Presenilin-1 Maintains a Nine-Transmembrane Topology throughout the Secretory Pathway

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Presenilin-1 is a polytopic membrane protein that assembles with nicastrin, PEN-2, and APH-1 into an active γ-secretase complex required for intramembrane proteolysis of type I transmembrane proteins. Although essential for a correct understanding of structure-function relationships, its exact topology remains an issue of strong controversy. We revisited presenilin-1 topology by inserting glycosylation consensus sequences in human PS1 and expressing the obtained mutants in a presenilin-1 and 2 knock-out background. Based on the glycosylation status of these variants we provide evidence that presenilin-1 traffics through the Golgi after a conformational change induced by complex assembly. Based on our glycosylation variants of presenilin-1 we hypothesize that complex assembly occurs during transport between the endoplasmic reticulum and the Golgi apparatus. Furthermore, our data indicate that presenilin-1 has a nine-transmembrane domain topology with the COOH terminus exposed to the lumen/extracellular surface. This topology is independently underscored by lysine mutagenesis, cell surface biotinylation, and cysteine derivation strategies and is compatible with the different physiological functions assigned to presenilin-1.

γ-Secretase is a multisubunit protease requiring the coordinated action of presenilins (PSs), nicastrin (NCT), PEN-2, and APH-1 and is crucial for the intramembrane proteolysis of type I membrane proteins such as the amyloid precursor protein (APP) and Notch. The catalytic component, PS1, is a polytopic membrane protein that undergoes endoproteolysis resulting in stable PS1 NH2- and COOH-terminal fragments (PS1-NTF and -CTF). According to the Kyte-Doolittle plot, PS1 has ten hydrophobic regions (HR) (5), but it is unclear how many of these cross the lipid bilayer as transmembrane domains (TMDs) (6). A widely accepted model proposes eight TMDs (HR I to VI, VIII, and IX) with the NH2-COOH terminus and the hydrophilic loop domain between TMD 6 and 7, all facing the cytosol (Fig. 1A). All published models agree that the first six HR cross the membrane, implying a consensus for the topology of the PS1-NTF. In contrast, divergent proposals exist for the number of TMDs in the PS1-CTF (7–10). Several of these models are difficult to reconcile with the different physiological roles assigned to PS1, such as the location of the aspartate residues in HRV1 and VIII or the cytosolic-oriented loop domain required for β-catenin binding (4, 11). Knowledge of the exact topology of PS1 is therefore of pivotal importance to understanding its multiple roles.

In this report, we revisited the trafficking and topology of PS1 using glycosylation consensus sequences inserted at different positions in human PS1 (hPS1). Expression of these mutants in PS1 and 2 knock-out (KO) mouse embryonic fibroblasts (MEFs) allowed us to evaluate the glycosylation status of these variants, and hence the topology, without interference of endogenous PS. Combined with a cysteine derivation strategy we provide strong evidence for a 9-TMD model (Fig. 1B) that includes three TMDs in the PS1-CTF and a luminal/extracellular COOH terminus. This model is not only in agreement with recent findings (12, 13) but is also more consistent with functions assigned to PS1 thus far. We also demonstrate that this topology is maintained throughout the secretory pathway. Finally, our data lead us to suggest that γ-secretase complex formation precedes passage through the Golgi.

EXPERIMENTAL PROCEDURES

Cloning of the PS1 Constructs and Generation of Stable MEF Lines—A pCDNA3 vector encoding human PS1 with an Escherichia coli leader peptidase sequence inserted at codon 241 was obtained from Dr. Nakai (9). This sequence, coding for amino acids FSRRNGGEATSGFEEVKNETKENGIRSEKERKETLGDVTL and bearing a glycosylation consensus sequence (indicated in bold), was PCR amplified using primers with Nhel and BglII restriction sites. Next, Nhel or BglII sites were introduced at codon positions 128, 192, 370, 400 of human PS1 by in vitro mutagen-
esis (Stratagene) and used to insert the *E. coli* leader peptidase sequence. These PS1glyc cDNAs were BamHI/SalI subcloned from pBluescript into a pMSCV vector (Clontech) with an extended poly linker. Alternatively, NGT sites at codon positions 52 and 463 or L429-L430 to HH substitution were introduced in pMSCV/human PS1 using site-directed XL mutagenesis (Stratagene). All mutants were verified by sequencing.

pMSCV constructs were co-transfected with helper plasmid pIK Ecopac in human embryonic kidney 293 cells for packaging in retroviruses. Retroviruses were harvested, and snap-frozen aliquots were stored at −80 °C until use. MEF PS1/PS2 cells were transduced with retrovirus for 24 h followed by puromycin selection in Dulbecco’s modified Eagle’s medium/F12 supplemented with 10% fetal calf serum until stable lines were obtained.

