Membrane Topology of Alzheimer's Disease-related Presenilin 1

EVIDENCE FOR THE EXISTENCE OF A MOLECULAR SPECIES WITH A SEVEN MEMBRANE-SPANNING AND ONE MEMBRANE-EMBEDDED STRUCTURE*

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Toshiki Nakai‡, Aya Yamasaki§§, Masao Sakaguchi‖, Kenji Kosaka§, Katsuyoshi Mihara¶, Yoshihiro Amaya** and Satoshi Miura‡‡

From the ‡Radiosotope Research Center, §Department of Psychiatry, ||Department of Biochemistry, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 226-0004 and the *Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

A significant member of early-onset familial type of Alzheimer's disease cases has been shown to be caused by dominant mutations in either of the two genes encoding presenilin 1 (PS1) and presenilin 2 (PS2). These two proteins are highly homologous to each other and have been reported to be mainly localized to the membranes of intracellular compartments such as the endoplasmic reticulum. Information about the membrane topological structures of these proteins is indispensable for understanding their physiological and pathological roles. Although several models have been proposed previously, their precise membrane topologies remain unknown. In this study, we examined this issue in detail by expressing a series of C-terminally deleted PS1 mutants fused to the hydrophilic portion of Escherichia coli leader peptidase in vitro using a reticulocyte lysate in the presence of microsomal membranes. Our results predict that PS1 exists mainly in a seven membrane-spanning structure with its C-terminal end exposed to the luminal space. This was also confirmed by expressing these fusion proteins in cultured cells. We further showed that a ninth hydrophobic segment is tightly bound to the membrane without spanning it. Based on the above observations, we propose a novel “seven membrane-spanning and one membrane-embedded” topological model for presenilins.

A significant portion of inheritable familial Alzheimer's disease (FAD)† is caused and transmitted in a dominant manner through mutations in either of the two highly homologous genes encoding presenilin 1 (PS1) and presenilin 2 (PS2) on chromosomes 14 and 1, respectively (1–5). These proteins have been shown to be mainly localized to the endoplasmic reticulum (ER), the Golgi apparatus, and the ER-Golgi-intermediate compartment (ERGIC) (6–8), although their localization to other compartments such as the plasma membrane has also been suggested (9, 10). One of the physiological roles of presenilins has been suggested through genetic analysis of Caenorhabditis elegans sel-12, which codes for a protein highly homologous to mammalian presenilins (11), and through analysis of PS1-knockout mice (12, 13). These studies have suggested that presenilins are involved in the Notch signaling pathway, which is known to be involved in many different aspects of the regulation of cell differentiation in C. elegans and higher eukaryotes. Several roles of presenilins in the pathogenesis of FAD have also been suggested. First, in the cerebrospinal fluid of FAD patients or in the medium of cultured cells expressing FAD-linked mutant presenilins, elevation of the level of amyloid β protein consisting of 42 amino acid residues (Aβ42), which is thought to play a key role in the pathological progression of Alzheimer’s disease, especially for senile plaque formation, has been observed (14–17). This suggests that FAD-associated mutations cause the disease by elevating the production of Aβ42 either intracellularly or extracellularly. Furthermore, analysis of neuronal cell primary cultures derived from PS1 knockout mouse embryos implied that PS1 is required for γ-secretase cleavage of Aβ from amyloid precursor protein (18). Second, several works have shown that wild-type or mutant presenilins are involved in the regulation of the cellular apoptotic pathway (19–22), implying the possibility that they may be related to the neural cell loss or degeneration commonly observed in this disease. However, the mechanisms underlying both the physiological and pathogenic functions of presenilins at the molecular level remain to be elucidated.

To understand the function(s) of presenilins at the molecular level, conformational information on these proteins in membranes is indispensable. Several previous reports have suggested different models for the membrane topologies of these or related proteins differing in the number of membrane-spanning segments (six to eight), but in all of them the C terminus is exposed to the cytosolic space (23–27). In this study, we re-examined this issue using a series of fusion proteins consisting of C-terminally deleted PS1 and a reporter sequence, with special emphasis on the C-terminal half region, where significant discordance still exists. Although two previous studies involved similar strategies (25, 26), in our study special attention was paid when choosing the reporter fragment to be fused because we consider it possible that the relatively hydrophobic nature of the extreme C-terminal region of PS1 would affect the results if the N-terminal portion of the reporter is also hydro-

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** Present address: Dept. of Biochemistry, School of Dentistry, Niigata University, 2-5274, Gakkocho, Niigata 951-8514, Japan.

†† To whom correspondence should be addressed: Radiosotope Research Center, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 226-0004, Japan. Tel.: 81-45-787-2758; Fax: 81-45-782-1251; E-mail: smiura@med.yokohama-cu.ac.jp.

‡ The abbreviations used are: FAD, familial Alzheimer's disease; Aβ, amyloid beta protein; CTF, C-terminal fragment; ER, endoplasmic reticulum; ERGIC, ER-Golgi-intermediate compartment; GH, growth hormone; HR, hydrophobic region; LPase, E. coli leader peptidase; MS, dog pancreas microsomal membranes; PS1, presenilin 1; PS2, presenilin 2; PAGE, polyacrylamide gel electrophoresis; aa, amino acid; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary.
phobic. The results we obtained using a series of such fusion proteins confirmed the notion that the N-terminal and the large hydrophobic loop regions of PS1 face the cytosol, as suggested by several previous studies. However, by using a reporter sequence that does not include hydrophobic residues in the vicinity of the N-terminal portion of the reporter region just after the fused point, we unexpectedly observed that a significant fraction of the molecules expressed in vitro or in cultured cells exposed their C-terminal ends to the luminal space on the ER membrane. In addition, we show here that the eighth and ninth hydrophobic segments do not span the membrane and that, despite this, the latter is tightly bound to the lipid bilayer from the cytosolic face. Based on the data presented in this study, we here propose a “seven membrane-spanning and one membrane-embedded” model for the topological structures of presenilins. Possible causes for the discrepancy between our results and the models proposed by several other groups are also discussed.

