproteolysis of PS1_optC was glycosylated, implying that PS molecules with a luminal orientation of the C-terminal tail can form γ-secretase complexes. To further analyze the functionality of CTFs with a luminal C-terminal tail, we performed co-immunoprecipitations under γ-secretase-preserving conditions. We showed that glycosylated CTF generated from PS1_optC interacted with nicastrin in transiently transfected cells (Fig. 2D). In all cases, no non-glycosylated CTF derived from PS1_opt was seen (Fig. 2, C and D). Collectively, our data obtained with the PS1_optC construct strongly support the notion that functional PS1 can adopt a membrane topology in which the C terminus of the protein faces the luminal space.

A Nine-TM Topology Model for PS1—The results from the glycosylation acceptor site assay are in keeping with both a 7 TM domain model, as proposed by Nakai et al. (31), and a 9 TM model. To distinguish between these two models, we first used a computer-based

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method for topology predictions of TM proteins (34). The prediction method is based on the TMHMM method (35), which has been further developed to allow for incorporation of experimental data (34), thus constrained predictions can be performed (www.sbc.su.se/~karin/predTM.html). An unconstrained prediction solely based on the primary sequence of PS1 suggested a 9-TM domain topology that positioned the large hydrophilic loop and HD 8, containing the Asp-385 residue, in the lumen. When constrained by our experimental data, so that the first hydrophilic loop and the C terminus were restricted to the luminal space, and the large hydrophilic loop to the cytosol, a topological model with 9 TMs (Fig. 3) was predicted.

To test the nine-TM domain model experimentally, we made constructs with a glycosylation acceptor site in the C terminus, where one or two potential TM domain regions (i.e. HDs) were deleted. This approach has been used by others (17) and is based on the notion that deletion of a TM domain will reverse the topological orientation of all TM domains located C-terminal to the deletion during the translocation process. We generated two constructs that had glycosylation acceptor sites in the C terminus and HD 8 or HD 8–9 deleted (Fig. 4A). Deletion of HD 8 (PS1_CΔHD8) reversed the topology of the C terminus as shown by the appearance of a single band on a Western blot (Fig. 4B, lane 3). The construct lacking both HD 8 and HD 9 (PS1_CΔHD8–9) migrated as a double band (Fig. 4B, lane 5). The slower migration of the upper band of PS1_CΔHD8–9 was due to glycosylation, as confirmed by endoglycosidase H treatment (Fig. 4B, lane

![FIGURE 3. Revised topological model for PS1](image_url)
6). In conclusion, these data suggest that HD 8 and HD 9 are TM regions and emphasize that HD 10 has the potential to span the membrane. Both deletion constructs lack the essential Asp-385 residue in HD 8 and were as expected non-functional with respect to \( \text{H9253} \)-secretase activity and endoproteolysis (Fig. 4C, lanes 3 and 4). We have previously shown that PS1 CTF can restore endoproteolysis and \( \gamma \)-secretase activity of endoproteolysis-defective C-terminally modified PS1 molecules upon co-expression (23). Here we utilized this finding and showed that co-expression of the mutants with native CTF rescued both PS1 endoproteolysis and \( \gamma \)-secretase cleavage of C99 and Notch \( \Delta E \) (Fig. 4C, lanes 6 and 7). This indicated that the NTF-forming parts of PS1_C\( \Delta \)HD8 and PS1_C\( \Delta \)HD8–9 were in native conformations. We also noted that the full-length form of the construct with a reversed topological orientation of the C terminus (PS1_C\( \Delta \)HD8) appeared to have a reduced stability, probably due to impaired interaction with other \( \gamma \)-secretase components.