**Antibodies and Chemicals**—pAb against mouse PS1 (B19.2, B32.1), human PS1 (B14.5), APP (B63.1), PEN-2 (B95.1), and NCT (B59.1) and mAb against NCT (9C3) are described (14). pAb SB129 (against amino acids 3–18 of PS1) cross-reacts with human/mouse PS1 and mAb 5.2 (amino acids 307–331 of hPS1) were provided by C. Van Broeckhoven (Antwerp, Belgium) and B. Cordell (Scios), respectively. B104.1 recognizes the 17 NH₂-terminal amino acids of Aβ. Guinea pig anti-NCT and mAb 5232 against PS1-CTF were from Chemicon; anti-KDEL was from Stressgen. Antigalactosyltransferase mAb was from E. Berger (University of Zürich). Kifunensine and swainsonine were from Calbiochem and Sigma, respectively.

**Deglycosylation Experiment**—Glycoproteins (like NCT) were differentially deglycosylated by treatment with either endoglycosidase F or endoglycosidase H (endoH) to distinguish immature from mature glycosylation. MEFs were harvested in phosphate-buffered saline, centrifuged (800 × g, 10 min), and lysed in 100 mM phosphate buffer (0.1% SDS, 0.5% Triton-X100, 0.5% -mercapto-ethanol, protease inhibitors), pH 5.7 or pH 7.4 (for endoH or endoglycosidase F treatment (Roche Applied Science), respectively). Lysates were denatured (10 min, 70 °C) and incubated with endoH (1 unit/50 µl, 18 h, 37 °C) or endoglycosidase F (1 unit/50 µl) and analyzed by SDS-PAGE on 4–12% Tris-acetate gels in MES buffer (Invitrogen) and Western blotting. Detection was with chemiluminescence (PerkinElmer).

**Cell Surface Biotinylation**—NHS-SS-biotin (0.5 mg/ml; Pierce) was used for cell surface biotinylation, and biotinylated proteins were isolated using streptavidin-Sepharose (Amersham Biosciences) followed by Western blotting. Analysis of APP processing using metabolic labeling and blue native gel electrophoresis was done as described (15).

**Immunofluorescence**—HeLa cells were incubated at 37 or 20 °C (3 h) (to block TGN exit) and fixed (20 min) in 4% paraformaldehyde, followed by immunocytochemistry and confocal microscopy using a Zeiss Radiance 2100 connected to an upright Nikon Eclipse E800 microscope. Acquisition was

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**FIGURE 1.** A, 8-TMD topology of PS1 with 8 HRs (I–VI and VIII–IX) crossing the lipid bilayer as TMD. Sites where a leader peptidase or NGT sequence was inserted are indicated with thick bars and thin bars, respectively (amino acid numbering of hPS1). Lys to His mutations at codon 429–430 (KK/HH) and antibody epitopes are indicated. B and C, 9-TMD model with the cytosolic NH₂ terminus, terminus and loop domain, the luminal COOH terminus, and three TMDs in PS1-CTF (HR VIII–IX). Arrows point to key experiments that revealed the topology of this region. Cys to Ala mutations at positions 92, 158, 263, 410, and 419 (*) and re-introduced Cys residues (●) are indicated (see Fig. 4, D and E).
Cysteine Derivation—The Cys7/H11002PS1 and subsequent cysteine re-introductions were done using the (Multi) site-directed mutagenesis kit (Stratagene). For labeling with thiol-reactive reagents, MEFs were incubated on ice with either 200 μM (in phosphate-buffered saline, pH 7.4) membrane-permeable EZ-Link Biotin-HPDP (Pierce), or 300 μM membrane-impermeable TS Biotin-X ethylenediamine (Biotium) for 45 min. After washing, cells were lysed in solubilization buffer containing 25 mM Heps, pH 8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and protease inhibitors. Biotinylated proteins were captured using immobilized NeutrAvidin beads (Pierce) at 4 °C overnight, and the bound material was eluted by heating in Nu-Page sample buffer followed by SDS-PAGE on a 4–12% BisTris gel (Invitrogen).