MATERIALS AND METHODS

Cloning of the PS1 Gene and Plasmid Construction—cDNAs for four contiguous parts spanning the whole coding region of human PS1 were amplified from a human fetal brain cDNA library (Stratagene) by PCR using four sets of oligonucleotides as primers (Table I). The four PCR products were digested with appropriate restriction enzymes, then subcloned into appropriate vectors, and finally recombined between the EcoRI and XhoI sites of the Bluescript II vector (Stratagene) to reconstitute the whole PS1 coding region. For the most N-terminal region, we subcloned the cDNAs for both of the two known variants in which VRSQ was excised and subcloned between the 26th and 29th amino acid residues is retained or deleted, respectively. Throughout this study, the VRSQ-deleted variant was used. However, the amino acid residue number of PS1 used in this study is based on that of the VRSQ-containing variant. The 1.4-kilobase fragment of the EcoRI-XhoI fragment containing the whole PS1 coding region was excised and subcloned between the EcoRI-XhoI sites of the Bluescript II vector (Stratagene) as a template (29). Primers used for amplification were GGCGGATCCGGCGACGTCGTAGGGGTA and CGCTGTCCGGAGCTAGATATAAAATTGATGGAc.

Antibodies and Immunological Procedures—Anti-LPase polyclonal antibody LP-1 was raised against N-terminally 63 amino acids of dog microsomal LPase (Novagen). Polyclonal antibody LOOP1 against the mouse PS1 loop region (332–371 aa) was raised by injecting a rabbit with glutathione S-transferase fusion protein containing this region expressed in E. coli using pGEX-5X-1 (Amersham Pharmacia Biotech) and was used after purification using an Affi-Gel matrix (Bio-Rad) to which a fusion protein consisting of maltose-binding protein and 263–407 aa of human PS1 expressed using pMAL-c2 (New England Biolabs) was attached. This antibody cross-reacts with the corresponding region of human PS1. For immunoprecipitation, samples were diluted with immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 0.1% SDS, 0.1% Triton X-100, 2 mM EDTA) and then incubated with the antibody for 1 hour at room temperature and further for 1 hour in the presence of Pansorbin cell, fixed Staphylococcus aureus cells (Calbiochem). After centrifugation, the pellets were washed four times with immunoprecipitation buffer, and proteins were extracted with 2× SDS-PAGE sample buffer (30) for 30 min at room temperature. Western blotting was performed after the proteins had been separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). Proteins were detected using an ECL chemiluminescence detection kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

In Vitro Transcription and Translation—Linearized plasmids were transcribed in vitro from the T7 promoter located upstream of the multicloning site of pcDNA3.1(+) using T7 RNA polymerase (Stratagene) according to the manufacturer’s instructions. The transcribed mRNAs were used for translation in vitro using a rabbit reticulocyte lysate (31) in the presence or absence of dog pancreas microsomal membranes (MS) prepared as described previously (32).

### Table I

| Amino acid residue | Primer |
|--------------------|--------|
| 1–156              | Sense  |
| 157–269            | Sense  |
| 270–401            | Sense  |
| 402–467            | Sense  |

### Table II

| Construct | Primer* |
|-----------|---------|
| ΔH1-C     | GGCGGATCCTGCTGGTTGGGCAATTTTCAAA |
| ΔH2-C     | GGCGATCCGACAGGCTCTGTCGCC |
| ΔH4-C     | GGCGGATCTGCAACGCTTATIGGTT |
| ΔH5-C     | GGCGGATCGTCAAGGTAGCCCTTCAGGTTG |
| ΔH6-C     | GGCGGATCTGCAAGGTACTGGATTAAATA |
| ΔH7-C     | GGCGGATCCTTCCTTCTCTTCTGAGG |
| ΔH8-C     | GGCGGATCCTTTTACCTCCCTTTCCTC |
| ΔH9-C     | GGCGGATCTGCAAGGTACTGGATTAAATA |
| ΔH10-C    | GGCGGATCCTTCCTTCTCTTCTGAGG |
| Full-C    | GGCGGATCCTTTTACCTCCCTTTCCTC |

* A BamHI restriction site (underlined) was introduced next to the C-terminal end of each deletion mutant.

GACTGGG and GATCCCGAGTCG, respectively.

For PS1-newt growth hormone (GH) fusion construction, a portion of newt GH coding sequence spanning the region from the 3rd amino acid residue of the mature protein to the C terminus was amplified by PCR using its cDNA subcloned in a plBluescript II vector (Stratagene) as a template (29). Primers used for amplification were GGCGGATCCGGCGACGTCGTAGGGGTA and CGCTGTCCGGAGCTAGATATAAAATTGATGGAc.

Construct Primer

| Construct | Primer* |
|-----------|---------|
| ΔH1-C     | GGCGGATCCTGCTGGTTGGGCAATTTTCAAA |
| ΔH2-C     | GGCGATCCGACAGGCTCTGTCGCC |
| ΔH4-C     | GGCGGATCTGCAACGCTTATIGGTT |
| ΔH5-C     | GGCGGATCGTCAAGGTAGCCCTTCAGGTTG |
| ΔH6-C     | GGCGGATCTGCAAGGTACTGGATTAAATA |
| ΔH7-C     | GGCGGATCCTTCCTTCTCTTCTGAGG |
| ΔH8-C     | GGCGGATCCTTTTACCTCCCTTTCCTC |
| ΔH9-C     | GGCGGATCTGCAAGGTACTGGATTAAATA |
| ΔH10-C    | GGCGGATCCTTCCTTCTCTTCTGAGG |
| Full-C    | GGCGGATCCTTTTACCTCCCTTTCCTC |

* A BamHI restriction site (underlined) was introduced next to the C-terminal end of each deletion mutant.
Membrane Topology of Presenilin 1

Human Placenta Homogenate Preparation and Na₂CO₃ Extraction—
Human placenta tissue was homogenized in PBS(−)(0.2 g/liter KH₂PO₄, 2.16 g/liter Na₂HPO₄, 7H₂O, 8 g/liter NaCl, 0.2 g/liter Na₂CO₃) in the presence of 1 mM phenylmethylsulfonyl fluoride and then centrifuged at 100,000 g for 1 h. The pelleted membrane fraction was resuspended in 0.1 M Na₂CO₃ and then incubated on ice for 30 min. One-half of it was further centrifuged at 100,000 × g for 1 h, and then the supernatant and pellet fractions were analyzed, together with the uncentrifuged half, for the presence of C-terminal fragment (CTF) by SDS-PAGE and Western blotting.