Finally, we examined the topological properties of PS1 CTF using cell surface biotinylation assays. It has previously been shown that PS1 CTF can become biotinylated in a cell surface biotinylation experiment (36). Having established that the large hydrophilic loop resides in the cytoplasm, we reasoned that the amino acid residue(s) accessible for biotinylation must lay C-terminally of HD 8. We examined the PS1 CTF and found three amino acid residues C-terminally of HD 8 that are potential sites for covalent attachment of biotin, namely the lysines in positions 395, 429, and 430 (Fig. 5A). K395 is located between HD 8 and HD 9, whereas Lys-429 and Lys-430 both are located between HD 9 and HD 10. If HD 8 spans the membrane, Lys-395 should face the extracellular space and thus be accessible for biotinylation. Accordingly, if both HD 8 and HD 9 span the membrane, as predicted by our 9-TM topology model, Lys-429 and Lys-430 would be positioned on the cytosolic face of the membrane, inaccessible for biotin. Conversely, in any topological model suggesting that only one of HDs 8 and 9 spans the membrane, Lys-429 and Lys-430 would face the extracellular space and thus be accessible for biotinylation. Finally, our 9-TM topology model predicts that a lysine residue inserted C-terminal of HD 10 is accessible for biotin.

We generated two PS mutant constructs: PS1_K395E, where the lysine at position 395 is exchanged for a glutamic acid residue, and PS1_K395E,Q464K, carrying the mentioned lysine to glutamic acid mutation as well as a mutation where the glutamine in position 464 has been changed to lysine (Fig. 5A). K395 is located between HD 8 and HD 9, whereas Lys-429 and Lys-430 both are located between HD 9 and HD 10. If HD 8 spans the membrane, Lys-395 should face the extracellular space and thus be accessible for biotinylation. Accordingly, if both HD 8 and HD 9 span the membrane, as predicted by our 9-TM topology model, Lys-429 and Lys-430 would be positioned on the cytosolic face of the membrane, inaccessible for biotin. Conversely, in any topological model suggesting that only one of HDs 8 and 9 spans the membrane, Lys-429 and Lys-430 would face the extracellular space and thus be accessible for biotinylation. Finally, our 9-TM topology model predicts that a lysine residue inserted C-terminal of HD 10 is accessible for biotin.
tated proteins revealed that mature nicastrin and PS1 NTF were biotinylated in BD8 cells expressing either of the three PS1 variants, but not in naïve BD8 cells (Fig. 5C, upper right panel). Importantly, biotinylated PS1 CTF species could only be detected in BD8 cells expressing wild type PS1 or PS1_K395E.Q464K (Fig. 5C, upper right panel). This shows that the lysine between HD 8 and HD 9 (Lys-395), and the inserted lysine three amino acids from the C terminus (Lys-464) have a luminal/extracellular location. Moreover, the fact that the two lysine residues located between HD 9 and HD 10 could not be biotinylated (Fig. 5C, construct PS1_K395E, upper right panel) suggests that they reside in the cytoplasm. Taken together, the results from the biotinylaton assay strongly favor the nine-TM topology model for PS.

**DISCUSSION**

To determine the topology of PS is important for a deeper understanding of the function of γ-secretase. This is of relevance from an Alzheimer disease perspective and to better understand how proteases of the intramembrane-cleaving protease type conduct proteolytic processing in a hydrophobic environment (1). Hydropathy analysis of the PS primary sequence identifies ten HDs, which can all theoretically traverse the membrane. Over the years a number of models for PS topology have been put forward. A topology with eight TM domains has also been reported (29, 31, 37, 38). However, all previous studies relied on extensive manipulation of the PS protein by fusion of reporter probes to C-terminally truncated variants of PS1, or on immunocytochemical labeling of intact or semi-permeabilized cells. To overcome problems with bulky probes, and non-functionality of C-terminally truncated PS1 molecules and the limitations of immunchemistry, we opted for the use of small glycosylation acceptor sites as a less invasive strategy to track topology. To reduce background from endogenous PS, we also conducted the experiments in BD8 cells, which are devoid of endogenous PS expression.

Our data support a nine-TM domain model for PS1, in which the N terminus and the large hydrophilic loop are in the cytosol, whereas the C terminus locates to the lumen/extracellular space (Fig. 3). The key evidence for this model are: (i) glycosylation acceptor sequences in the first hydrophilic loop and the C terminus are glycosylated, whereas glycosylation acceptor sequences in the N-terminal tail, the large hydrophilic loop and HD 10 are not; (ii) deletions of HD 8 and HD 8–9 demonstrated that both HD 8 and 9 have the ability to span the membrane, and (iii) lysine residues between HD 8 and HD 9 as well as in the C-terminal tail can be biotinylated on the cell surface, whereas lysine residues between HD 9 and HD 10 cannot.