On-line Supplemental Material—Supplemental Fig. S1 demonstrates a cytosolic-oriented NH2 terminus and hydrophilic loop between HR VI and VIII of PS1.

FIGURE 2. hPS1glyc241 is N-glycosylated and restores γ-secretase activity. A, cell extracts of MEF WT, PS1 and 2 KO MEF, hPS1glyc241, and hPS1WT rescued lines were not (−), endoH-resistant (H), and endoglycosidase F-treated (F). Endoglycosidase F treatment completely removes oligosaccharide chains from glycoproteins, whereas endoH only removes oligosaccharides that still bear free high mannose residues and are not yet further modified by mannosidase I and II and N-acetylglucosamine transferase. Modification by these enzymes renders the bond between the two N-acetylglucosamines in the core resistant to endoH treatment. Because these modifications occur beyond cis-Golgi cisternae, endoH treatment provides a readout for the ratio of mature versus immature glycosylation and hence the transport between ER and Golgi. Western blot revealed that hPS1glyc241 is N-glycosylated but remains endoH sensitive, is partially endoproteolysed (*), and partially rescues NCT maturation, PEN-2 stability, and APP-CTF processing. Treatment with 5 μg/ml brefeldin A (3 h) (right) generates an endoH-resistant fraction (*), proving that the consensus sequence is sufficient for complex glycosylation. B, PS1 and 2 KO, hPS1WT, and hPS1glyc241 MEFs were virally transduced with APPswedish, followed by metabolic labeling and immunoprecipitation of APP and secreted Aβ. Phosphorimaging analysis indicates a partial rescue of Aβ secretion by hPS1glyc241. C, blue native gel electrophoresis shows the incorporation of hPS1glyc241 and hPS1WT in a 440-kDa complex. D, cell surface biotinylation showing that endoH-sensitive hPS1glyc241 resides at the cell surface.
RESULTS AND DISCUSSION

PS1glyc241 is N-Glycosylated and Restores γ-Secretase Activity—Because PS1 is not post-translationally modified by N-glycosylation, we introduced glycosylation consensus motifs into hPS1 and expressed these variants in PS1 and 2 KO MEFs using retroviral transduction (15). Importantly, selected stable cell lines express mutant PS1 at levels equal or even lower than endogenous PS1, avoiding artifacts caused by high overexpression systems (data not shown, and e.g. Fig. 4A). Moreover, by using PS1 and 2 KO MEFs, no interference is expected from endogenous PS, which could otherwise be a concern because PS1 fragments may exist as dimers and therefore give rise to hybrid PS1 complexes (17). The minimal requirement for N-glycosylation is an NXT/S motif. Most hPS1 variants with this motif introduced in a luminal loop domain did not show N-glycosylation. This can be explained by the fact that a distance of at least 10 amino acids from any TMD is required for efficient glycosylation (18), a requirement that is not reached in most hPS1 luminal loop domains. Alternatively, we used a hydrophilic 40-amino acid glycosylation consensus sequence derived from the E. coli leader peptidase (hPS1glyc variants, thick bar in Fig. 1) that becomes efficiently glycosylated when fused to PS1 fragments (9). This sequence was introduced at codon 241 (Fig. 1A, hPS1glyc241). Stable expression resulted in partial endoproteolysis of hPS1glyc241 (Fig. 2A). Both PS1-FL (full-length) and PS1-NTF have a lower mobility in SDS-PAGE, as expected from the 40-amino acid insertion, but treatment with endoH resulted in a shift that is not observed in wild-type (WT) or PS1 and 2 KO MEFs rescued with hPS1. Thus, hPS1glyc241 is N-glycosylated, implying that the loop between HR V and VI is luminal. hPS1glyc241 partially rescued NCT maturation, largely stabilized PEN-2 levels, reduced APP-CTF accumulation to WT levels (Fig. 2A), and partially restored AB secretion (Fig. 2B), demonstrating that hPS1glyc241 is functional. Blue native gel electrophoresis demonstrated that hPS1glyc241, like WT hPS1, assembles in a 440-kDa complex (Fig. 2C). In contrast to NCT, hPS1glyc241 remained fully endoH sensitive, indicating that it did not acquire complex glycosylation in the Golgi. When we treated hPS1glyc241 MEFs with brefeldin A, a drug known to redistribute Golgi enzymes to the ER, NCT as well as hPS1glyc241-FL and -NTF (Fig. 2A, inset) became mature glycosylated. Thus, the incorporated leader peptidase sequence is sufficient for complex glycosylation. One possible explanation is that the hPS1glyc241 fraction that passes the Golgi is below the detection limit in total extracts. Because a small post-Golgi pool of PS1 resides at the plasmalemma (19, 20), we analyzed this fraction by cell surface biotinylation on hPS1glyc241 MEFs. As for WT PS1, a fraction of hPS1glyc241 (8 ± 2%, n = 3) binds to streptavidin beads (Fig. 2D), indicating that this insertion does not disturb the trafficking of hPS1glyc241 to the cell surface. Surprisingly, this pool remained endoH sensitive, suggesting that the immature N-glycosylated chain generated in the ER is not modified further by glycosylation enzymes present in the Golgi apparatus. There are two main possibilities that could explain these observations. First, the N-glycosylation site on codon 241 is not accessible for further modification and complex glycosylation once hPS1glyc241 passes through the Golgi apparatus. The brefeldin A experiments suggest that hPS1glyc241 can acquire mature glycosylation as long as it is located in the ER. It is tempting to speculate that this masking of the N-glycosylation site could be caused by the incorporation of hPS1glyc241 in the γ-secretase complex. In this case, endoH-sensitive glycosylation in the ER occurs, but complex assembly prior to reaching the medial/trans Golgi cisternae prevents further glycosyl modification. This is reminiscent of NCT, which undergoes a conformational change associated with complex assembly (21).