Cell Culture and Plasmid Transfection—
COS-1 and CHO-K1 cells were cultured in Dulbecco’s modified Eagle’s medium/high glucose medium and F12 medium, respectively, both of which were supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Cells were transfected with the indicated plasmids using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions.

Cell Labeling—
Cells cultured on 35-mm dishes were washed once with PBS(−) and then preincubated for 2 h in Dulbecco’s modified Eagle’s medium/high glucose without cysteine and methionine but supplemented with diazylated 10% fetal bovine serum at 37 °C under a 5% CO₂ atmosphere. Labeling was started by the addition of 100 Ci of Met-³⁵S-Label (mixture of L-³⁵S-methionine and L-³⁵S-cysteine, >97 TBg/mmol; American Radiolabeled Chemicals, Inc.) to the culture. After 5 h labeling, the cells were washed three times with PBS(−), and then the proteins were extracted with extraction buffer (PBS(−) supplemented with 0.5% Nonidet P-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μM antipain, 10 μM leupeptin, 10 μM chymostatin, and 10 μM pepstatin) and processed for immunoprecipitation.

RESULTS

Strategies for Membrane Topology Analysis—
Hydropathy analysis of the predicted primary structure of PS1 revealed 10 distinct hydrophobic regions (HR1 to HR10) that are candidate membrane-spanning segments (Fig. 1A). In order to determine which of these regions actually span the membrane, we adopted a deletion strategy based on the assumption that polypeptide membrane proteins are inserted into the membrane sequentially from the N terminus without the requirement of a more distal C-terminal region (33). According to this assumption, when a series of C-termi nally deleted mutants for a polypeptide membrane protein is expressed in the presence of ER membrane, localization of the C-terminal end of each deletion mutant should reflect the topology of the corresponding point when the full-length protein is expressed. Thus, when a reporter protein is fused to the C terminus of such a deletion mutant, the localization of the reporter protein according to the membrane should reflect that of the fusion point in the wild-type protein if the reporter region is neutral as to membrane translocation. As a reporter protein, we used the C-terminal hydrophilic region of E. coli leader peptidase (LPase). In E. coli, LPase is expressed as an inner membrane protein, the C-terminal region of which contains an active site located in the periplasmic space (34). Thus, this portion should not interfere with its translocation across the membrane. Moreover, it is known that, when expressed in vitro in a eukaryotic system in the presence of dog pancreas microsomes, this protein retains its activity and becomes inserted at the C-terminus of the leader peptidase E. coli leader peptidase (LPase). 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Moreover, it is known that, when expressed in vitro in a eukaryotic system in the presence of dog pancreas microsomes, this protein remains the same topogenic ability as in E. coli, and the C-terminal hydrophilic region localized in the luminal space undergoes N-glycosylation efficiently (35). The latter property also enabled us to determine whether or not this portion is localized in the luminal space.

Construction of C-termi nally Deleted PS1 Fusion Genes—
For the analysis of PS1 topogenicity, based on the results of hydropathy analysis, a series of C-terminally deleted PS1 mutant cDNAs truncated at the points just prior to the hydrophobic regions (PS1-ΔH1-C to PS1-ΔH10-C, Fig. 1B) and just after HR10, the most C-terminal hydrophobic segment (PS1-ΔC), were constructed, and they, together with the whole PS1 coding
region (PS1-full), were fused in frame to the sequence encoding the C-terminal region of LPase corresponding to 104–323 amino acid residues. When choosing a reporter, especially for the construction of a fusion protein with full-length PS1, the hydrophilic property of the N terminus of the reporter fragment is important to prevent fortuitous production of a hydrophobic segment at the junction, because the extreme C-terminal portion of PS1 is relatively rich in hydrophobic residues. The N terminus of the above LPase region is hydrophilic enough to prevent the creation of such hydrophobic segments together with the C terminus of PS1, as judged by hydrophyty analysis (data not shown). These fusion constructs were transcribed in vitro, and the synthesized mRNAs were translated in an in vitro translation system using a rabbit reticulocyte lysate and proteinase K protection assaying of the translation products. The plasmids indicated below each panel were linearized with XbaI, and mRNA was synthesized using T7 RNA polymerase. Transcripts were translated in a rabbit reticulocyte lysate in the presence of [35S]methionine and the presence (ER+, lanes 2–5 in each panel) or absence (ER−, lane 1 in each panel) of dog pancreas microsomal membrane. The translation products obtained in the presence of the membrane were divided into four parts, three of which were treated with proteinase K (20 μg/ml) in the presence (X100+, lane 4 in each panel) or absence (X100−, lanes 3 and 5 in each panel) of 1% Triton X-100. A part of the latter was further immunoprecipitated with anti-LPase polyclonal antibody, LP-1 (ImPpt+; lane 5 of each panel). The proteins in all samples were separated by 10% SDS-polyacrylamide gel electrophoresis, and isotopically labeled proteins were detected by fluorography. The positions for the bands of unglycosylated or glycosylated proteins are indicated by open arrowheads, and the positions for the bands of prestained marker proteins (New England Biolabs) are indicated on the left panel. The bands for proteinase K-protected fragments containing the LPase region are indicated by closed arrowheads on the right of the panels for PS1-DH2-C-LP, PS1-DH4-C-LP, PS1-DH6-C-LP, PS1-ΔC-LP, and PS1-full-LP. The positions of prestained marker proteins (New England Biolabs) are indicated on the left of the fluorograms.