We observed only partial glycosylation when a glycosylation acceptor site was placed in the absolute C terminus of PS1 (PS1_C). This is in keeping with previous data on the location of glycosylation acceptor sites, which are known not to function optimally when placed very close to the C-terminal end of proteins (33).

Moreover, we could not detect any glycosylated CTF for the PS1_C construct, and no interaction between nicastrin and glycosylated CTF. In agreement with the importance of the C terminus (see below), this may indicate that glycosylation at the glycosylation acceptor site at the C terminus is incompatible with incorporation into the γ-secretase complex. One interpretation would be that introduction of the glycosylation site per se is compatible with function, but that the addition of sugar moieties apparently compromises the functionality of the molecule. However, moving the glycosylation acceptor site three amino acids away from the C terminus resulted in full glycosylation and generation of a glycosylated CTF. Importantly the construct with an optimized glycosylation acceptor site, PS1_optC, appeared to have intact γ-secre-
tase activity as assessed by APP- and Notch-based assays. In cells transfected with PS1_optC only glycosylated CTF was detected, and given that only glycosylated CTF from this mutant was found to interact with nicastrin, the most straightforward interpretation of our results is that a luminal orientation of the PS1 C terminus is compatible with γ-secretase function.

Similarly, we believe our biotinylation experiments reflect the topology of biologically active PS proteins. γ-Secretase complexes containing NTF/CTF heterodimers have been shown to be present at the cell surface, where they can interact with Notch (24) as well as APP (39). Moreover, given that only PS molecules incorporated in a complex with nicastrin, Aph-1, and Pen-2 undergo endoproteolysis to generate NTF and CTF, whereas PS molecules that do not associate with other γ-secretase components are highly unstable and rapidly degraded (10), any PS fragments found at the cell surface are likely to be part of active γ-secretase complexes.

In sum, we believe our results conclusively show that PS1 proteins with a nine-TM topology enter functional γ-secretase complexes mediating regulated intramembrane proteolysis of APP as well as Notch. However, we cannot rule out that PS1 proteins with other topologies can form functional γ-secretase complexes as well.

In the nine-TM domain topology model the C terminus localizes to the luminal side. This is of interest in terms of the importance of the C terminus for PS function and for its interaction with other proteins. We recently demonstrated that the C terminus of PS1 is critical for γ-secretase complex assembly (40), which suggests that the absolute C terminus could be embedded in the complex. Similarly, Kaether et al. (41) showed that the absolute C terminus of PS1 interacts with the TM domain of nicastrin, suggesting that the C terminus of PS1 either protrudes into the membrane or penetrates it completely. An antibody raised against the last ten residues of PS1, 4627 (42), detects PS1 CTF on a Western blot but is not efficient for co-immunoprecipitations under conditions that keep the γ-secretase complex intact (43), supporting the notion that the epitope could be hidden in the complex. In addition to the other known γ-secretase components, a number of proteins have been suggested to bind to the C terminus of PS1, including the brain G-protein Gα (44), the calcium-binding protein calsenilin (45), the PS1-associated protein (46), the neuronal adaptors proteins X11α and X11β (47), and telen cephalin (48). Most of these studies have identified PS1-binding proteins by using peptides corresponding to the last 39–79 residues of the PS1 C terminus for glutathione S-transferase pull-down experiments or yeast two-hybrid screens. Hence, the PS1 fragments used include hydrophilic residues flanking both sides of HD 10. However, Annaert et al. (48) identified an interaction between PS1 and telen cephalin by using only the eight most C-terminal residues of PS1 in a yeast two-hybrid system. Importantly, their findings do not contradict a membrane spanning topology for PS1 HD 10.

It follows from our nine-TM model as well as from the eight-TM model (15–17), that the catalytic aspartates (Asp-257 and Asp-385) are situated that only glycosylated CTF from this mutant was found to interact with nicastrin, Aph-1, and Pen-2 undergo endoproteolysis to generate NTF and CTF, whereas PS molecules that do not associate with other γ-secretase components are highly unstable and rapidly degraded (10), any PS fragments found at the cell surface are likely to be part of active γ-secretase complexes.

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A Nine-transmembrane Domain Topology for Presenilin 1
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