Evidence for a Six-transmembrane Domain Structure of Presenilin 1*

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Sylvain Lehmann‡, Roberto Chiesa§, and David A. Harris¶

From the Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

Mutations in genes encoding presenilin 1 and presenilin 2 account for the majority of cases of early-onset familial Alzheimer's disease. The presenilins have been localized to the endoplasmic reticulum and Golgi, but which of the multiple hydrophobic segments of the polypeptide chain span the lipid bilayer is unclear. To address this question, we have constructed a series of chimeric molecules in which a topologically neutral reporter protein (a C-terminal fragment of prolactin) containing three artificial glycosylation sites is fused to presenilin 1 following each of the 10 potential transmembrane domains identified in hydropathy plots. We have expressed these chimeras by translation in reticulocyte lysate containing canine pancreatic microsomes and by synthesis in transfected COS cells. Based on utilization of the glycosylation sites and sensitivity of the reporter to protease digestion, we provide evidence that presenilin 1 has six transmembrane segments with the N and C termini in the cytoplasm. This model provides important clues to the potential functions of different parts of the presenilin molecule and how these might relate to the pathogenesis of Alzheimer's disease.

Alzheimer's disease (AD)† is a debilitating neurodegenerative disorder that is characterized by dementia, neuronal loss, and accumulation in the brain of extracellular amyloid plaques and intraneuronal neurofibrillary tangles (1). Familial forms of AD have been linked to mutations in three different genes, encoding the amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2) on chromosomes 21, 14, and 1, respectively (2–5). Over 50% of the cases of early-onset familial AD are attributable to mutations in the presenilin genes. The mutations that have been described include over 35 different amino acid substitutions, as well as an in-frame deletion of exon 9 (reviewed in Ref. 6).

PS1 and PS2 are polypeptides of 467 and 448 amino acids, respectively, that have multiple hydrophobic segments (2, 4, 5). These proteins are expressed in a number of tissues in addition to brain and are encoded by several alternatively spliced mRNAs (4, 7, 8). The presenilins exhibit significant homology to a protein from Caenorhabditis elegans called SEL-12, which, based on genetic evidence, modulates signaling by the LIN-12/Notch pathway (9). They show weaker homology to SPE-4, a membrane protein of C. elegans sperm (10). Immunofluorescence localization studies of transfected cells indicate that the protein is present at steady-state primarily in the ER, and perhaps in the Golgi also, but probably not on the plasma membrane (11). In a neuronal cell line, PS1 expressed using a viral vector is localized to the cell body and dendrites (12).

Recent evidence indicates that PS1 is proteolytically cleaved between amino acids 260 and 320 in transfected cells as well as in brain tissue, although the significance of this cleavage is unclear (13). There are reports that recombinant forms of PS2 can positively regulate apoptosis in lymphocytes and PC12 cells, but whether the endogenous protein normally functions in this way is unclear (14, 15). Taken together, none of the available evidence clearly identifies the physiological role of the presenilins. However, analysis of patient fibroblasts, transfected cells, and transgenic mice that co-express PS1 and APP indicate that pathogenic mutations in the presenilin protein enhance production of the amyloidogenic Aβ42 fragment by 1.5–2-fold (16–19).

Determination of the membrane topology of the presenilins, in other words defining which parts of the polypeptide chain traverse the lipid bilayer, is of enormous importance in understanding the function of these proteins and their role in AD. There are several reasons for this. First, knowing which domains of the polypeptide lie in the cytoplasm and which in the lumen of the ER or Golgi will dictate hypotheses about the function of these domains. Second, experimental attempts to identify cytoplasmic proteins that interact with the presenilins using screening procedures such as the yeast two-hybrid system or interaction cloning require a knowledge of which segments of the polypeptide chain lie in the cytoplasm. Third, the membrane topology of the presenilins can be correlated with the positions of pathogenic mutations, providing clues to how these mutations alter the function of the proteins. Finally, knowledge of the membrane topology of the presenilins will allow a more detailed comparison with possible homologues than is possible simply on the basis of hydropathy plots.

We report here our analysis of the membrane topology of PS1 using both in vitro and in vivo expression systems. Our results suggest that the protein spans the membrane six times, with both N and C termini located in the cytoplasm. This model stands in marked contrast to ones originally proposed on the basis of hydropathy plots that hypothesized either seven or nine transmembrane domains for PS1 and PS2 (2, 4, 5, 20). Our conclusions are consistent with and significantly extend those of a recent report, suggesting that PS1 possesses either six or eight transmembrane segments (21), but they differ from those...
of a second study proposing an eight-transmembrane topology for the presenilin homologue SEL-12 (22).