**FIG. 2.** *In vitro* translation of the fusion genes in a rabbit reticulocyte lysate and proteinase K protection assaying of the translation products. The plasmids indicated below each panel were linearized with XbaI, and mRNA was synthesized using T7 RNA polymerase. Transcripts were translated in a rabbit reticulocyte lysate in the presence of [35S]methionine and the presence (ER+, lanes 2–5 in each panel) or absence (ER−, lane 1 in each panel) of dog pancreas microsomal membrane. The translation products obtained in the presence of the membrane were divided into four parts, three of which were treated with proteinase K (20 μg/ml) in the presence (X100+, lane 4 in each panel) or absence (X100−, lanes 3 and 5 in each panel) of 1% Triton X-100. A part of the latter was further immunoprecipitated with anti-LPase polyclonal antibody, LP-1 (ImPpt+, lane 5 of each panel). The proteins in all samples were separated by 10% SDS-polyacrylamide gel electrophoresis, and isotopically labeled proteins were detected by fluorography. The positions for the bands of unglycosylated or glycosylated proteins are indicated by open arrowheads, and the positions for the bands of prestained marker proteins (New England Biolabs) are indicated on the left panel. The bands for proteinase K-protected fragments containing the LPase region are indicated by closed arrowheads on the right of the panels for PS1-DH2-C-LP, PS1-DH4-C-LP, PS1-DH6-C-LP, PS1-ΔC-LP, and PS1-full-LP. The positions of prestained marker proteins (New England Biolabs) are indicated on the left of the fluorograms.

**PS1 Spans the Membrane Seven Times—**We assessed the membrane orientation of the reporter portion of each fusion protein expressed *in vitro* in the presence of MS using two distinct criteria as follows: 1) susceptibility of the reporter portion to proteinase K added via the cytosolic space; and 2) N-linked glycosylation, specific for the luminal space of ER, of the consensus sequence present in the reporter portion. Fig. 2 shows a fluorogram of the *in vitro* translation product for each fusion construct. These data indicate first that, in the presence of MS, translation products with slightly reduced electrophoretic mobility, as compared with those in the absence of the membrane, were predominant for PS1-DH2-C-LP, PS1-DH4-C-LP, PS1-DH6-C-LP, PS1-ΔC-LP, and PS1-full-LP (lanes 1 and 2 for each set in Fig. 2); however, in the cases of PS1-ΔH1-C-LP, PS1-ΔH3-C-LP, PS1-ΔH5-C-LP, PS1-ΔH7-C-LP, PS1-ΔH8-C-LP, PS1-ΔH9-C-LP, and PS1-ΔH10-C-LP (lanes 1 and 2 for each set in Fig. 2), no such mobility shift was observed. These results suggest that, for the former constructs, an N-linked glycosyl chain was attached to the reporter portion as a result of its translocation through the membrane and subsequent exposure to N-glycosyltransferase, whose active site resides in the luminal face of ER. This was confirmed by endoglycosidase H treatment, which is known to remove N-linked glycosyl chains from a polypeptide. Such treatment effectively restored the mobility to the extent that was observed for the translation products in the absence of microsomal membranes for all of the five constructs above (Fig. 3).

Second, when proteinase K is added via the cytosolic space to the translation products inserted into MS, the reporter portion located in the luminal space is expected to be protected from digestion because the protease cannot go into the luminal space due to the lipid bilayer of the microsomal vesicles. As shown in Fig. 2, only when a shift of the electrophoretic mobility was observed in the presence of MS (PS1-DH2-C-LP, PS1-DH4-C-LP, PS1-DH6-C-LP, PS1-ΔC-LP and PS1-full-LP) were protected fragments also observed (lane 3), which can be immunoprecipitated with LP-1 specific for LPase (lane 5). These fragments were abolished when proteinase K was added in the presence of Triton X-100, which disrupts the membrane (lane 4). These results, together with the presence of N-linked glycosylation, indicate that the reporter LPase portion of these products derived from PS1-DH2-C-LP, PS1-DH4-C-LP, PS1-DH6-C-LP, PS1-ΔC-LP, and PS1-full-LP is sequestered on the luminal side of microsomal vesicles, whereas that in the fusion proteins expressed by other constructs (PS1-ΔH1-C-LP, PS1-ΔH3-C-LP, PS1-ΔH5-C-LP, PS1-ΔH7-C-LP, PS1-ΔH8-C-LP, PS1-ΔH9-C-LP, and PS1-ΔH10-C-LP) is located on the cytosolic face.

To confirm the above results, we further examined two additional distinct series of constructs. On the one hand, in order to determine whether or not the above results hold true when another reporter protein, especially with a different N-terminal sequence, is used, a series of distinct fusion constructs corresponding to PS1-ΔH6-C, PS1-ΔH10-C, and PS1-ΔC were constructed using a shorter region of LPase (142–323 aa) having a
different N-terminal sequence as a reporter. Experiments involving these constructs gave results consistent with those described above (data not shown). On the other hand, when an extra membrane-spanning segment corresponding to TM2 of LPase (58–76 aa), which spans the ER membrane from the cytosol to the lumen when full-length LPase is expressed in a eukaryotic system (34), was inserted between the presenilin region and the reporter region, a complementary pattern of N-linked glycosylation was observed (Fig. 4). For constructs derived from PS1-ΔH7-C to PS1-ΔH10-C, the translation products were mainly N-glycosylated, whereas for those from PS1-ΔH6-C, PS1-ΔC, and PS1-full, the translation products were predominantly unglycosylated, although minor glycosylated products were observed for the last two of the latter. The existence of glycosylated species for PS1-ΔC-TM-LP and PS1-full-TM-LP may be due to incompleteness of the translocation efficiency of HR10. In such minor molecular species with cytosolic HR10, the extra membrane-spanning segment could span the membrane from the cytosol to the lumen and hence result in the translocation of the reporter portion to the luminal space.