Alternatively, we cannot exclude the possibility that hPS1glyc241 “bypasses” the Golgi on its way to the cell surface, as suggested for instance for cystic fibrosis transmembrane conductance regulator (22), the paranodal complex (23), and more recently the yeast protein Ist2p (24). To investigate this, we introduced a glycosylation consensus sequence in every predicted luminal domain of PS1. A hPS1glyc variant displaying endoH resistance would then confirm the first model, i.e. the trafficking of PS1 via the conventional route through the Golgi.

hPS1 Glycosylation Variants Necessitate a Re-evaluation of PS1 Topology—In Fig. 3, A and B, we analyzed the PS1 and 2 KO MEF lines expressing hPS1glyc or hPS1NTG variants. Next to codon 241 we introduced the leader peptidase sequence in the first (codon 128) and second (codon 192) luminal loop domain and the NGT motif in the hydrophilic PS1 NH2 terminus (codon 52). Insertions in the first luminal loop dramatically interfered with PS1 stability, because protein expression (including FL) was never detected despite a stable introduction of the viral vector (data not shown). Interestingly, this region bears FAD-linked mutations and is, together with the HR I, critical for the interaction with APP and telencephalin (25). Interference with the structural integrity of this region may therefore not be tolerated. For all other variants, we compared control and endoH-treated extracts and analyzed their effect on NCT maturation and APP-CTF as a correlate for rescue of γ-secretase assembly and activity. hPS1NTG52 is fully endoproteolysed and restores NCT maturation and APP-CTF processing. However, no glycosylation was observed in agreement with the cytosolic location of the NH2 terminus. The glycosylation variant hPS1glyc192 is mainly