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The PS1 segments of HR-PRL chimeras were generated by PCR, using as a template a PS1 cDNA derived from the RNA splice variant lacking exon 4 (which encodes the sequence VSRQ) (provided by Dr. Alison Goate (Ref. 8)). The 5′ primer was GATCAGGAAT-CCGTTAGTGCAGTTGTTGTTGTAGATGAT. The PS1 fragments were digested with EcoRI and BstEII, and the reporter fragment with BstEII and XbaI. The fragments were ligated into EcoRI/XbaI-digested pcDNA3 (Invitrogen). All constructs were sequenced in their entirety.

In Vitro Transcription and Translation—Plasmids were linearized with XbaI and capped mRNAs were synthesized on T7 RNA polymerase using a kit from Ambion. Rabbit reticulocyte lysate and canine pancreatic microsomes were obtained from Promega and used to translate mRNAs in the presence of [35S]methionine as described by the manufacturer. Protease K treatment of translation reactions was performed at final concentration of 50 μg/ml for 1 h on ice in the presence or absence of 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.5% SDS) supplemented with protease inhibitors (peptatin and leupeptin, 1 μg/ml; phenylmethylsulfonyl fluoride, 0.5 mm; EDTA, 2 mM). After 5 min at 25 °C, 160 μl of dilution buffer (50 mM NaCl, 0.5% Triton X-100) containing protease inhibitors was added, and the samples were precleared with protein A-Sepharose beads. Supernatants were then incubated with one of the following rabbit antibodies: anti-HA (Babco), anti-bovine prolactin (Dr. A. F. Parlow), or anti-PS1 (prepared by Dr. Jeanne Nerbonne against a synthetic peptide encompassing residues 26–42). Immunocomplexes were then isolated with protein A-Sepharose beads and analyzed by SDS-PAGE and autoradiography. Samples were heated at 60 °C for 5 min prior to SDS-PAGE, since boiling results in aggregation of PS1 proteins (23).

**COS Cells**—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 1 mM pyruvate, and penicillin/streptomycin in an atmosphere of 5% CO2, 95% air. Cells were transfected with LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s directions and 48 h later were labeled for 3 h with [35S]methionine. Labeled cultures were extracted with lysis buffer and PS1-PRL proteins immunoprecipitated with anti-HA antibody as described above. For protease digestion experiments, labeled cells were collected in phosphate-buffered saline and gently homogenized by passage eight times through 0.25-mm diameter plastic tubing attached at each end to a 27-gauge needle on a syringe. After centrifugation at 700 × g for 3 min, the postnuclear supernatant was treated with proteinase K in the presence or absence of Triton X-100 and proteins immunoprecipitated as described above.

**N-Glycosidase F**—After addition of lysis and dilution buffers as described above, reticulocyte lysate was treated with 0.025 unit/ml of N-glycosidase F (Boehringer Mannheim) for 6 h at 37 °C. Proteins were then precipitated with 4 volumes of methanol and analyzed by SDS-PAGE and autoradiography. Diluted lysates of COS cells were treated with 0.01 unit/ml of the enzyme for 16 h at 37 °C prior to immunoprecipitation.

**RESULTS AND DISCUSSION**

Hydrophobicity plots identify 10 segments of at least 15 amino acids in the sequence of PS1 that could potentially span the membrane (Fig. 1A). We have constructed chimeras in which a prolactin reporter is fused after each of these hydrophobic regions (Fig. 1B). The reporter is a C-terminal fragment of bovine prolactin (residues 82–229) onto which we have appended three consensus sites for N-glycosylation, as well as an HA tag so that the proteins can be recognized by anti-HA as well as anti-prolactin antibodies. A number of studies have utilized this portion of prolactin as a reporter, since it lacks any signals that might influence the membrane orientation of the adjacent polypeptide (24–26). When the fusion proteins are synthesized on membrane-attached ribosomes, the reporter will be glycosylated and protected from proteases applied from the cytoplasmic surface only if it resides in the lumen of the ER, which is topologically equivalent to the extracellular space. The two techniques we have combined here, tagging a protein with artificial glycosylation sites and fusing it to a reporter molecule that can be recognized by antibody binding or enzymatic activity, have been used individually to map the topology of a number of other membrane proteins (27, 28). We recognize that although these techniques can provide evidence in support of a specific model of membrane topology, it is always possible that experimental modifications of the polypeptide chain may perturb its interaction with the lipid bilayer.

We first translated synthetic mRNAs encoding the PS1-prolactin chimeras in a reticulocyte lysate system. In the absence of canine pancreatic microsomes, proteins ranging in size from 33 to 70 kDa were recovered after immunoprecipitation with anti-HA antibody, corresponding to the unglycosylated fusion proteins (Fig. 2A, lane 1). When translation was performed in the presence of microsomes, HR1-PRL, HR3-PRL, and HR5-PRL (but not the other constructs) yielded additional products...
of slightly higher molecular weight (Fig. 2A, lane 2). These larger products represent glycosylated forms of the proteins, since they are eliminated when samples are treated with N-glycosidase F (Fig. 2B, lanes 3 and 4). As has been observed by others (25, 26), glycosylation in the in vitro system is inefficient, resulting in the presence of some unglycosylated protein even when translation is performed with microsomes (Fig. 2A, lane 2; Fig. 2B, lane 3). We noted that PS1-PRL fusion proteins often migrated as closely spaced doublets on SDS-PAGE (see Fig. 2B, lanes 1 and 2); this phenomenon has been observed for full-length PS1 synthesized both in vitro and in transfected cells and is likely to result from an uncharacterized posttranslational modification (11, 12, 29).