The consistent results obtained with the above three series of constructs strongly suggest that PS1 spans the membrane seven times with the portions of the PS1 molecule just proximal to HR2, HR4, and HR6, the region just distal to HR10, and the C terminus exposed to the luminal space, and the portions just proximal to HR1, HR3, HR5, HR7, HR8, HR9, and HR10 exposed to the cytosolic space.

We also examined a series of PS1-GH fusion constructs (Fig. 5). When GH was fused just prior to HR10 (PS1-ΔH10-C-GH), or just after HR10 (PS1-ΔC-GH), results were consistent with those in the case when LPase was used as a reporter, although N-glycosylation and protection against proteinase K in PS1-ΔC-GH were less efficient than those in PS1-ΔC-LP. When GH was fused after the C terminus of PS1 (PS1-full-GH), the results were contradictory. N-Glycosylation and protection against proteinase K were very inefficient as compared with the case with the corresponding LPase fusion construct. As the C-terminal portion of PS1 and the N-terminal portion of the GH region fused to PS1 are immediately hydrophobic, we think the results of PS1-full-GH as an artifact of synthetic hydrophobicity of the region around the fusion point (see “Discussion”).

Topological analysis of PS1 in Cultured Cells—To confirm the results obtained with the in vitro experimental system, we transiently expressed PS1-ΔH6-C-LP to PS1-ΔH10-C-LP, PS1-ΔC-LP, and PS1-full-LP in COS-1 cells in the presence of a mixture of [35S]methionine and [35S]cysteine to label newly synthesized proteins isotopically. As shown in Fig. 6, the electrophoretic mobilities of the major translation products for PS1-ΔH6-C-LP, PS1-ΔC-LP, and PS1-full-LP decreased when the cell extracts were treated with endoglycosidase H prior to immunoprecipitation, indicating that in the majority of the fusion proteins expressed in living cells, the reporter regions underwent N-linked glycosylation and were therefore translocated to the luminal space. Note that significant portions of them were unglycosylated, implying the existence of PS1 molecules with the C terminus exposed to the cytosol in living cells. This may explain the discrepancy between our results and
那些从其他群体（见“讨论”）。PS1-ΔH7-C-LP到PS1-ΔH10-C-LP没有改变由内源性糖基化处理。表达这些蛋白质在CHO-K1细胞表现出类似结果（数据未显示）。上述结果表明，膜拓扑上的PS1预测从体外实验系统中准确反映其在细胞内的状态。

CTF表达单独可被组装到膜中相同拓扑结构的体外体外翻译产物——它已被报道，PS1和PS2在剪接时被转录和翻译在两段（N端段和CTF）的体外。为了确定每个片段的特定功能中的每个两个片段，不同的序列表达每个片段将是一个强大的方法。然而，当结果的类似方法在CTF，它是重要的知道是否或者这种片段表达分别可以被组装到膜中的体内的相同拓扑结构。因为完整的PS1分子。此外，C-端片段的PS2表达单独已显示可以有生物能力，抑制凋亡在几个实验环境（21, 22）。所以，我们研究使用一个类似的策略，然后采用上述的体外PS1。我们构建了一系列融合基因中，其中PS1编码序列由289个氨基酸组成，分配的序列表达已被报告为发生（38），并结合到的两个片段中对应的PS1-ΔH8-C到PS1-ΔH10-C, PS1-ΔC,和PS1-full, 被转录和翻译在两段（N端段和CTF）的体外。这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和翻译在两段（N端段和CTF）的体外。然后，这些片段将被转录和译
having five membrane-spanning hydrophobic segments was co-translated with each of the constructs used. As expected, the product of PS1-ΔH6-C-LP was partitioned mainly into the insoluble fraction with both sodium carbonate and the neutral buffer in the absence of the detergent. Taken together, these results suggest strongly that HR9 is embedded in the membrane with both of its flanking regions exposed to the cytosolic space (see Fig. 9 for our model).

**DISCUSSION**

Detailed knowledge of the membrane topological structures of presenilins is crucial for understanding their molecular functions in normal cells and in the pathogenesis of Alzheimer’s disease. Earlier, based only on the information derived from the primary structures of presenilins, a seven membrane-spanning model, in which HR1 to HR6 span the membrane, was proposed (5). Since then, based on at least some experimental results, several groups have proposed distinct topological models for these proteins (23–26, 40), although significant disagreement exists among these models, especially with regard to the C-terminal half portion. In this study, we addressed this controversial subject in detail using a series of fusion proteins in which C-terminally deleted PS1 is fused to a reporter protein. We expressed such fusion proteins either in vitro in the presence of the microsomal membrane (MS) or in vivo using cultured cells, and we determined the localization of the reporter portion by examining the N-glycosylation of this portion and its susceptibility to cytosolically added proteinase K. Based on the results of such experiments, together with several lines of evidence also described in this study, we here propose a novel model for the membrane topology of presenilins with a seven membrane-spanning and one membrane-embedded structure, as illustrated in Fig. 9A. According to this model, (a) the N-terminal region is located in the cytosolic space and the first six (HR1 to HR6) hydrophobic regions span the membrane in alternating directions; (b) HR7, HR8, and the large hydrophilic region between them are exposed to the cytosolic space; (c) HR9 does not span the membrane, but it is tightly associated with, most probably being embedded in, the lipid bilayer; and (d) HR10 spans the membrane and the C-terminal portion after this region is located in the luminal space.