FIGURE 3. Scanning hPS1 topology using various glycosylation inserts. A, PS1-NTF glycosylation variants. hPS1NTG52 is endoproteolysed and rescues PS1 and 2 deficiency but is not glycosylated. The hPS1glyc192 is endoH-sensitive glycosylated but displays very moderate endoproteolysis and no clear rescue of NCT maturation and APP-CTF processing. PS1-NTFs of hPS1glyc241 are weakly detected with pAb SB129 (°). B, PS1-CTF glycosylation variants. hPS1glyc370 is not glycosylated as expected from the cytosolic orientation of the large loop domain. The consensus sequence inserted after HR VIII (hPS1glyc400) is glycosylated and acquires partial endoH resistance (°). NGT at the COOH terminus (hPS1NTG463) is also glycosylated. The different glycosylation variants are endoproteolysed but display limited rescue of NCT maturation and APP processing. Note: in panels A and B human and mouse PS1 fragments were detected with different species-specific antibodies. C, treatment of hPS1glyc400 MEF cells for 48 h with 1 μg/ml kifunensine or swainsonine abrogated the endoH-resistant band (°). D, hPS1glyc463 rescues complex formation (left) and Aβ secretion (right). E, a 20 C-block causes PS1 and NCT to accumulate in the TGN. Triple staining of HeLa cells was with Alexa488 (PS1, pAb sb129), -568 (anti-galactosyltransferase (GalT), labeling TGN) and -647 (guinea pig anti-NCT) secondary antibodies. Bar, 10 μm.
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expressed as a FL protein with very low endoproteolysis and no significant aspartate mutants (15). Nevertheless, the holo- and PS1-NTF are (endoH-sensitive) glycosylated, indicating luminal orientation in the ER. Overall, the results with the 52, 192, and 241 mutants are entirely consistent with previous models proposing the existence of six TMDs and a cytosolic NH2-terminus for PS1-NTF.

Next we visited the topology of the PS1-CTF. First, glycosylation sequences introduced in hPS1-CTF (Fig. 3, hPS1glyc370, glyc400, and -NGT463) did not compromise endoproteolysis (panel B versus panel A), suggesting that “presenilinase” activity depends more on the structural integrity of the NTF. Modifying this integrity may, for instance, affect its interaction with PEN-2, which is suggested to trigger endoproteolysis (26). Second, and despite complete endoproteolysis, NCT maturation and APP-CTF processing were weakly restored (Fig. 3B). For hPS1glyc370 this is unexpected because the hydrophobic loop domain is dispensable for γ-secretase activity (11). We assume that the very low expression levels of hPS1glyc370 are insufficient for rescue. More importantly, hPS1glyc370 mobility was not affected by endoH treatment, confirming the cytosolic orientation of the large loop region. Finally, hPS1glyc400 was particularly informative as it is the only variant that acquired partial endoH resistance, thus suggesting Golgi passage.

We next treated hPS1glyc400 MEFs with 1 μg/ml kifunensine or swainsonine, drugs that inhibit cis- or medial-Golgi-localized mannosidase I and II, respectively. As shown in Fig. 3C, the endoH-resistant fraction disappeared after each treatment, demonstrating that hPS1glyc400 passes through cis- and medial-Golgi. Further proof came from experiments in which we incubated HeLa cells at 20 °C to block exit from the TGN (27). In controls (37 °C), hardly any endogenous PS1 and NCT colocalized with galactosyltransferase, a trans Golgi enzyme (Fig. 3E, 37 °C). However, after a 3-h incubation at 20 °C, small amounts of endogenous PS1 and NCT colocalized clearly with galactosyltransferase in the TGN (Fig. 3E, 20 °C). Thus, a first major conclusion is that PS1 travels via the conventional route from the ER via the Golgi apparatus to the cell surface. This further supports our hypothesis that the incorporation of PS1 into the γ-secretase complex occurs prior to entering the Golgi. Because proper assembly of the γ-secretase complex is a prerequisite for its function in post-Golgi compartments, it is not unlikely that this assembly is tightly regulated both spatially and temporally. The secondary quality control system in ER and Golgi compartments includes such a mechanism that retrieves unassembled subunits or incompletely assembled complexes from the Golgi through interactions with sorting motifs/signals on individual subunits (28). During multimeric assembly, such signals become masked, allowing transport of the assembled complex through the Golgi as has been demonstrated for potassium channels (29).

Although such sorting signals have not yet been clearly identified for γ-secretase components, with the possible exception of PS1 (30), several findings may link ER-Golgi sorting to assembly. First, the slow kinetics of complex glycosylation of NCT (31) and the enrichment of endogenous PS1 in cotransporter protein complex I (COPI)-coated membranes (20) already suggest that these components reside and/or recycle in early compartments for an extended period. Furthermore, using a cell-free ER budding assay, Kim et al. (32) demonstrated that the PS1 holoprotein is the principle PS1 species packaged into COPII vesicles, suggesting that PS1 processing, an event likely to precede complete complex assembly, occurs in distal compartments such as the intermediate compartment and cis-Golgi. This seems to contradict the findings of Capell et al. (33) who argued, solely based on introducing a dilysine motif in NCT, that complete assembly of the γ-secretase complex occurs within the ER. We believe, however, that this is not correctly interpreted because a -KKXX motif does not mediate ER retention but rather retrieval from cis-Golgi and other compartments through binding to COPI coat components (34). Hence, this extends the sites for assembly to the intermediate and cis-Golgi compartments.