The topology of the N-terminal half portion up to HR6 in our model is consistent with most of the others (24–26, 40), further confirming that the N terminus and the large hydrophilic loop region between HR7 and HR8 are exposed to the cytosol and that HR1 to HR6 span the membrane. Although our results demonstrate that HR7 does not span the membrane and is localized on the cytosolic face, it is still unclear from our results whether or not a part of this portion is associated with the membrane, as in one of the models proposed by Doan et al. (24). Based on the accessibility of region-specific antibodies to presenilin molecules expressed on the surface of unfixed living Drosophila cells, one group proposed that the N termini of cell surface presenilins are extracellular and that HR1 to HR6 span the membrane in opposite directions as compared with other models including ours (23). The reason for this discrepancy is unclear at present. Although it might be that presenilin molecules expressed on the cell surface show a distinct membrane topological structure from those retained on ER or ERGIC, it is also possible that some misassembled PS1 molecules with a non-physiologically inverted membrane topology caused by overexpression leak out onto the cell surface because their retention signal(s) to ER, ERGIC, or the Golgi apparatus cannot function correctly in this membrane topology.

Meanwhile, regarding the topological structure of the portion from HR8 to the C terminus of PS1 or its homolog, SEL-12, several different models have been proposed (24–26). First, according to our model, HR10 spans the membrane in the direction from the cytosol to the lumen, and the C terminus is exposed to the luminal space, whereas in all other models it is exposed to the cytosolic space. In some models (24, 26), HR10 is exposed to the cytosol, and in others (24), it spans the membrane in the opposite direction to that in our model. Although we cannot completely exclude the possibility that the LPase sequence we used as a reporter artificially enhanced the insertion of a naturally cytosolic HR10 segment into the membrane, or that it interferes with the stop transfer activity of HR10 non-physiologically, we think this is unlikely for the following reasons. First, in our fusion constructs LPase does not abolish the stop transfer ability of HR2, HR4, and HR6 when fused after them. Second, our three different reporter sequences, long and short versions of LPase with different N-terminal sequences and LPase with an extra membrane-spanning segment at the N-terminal end, gave consistent results. Third, when the N-glycosylation consensus sequence and hemagglutinin tag sequence were introduced into the extreme C-terminal region of

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**FIG. 6.** Fusion proteins expressed in cultured cells are capable of being assembled into the membrane with the same topology as those translated in vitro. COS-1 cells were transfected with the indicated plasmids, and the newly synthesized proteins were isotypically labeled in the presence of [35S]methionine and [35S]cysteine for 4 h. After the cells had been washed twice with PBS(--), the proteins were extracted. The extract from each lot of transfected cells was divided into three parts. One of them was treated with endoglycosidase H and another one was treated with the enzyme reaction buffer only. These two samples together with the untreated sample were immunoprecipitated with LP-1 and then separated by SDS-PAGE on 7.5% gels. The 35S-labeled proteins were detected by fluorography. All samples were exposed for 24 h except for PS1-ΔC-LP and PS1-full-LP, which were exposed for 4 days because the labeling efficiency was very low for these constructs. Lanes 1–3, PS1-ΔH6-C-LP; lanes 4–6, PS1-ΔH7-C-LP; lanes 7–9, PS1-ΔH8-C-LP; lanes 10–12, PS1-ΔH9-C-LP; lanes 13–15, PS1-ΔH10-C-LP; lanes 16–18, PS1-ΔC-LP; lanes 19–21, PS1-full-LP. Lanes 1, 4, 7, 10, 13, 16, and 19, untreated; lanes 2, 5, 8, 11, 14, 17, and 20, buffer-treated; lanes 3, 6, 9, 12, 15, 18, and 21, endoglycosidase H-treated. The positions of unglycosylated or glycosylated proteins are indicated by closed or open arrowheads on the right of each panel, respectively. The positions of prestained marker proteins (New England Biolabs) are indicated on the left of the fluorogram.
PS1, such a protein still underwent N-glycosylation, indicating that the luminal localization of the C-terminal portion of PS1 is not specific for the LPase fusion constructs we employed. Fourth, no previous study has demonstrated that a hydrophilic region interferes with the stop transfer activity or enhances the membrane anchoring ability of a preceding hydrophobic segment.

Some of the cytosolic C-terminal models are based on results obtained in experiments involving fusion proteins, as in this study. On the one hand, Lehmann et al. (25) constructed a series of fusion proteins in which C-terminally deleted PS1 is fused to a portion of prolactin with several artificial N-glycosylation consensus sequences at the C terminus, and the fusion proteins were expressed either in an in vitro translation system in the presence of the microsomal membrane or in cultured cells. Based on their observation that neither N-glycosylation in the reporter region nor protection of this region against cytosolically added proteinase K was detected when a chimera protein containing full-length PS1 was examined, they concluded that the C terminus of PS1 is located in the cytosolic space. On the other hand, Li and Greenwald (26) expressed in C. elegans a series of C-terminally deleted SEL-12, the counterpart of presenilins in the worm, fused to β-galactosidase, and they examined β-galactosidase activity using 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) staining in situ. They observed that the activity was observed when β-galactosidase was fused to the C-terminal portion of full-length SEL-12 and that it was lost when an additional artificial hydrophobic segment was inserted between SEL-12 and β-galactosidase. As it is empirically known that β-galactosidase activity is lost when it is translocated to the luminal face of the ER membrane, they concluded that the C terminus end of SEL-12 is exposed to the cytosolic space.

So, what is the cause for such discrepancies between our results and others? We think that the relatively hydrophobic nature of the C-terminal region of PS1 or SEL-12 could lead to artificial results unless care is taken when choosing the reporter protein to be fused. In fact, nine or eight out of 16 amino acid residues of the C-terminal region after HR10 of human PS1 or SEL-12, respectively, are hydrophobic. Although this
region is not hydrophobic enough to constitute solely a membrane-spanning stop-transfer segment, it could create a new additional artificial membrane-spanning segment when it is fused to a protein with a short hydrophobic region at its N-terminal end, which is not hydrophobic enough to have membrane-spanning ability itself. In such a case, the reporter region would be exposed to the cytosolic space despite the luminal localization of the free C-terminal end of these proteins. To prevent this, care was especially taken not to use a reporter sequence with an N terminus of a hydrophobic nature. Indeed, hydrophathy analysis of the amino acid sequence around the junction point of the PS1-prolactin fusion protein expected from the construction in the literature (25) revealed an additional hydrophobic segment which could span the membrane (data not shown), although we could not examine the case of SEL-12 because of a lack of precise sequence information regarding the fusion constructs in their report. Such a possibility will be tested by examining constructs in which the reporter portion is fused to PS1 or SEL-12 just after HR10, like in our PS1-ΔC-LP. If our prediction is correct, such experiments should provide supporting this view, we actually observed that N-glycosylation of the reporter portion or protection against proteinase K was very inefficient when the whole PS1 was fused to a portion of newt GH, whose N terminus is relatively hydrophobic (Fig. 5, right). In contrast, when a fusion construct of PS1-ΔC, in which the C-terminal portion of PS1 after HR10 was lacking, and GH were used, the two indices for membrane translocation were clearly positive (Fig. 5, middle). These results suggest that the extreme C-terminal portion of PS1 after HR10 became an additional eighth membrane-spanning segment when fused to GH. In the case of SEL-12, an alternative possibility that its C-terminal membrane topological structure is different from those of mammalian presenilins cannot be excluded.

Another line of experimental evidence for the cytosolic localization of the C terminus is the accessibility of antibodies specific to the C-terminal region of PS1 from the cytosolic space in cultured cells. Doan et al. (24) observed positive staining of intracellular structures when CHO cells or N2a cells overexpressing PS1 were probed with anti-C-terminal-specific polyclonal antibodies after treatment with streptolysin O, which is known to permeabilize specifically the plasma membrane without disrupting the membranes of intracellular organelles. Under such conditions, extracellularly added antibody molecules

FIG. 8. Interaction of HR8 and HR9 with the membrane. A, a 100,000 × g membrane fraction of the human placenta homogenate was prepared and suspended in 0.1 M sodium carbonate as described under "Materials and Methods." A portion was fractionated by centrifugation at 100,000 × g. Each fraction was analyzed for the presence of PS1-CTF by SDS-PAGE and Western blotting with a polyclonal antibody; PS1-LOOP: lane 1, total membrane fraction before sodium carbonate extraction; lanes 2 and 3, supernatant and pellet fractions, respectively, obtained on 100,000 × g centrifugation after sodium carbonate extraction. The positions of CTF are indicated by closed or open arrowhead on the right of the panel. The upper bands (open arrowhead) may represent a phosphorylated molecular species. The full-length form is indicated by an asterisk on the right of the panel. The positions of prestained marker proteins (New England Biolabs) are indicated on the left of the fluorograms. B, PS1-CTF-ΔH9-C-LP, PS1-CTF-ΔH10-C-LP, and PS1-CTF-ΔC-LP were transcribed and translated in vitro as in Fig. 3 together with PS1-ΔH6-C-LP as an internal control in the presence of MS. Each sample was divided into four parts, three of which were extracted with solutions containing the following reagents: lanes 2 and 3, 20 mM HEPES, pH 7.6, 0.25 M sucrose, 1% Triton (buffer); lanes 4 and 5, 0.1 M Na2CO3 (Na2CO3); lanes 6 and 7, 20 mM HEPES, pH 7.6, 0.25 M sucrose, 1% Triton X-100 (buffer + Triton), for each panel, respectively. After centrifugation at 100,000 × g for 1 h, the supernatants (S, lanes 2, 4, and 6) and pellets (P, lanes 3, 5, and 7) were separated by SDS-PAGE together with total unextracted samples (T, lane 1), and labeled proteins were detected by fluorography. The positions of glycosylated or unglycosylated translation products for PS1-ΔH9-C-LP, PS1-CTF-ΔH10-C-LP, and PS1-CTF-ΔC-LP (open or closed arrowheads, respectively) as well as the doublet bands for glycosylated and unglycosylated translation products of PS1-ΔH6-C-LP (open and closed arrowheads, respectively) are indicated. The positions of prestained marker proteins (New England Biolabs) are indicated on the left of the fluorograms.
can diffuse into the cytosol through the plasma membrane but are not expected to go into the luminal space of intracellular organelles. Based on these observations, they concluded that the C terminus is exposed to the cytosolic space. However, in our view, although these data show the existence of PS1 molecules with the C terminus exposed to the cytosol in PS1-overexpressing cultured cells, they do not logically exclude the possibility of the existence of a molecular species whose C terminus is topologically exposed to the luminal space. So, we think it is highly possible that two topologically distinct molecular species co-exist in these cells. Supporting this possibility, when the PS1-LPase fusion protein was expressed transiently in COS-1 cells, we occasionally observed, in addition to a predominant band corresponding to the N-glycosylated form, a minor band corresponding to the unglycosylated form (for example, see Fig. 6, lanes 16–21). The latter may represent those that failed to translocate the C-terminal end of PS1, together with the reporter portion, to the luminal space and may correspond to the molecular species that Doan et al. (24) observed.

The molecular species of PS1 with the C-terminal region facing the cytosol might be an artifact of overexpression. In fact, although Doan et al. (24) also examined stably transfected cell lines, these lines may still overexpress PS1 when compared with cells expressing endogenous PS1. An alternative interpretation would be that both topological species exist physiologically. They might have distinct functions, as suggested for several other proteins (42–45). For example, the C-terminal region might interact with distinct cellular factors in the cytosol and luminal space of ER. Alternatively, cells might regulate the ratio of these two forms dynamically in a post-translational manner by actively translocating the C-terminal region to and fro across the membrane in response to some signals. For example, upon processing or interaction with other factors, one of the forms will be converted to the other. In fact, a protein exhibiting a change in its topology post-translationally has been recently reported (46). In this regard, it will be important to determine whether or not presenilins with the C terminus exposed to the cytosol can also be observed in cells physiologically without overexpressing them and to search for cytosolic or luminal factors interacting with the C terminus of PS1.