However, definitive proof that complex assembly in early compartments may drive forward transport through the Golgi is lacking. We anticipate that such evidence will emerge from the identification of sorting motifs in individual γ-secretase components or interactions with proteins regulating sorting between the ER and Golgi.

**A New Topology Model for PS1-CTF**—To further investigate PS1-CTF topology, we introduced an NGT motif in the extreme PS1 COOH terminus. This hPS1NGT463 rescued NCT maturation, complex formation, and APP-CTF processing (Fig. 3, B and D) and acquired full (endoH-sensitive) N-glycosylation. This observation precludes a cytosolic orientation of the COOH terminus, contrary to what is generally assumed (Fig. 1A), and now places the COOH terminus at the luminal side (Fig. 1B) in agreement with the proposal of Oh and Turner (35).

The N-glycosylation of both hPS1glyc400 and hPS1NGT463 precludes the existence of two TMDs in the PS1-CTF but pre-

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**FIGURE 4. Topology of PS1-CTF.** A and B, both hPS1glyc429 and -KK/KH are endoproteolysed, rescue NCT maturation, and restore APP-CTF processing and Aβ secretion moderately to strong, respectively. Also, both mutants become incorporated in an ~440-kDa complex. Anti-human/mousePS1 antibodies are indicated. The lower mobility of the hPS1glyc429-CTF is solely due to the inserted sequence and is not glycosylated, indicating a cytosolic orientation. C, Lys-429/430 are cytosolic. PS1 and 2 KO MEFs expressing hPS1WT and hPS1KK/KH (Lys-429 and Lys-430 to His mutation) were cell surface biotinylated and extracted in 2% CHAPS, 1% Triton X-100 (TX100), DSS, or 0.2% WT. Totals and bands were analyzed for NCT (mAb9C3), PS1-NTF (2B129), and PS1-CTF (mAb 5.2). For both cell lines equal amounts of CTF were recovered on streptavidin, proving that these Lys residues are not biotinylated. Bands in CHAPS are stronger because PS1-NTF/CTF heterodimers are preserved in this detergent and PS1-NTF/CTF heterodimers are preserved in this detergent. D, Cysless (Cys) PS1 and derived Cys variants rescue PS1 endoproteolysis, NCT maturation, PEN-2 stability, and APP-CTF processing. E, Cys derivation confirms three TMDs in PS1-CTF. Compared with Cys-less PS1 and WT, all three mutants are strongly labeled with the membrane-permeable reagent (left), whereas only the CWC and L460C mutants are labeled with the membrane-impermeable reagent (right). Because only the cell surface pool is measured, these Cys residues are located extracellularly. The PS1-NTF serves as a negative control. The signal for the WT PS1-NTF is due to a single accessible Cys residue in the first cytosolic loop domain that is not recognized by the membrane-impermeable reagent. NCT does not become labeled, demonstrating that all 12 Cys residues lack free thiols and form disulfide bridges as predicted by DISULFIND (39). PEN-2 is a positive control because it bears 1 luminal-oriented Cys and is hence recognized by both reagents.
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dicts either one TMD (HR VIII) or three TMDs (HR VIII, IX, and X) (Fig. 1). To distinguish between these two possibilities we first introduced the glycosylation sequence between HR IX and X. This hPS1glyc429, when stably expressed, rescued NCT maturation, complex formation, and catalytic activity but failed to become glycosylated (Fig. 4, A and B). Second, we tested the cell surface biotinylation of lysine residues. As shown in Fig. 4C, both WT hPS1-NTF and -CTF are recovered on streptavidin beads irrespective of the detergent used for extraction. With the exception of CHAPS, these detergents do not preserve the PS1-NTF/CTF heterodimer complex, indicating that PS1-NTF and -CTF are individually biotinylated. In PS1-NTF, several lysine residues indeed are localized in the predicted extracellular loop domains. For PS1-CTF, only three lysines are available, one on the edge of HR VIII (TMD 7) and two between HR IX and X (codon 429 – 430). When both Lys-429 and Lys-430 were mutated to His and stably expressed in PS1 and 2 KO MEFs (PS1KK/HH MEF), levels of biotinylated CTF were identical to WT levels (Fig. 4C). This allows us to conclude that Lys-429 and -430 do not contribute to cell surface biotinylation and hence are cytosolically oriented (13). Thus, both the biotinylation and glycosylation approach are in accordance with three TMDs in PS1-CTF and hence a 9-TMD model (Fig. 1B).