It is also an arguable point as to whether or not HR9 spans the membrane. Our results suggest strongly that this segment does not span the membrane, which is consistent with the results of Lehman et al. (25). Consistent results obtained with two different fusion strategies reinforce this idea and make it unlikely that this is caused by an unusual property of the reporter protein fused after this segment. Furthermore, we also showed that, at least when the C-terminal half region of PS1 from the 298th methionine is expressed separately, HR9 interacts with the membrane tightly. The N-glycosylation and proteinase K protection patterns of the fusion proteins starting from two different residues, the 1st and 298th methionines, suggest strongly that, in the full-length PS1, HR9 is also inserted into the lipid bilayer without spanning the membrane. Previously, similar structures have been implicated to exist in several polytopic membrane proteins such as subunits of several ion channels including glutamate receptors (47–49). Additionally, a glycine residue (Gly-417) is present at approximately the center of HR9. As a glycine residue is known to tend to form a turn in the secondary structure of a polypeptide (50), it is possible that HR9 forms a bending structure in the lipid bilayer which prevents it from spanning the membrane and facilitates the embedded topological structure, as illustrated in Fig. 9.

On the contrary, the experimental evidence in the literature supporting the membrane spanning of HR9 seems to be weak. On the one hand, Li and Greenwald (26) examined the activity of β-galactosidase as a reporter protein, which was fused after HR9 of SEL-12, and detected such activity, implying that the C-terminal flanking region of HR9 is exposed to the cytosol. In order to determine the location of the region between HR8 and HR9, they examined the activity of β-galactosidase when this enzyme was fused after HR8 or after an artificial extra membrane-spanning segment which was placed just after HR8, but no activity was detected for either of the fusion proteins. So, they further examined similar chimera proteins in which HR8 was deleted and detected such activity only in the presence of the artificial membrane-spanning segment. From this result, they argued that HR9 spans the membrane. However, although this result implies that HR9 could span the membrane from the cytosol to the lumen of ER in some situations, it does not necessarily prove that HR9 spans the membrane from the lumen to the cytosol as in their model. Their result implies that
HR9 spans the membrane from the cytosol to the lumenal space, which apparently contradicts our model. One interpretation explaining this would be that some of the missing sequence in their HR8-deleted construct is required for the correct topogenesis of HR9. For example, the whole HR8 region or a part of it might be needed for such an embedded structure of HR9 through its direct interaction with HR9. Another possibility would be that the newly exposed flanking sequence before HR9 in the HR8-deleted construct perturbed the topogenesis of HR9. We are now examining such possibilities. On the other hand, Doan et al. (24) proposed two possible models in which both HR9 and HR10 span the membrane. However, no description of the experimental evidence for such a topological structure of these segments could be found in their report.

As to HR8, their results suggest that it does not span the membrane. With PS1-ΔH9-C-LP as well as with PS1-CTF-ΔH9-C-LP, in which the reporter LPase was fused just after HR8, neither N-glycosylated nor protein K-protected LPase was observed in contrast to the case of PS1-ΔH9-C-TM-LP, where the two criteria for the translocation were clearly positive. Moreover, the results of sodium carbonate extraction experiments with PS1-CTF-ΔH9-C-LP strongly suggested that there is no physical interaction between HR8 and the membrane. On the contrary, Li and Greenwald (27) claimed that HR8 spans the membrane based on the observation that deletion of this segment of PS1 or SEL-12 (26) changes the localization of β-galactosidase fused after HR9 from the cytosol to the lumen. However, as discussed above, these results can also be explained by our model assuming that the N-terminal flanking region of HR9 affects its topology. More recently, in experiments involving an altered HR8 made more hydrophobic by the addition of some hydrophobic amino acid residues, the same group obtained results suggesting that this altered HR8 spans the membrane from the cytosol to the lumen (27). However, based on our results, we consider it highly likely that their results are artifacts caused by the alteration of HR8. Although they also showed that SEL-12 with this altered HR8 exhibits complementing ability as to the egg-laying defect of the SEL-12 mutant worm, it is also possible that whether or not HR8 spans the membrane is not important for the complementing activity of SEL-12.

To summarize, in this study, we obtained evidence for the existence of PS1 molecules with a seven membrane-spanning activity of SEL-12. HR8 spans the membrane is not important for the complementing ability as to the egg-laying defect of the egg-laying defect of the SEL-12 mutant worm, which apparently contradicts our model. One interpretation explaining this would be that some of the missing sequence in their HR8-deleted construct perturbed the topogenesis of HR9. For example, the whole HR8 region or a part of it might be needed for such an embedded structure of HR9 through its direct interaction with HR9. Another possibility would be that the newly exposed flanking sequence before HR9 in the HR8-deleted construct perturbed the topogenesis of HR9. We are now examining such possibilities. On the other hand, Doan et al. (24) proposed two possible models in which both HR9 and HR10 span the membrane. However, no description of the experimental evidence for such a topological structure of these segments could be found in their report.

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To summarize, in this study, we obtained evidence for the existence of PS1 molecules with a seven membrane-spanning and one membrane-embedded topology, in which tight binding of HR9 to the membrane without spanning it, membrane spanning of HR10 from the cytosolic to the luminal face, and luminal localization of the C-terminal novel features. Further study is necessary to elucidate the biological meaning of these structural aspects. For example, exposure of the C-terminals to the luminal space might be essential for this protein to undergo proper processing, which is known to occur normally in the cell, or for a specific interaction of this protein with an unknown cellular factor localized to the lumen of ER or the Golgi apparatus. Experiments are underway to assess these possibilities.

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