Because our proposed model strongly depends on the glycosylation variants, one can argue that the introduction of a 40-amino acid leader peptidase sequence may influence and alter the topology of PS1. Therefore, we decided to develop a third, least invasive, glycosylation approach are in accordance with three TMDs in PS1-CTF and hence a 9-TMD model (Fig. 1B).

In conclusion, we propose a 9-TMD topology for PS1 with a cytosolic NH$_2$ terminus and loop domain but extracellular COOH terminus, in line with recent findings (12, 13) but contradicting all previous topologies including the “accepted” 8-TMD (reviewed in Ref. 6). The 9-TMD model is here supported by three independent criteria, using glycosylation mutants, biotinylation assays, and cysteine derivation. The reason for the widely divergent topologies is, in our opinion, methodological. First, most studies have relied either on overexpression of chimeric proteins of truncated PS1 variants fused to different reporters (all of which can differentially affect the topology of individual HRs (9, 12) or on high overexpression of FL-PS1. In the latter case, this is not without risk because PS1 levels are in critical balance with the other $\gamma$-secretase complex components. Second, overexpression may lead to overloading of the translocation machinery as we experienced with glycosylation variants of PEN-2. Whereas strong overexpression resulted in two opposite topologies, low retroviral expression gave a single PEN-2 topology (data not shown). An alternative hypothesis is that PS1 may adapt to multiple topologies within the cell, as proposed for other proteins (discussed in Ref. 6). We believe that such complex interpretation is not needed. First, by selectively permeabilizing the cell surface using streptolysin-O followed by PS1-NH$_2$-terminal- and loop domain-specific antibodies, we confirmed their overall cytosolic orientation (supplemental Fig. S1). Second, the data from the endoH-sensitive glycosylation of PS1glyc variants, an early ER-associated event, are in line with the failure of Lys residues 429 – 430 to become biotinylated and with the differential Cys derivation of cell surface-associated PS1. This suggests that the same topology is maintained throughout the biosynthetic route. Third, our 9-TMD model reconciles many established PS1 interactions with other proteins and functions: (i) it preserves the juxtaposed position of the “catalytic” Asp-257 and -385 in TMDs 6 and 7 (4); (ii) several catenins interact with the cytosolic loop domain, and the role of PS1 in $\beta$-catenin turnover critically depends on this interaction (11); and (iii) the 9-TMD model is also compatible with the interaction of the PS1-COOH terminus with the TMD of APP (25) and NCT (30) as these hydrophobic COOH-terminal amino acids of PS1 may penetrate in the lipid bilayer. All this underscores that knowledge of the correct topology of PS1 is essential for the interpretation of experiments designed to understand its physiological function. For example, several two-hybrid screens identified PDZ-containing proteins interacting with the COOH terminus of PS (36) (37). These interactions can be explained by the hydrophobic nature of the final COOH-terminal amino acids that indeed resemble a binding module for PDZ domains. Likewise, the extracellular context of the PS1 COOH terminus was also not taken into account when proposing a cytoplasmic ER-export motif in PS1 and 2 (38) or when exploring the role of the COOH terminus in ER retention (30).

This is the first and most complete study on PS1 topology performed on physiological relevant expression levels of FL-PS1 variants in a KO background, arguing that this 9-TMD model most closely reflects the in vivo situation from its site of synthesis throughout the secretory route. Ultimate proof and confirmation have to come from structural work, but given the hydrophobic nature of PS1 this will not be easy to achieve. Finally, our unique approach strongly favors complex assembly after the ER but prior to Golgi passage.

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