HR1-PRL, HR3-PRL, and HR5-PRL were also the only three chimeras that produced a protected fragment reactive with anti-HA antibody after treatment of the microsome-containing translation mixture with proteinase K (Fig. 2A, lane 3). The protected fragment was glycosylated (not shown) and migrated approximately 7 kDa lower than the corresponding intact glycosylated protein (Fig. 2A, lane 2). The fragment was eliminated when microsomes were permeabilized with Triton X-100 prior to protease digestion (Fig. 2A, lane 4), demonstrating that it does not represent an intrinsically protease-resistant portion of the molecule. The protected fragment could be immunopre-
Membrane Topology of Presenilin 1

Fig. 5. Model for the membrane topology of PS1. Although our data indicate that HR7–HR10 do not span the membrane, one or more of these hydrophobic segments may associate closely with the lipid bilayer.

We expressed each of the PS1-PRL fusion proteins in COS cells. We again observed that only HR1-, HR3-, and HR5-PRL were glycosylated, as indicated by a reduction in the molecular weight of these proteins upon treatment with N-glycosidase F (Fig. 4A). In contrast to the situation in reticulocyte lysate, glycosylation of these chimeras in COS cells was essentially quantitative, making it unlikely that the proteins are heterogeneous with respect to localization of the reporter. Each of these three fusion proteins also produces a protected fragment that is ~7 kDa smaller than the intact protein following proteinase K digestion of a postnuclear supernatant derived from the transfected cells (Fig. 4B); the other seven fusion proteins do not yield any protected fragments (data not shown). Results similar to those shown in Fig. 4 have also been obtained in transfected Chinese hamster ovary cells (not shown).

Taken together, the results of glycosylation site utilization and protease protection both in vitro and in cells suggest the model of PS1 topology shown in Fig. 5. HR1, HR3, and HR5 span the membrane from the cytoplasmic to the luminal side and HR2, HR4, and HR6 from the luminal to the cytoplasmic side. Both the N and C termini are cytoplasmic. HR7–HR10 do not constitute transmembrane domains, although our analysis does not address the issue of whether these segments associate closely with the lipid bilayer in the manner of “reentrant loops” described for some channel proteins (25, 27, 30). This model places the two consensus sites for N-linked glycosylation (asparagine residues 275 and 401) in the cytoplasm, consistent with the fact that full-length PS1 expressed in cells or by in vitro translation is not glycosylated (12, 23, 29). Given the high degree of homology between PS1 and PS2, we would predict that PS2 has the same membrane topology shown in Fig. 5. Although our data support a six-transmembrane domain structure for PS1, it is of course possible that the membrane topology of the native protein is different from the one we have inferred here based on the properties of PS1-prolactin fusions. However, the fusion of a reporter segment or the introduction of ectopic glycosylation sites has not been found to disturb the topology of a number of other membrane proteins (27, 28).

Our results are consistent with those of Doan et al. (21) who concluded that PS1 has either six or eight transmembrane domains (their data did not distinguish between the two possibilities), with the N terminus, C terminus, and part of the “loop” region between residues 320 and 390 in the cytoplasm. However, our results differ from those of Li and Greenwald (22) who proposed a model for the C. elegans PS1 homologue SEL-12 having eight transmembrane segments, including HR1–HR6, HR-8, and HR-9. We think it unlikely on theoretical grounds that HR8 of PS1 spans the membrane, since it is short (16 amino acids) and is only marginally hydrophobic (see Fig. 4A). The identification of HR8 and HR9 as transmembrane segments in SEL-12 was inferred indirectly from the enzymatic activity of a pair of β-galactosidase fusions in which HR8 was deleted, and it is possible that this deletion modified the membrane topology of the protein or altered the activity of the galactosidase fusion. It is also conceivable that the membrane topologies of PS1 and SEL-12 are different.

Our model for the topology of PS1 has important implications for the function of the protein and its role in AD. We find that most of the polypeptide chain of PS1 is cytoplasmic, including the N-terminal region and a long C-terminal tail encompassing HR7–HR10. These segments may therefore be important in interactions with cytoplasmic proteins. Much of the cytoplasmic region from HR6 to the C terminus, exclusive of the segment between HR7 and HR8, is highly conserved in the presenilins and SEL-12, suggesting essential functions for this domain. A number of pathogenic mutations are found between the end of HR6 and the end of HR7 (reviewed in Ref. 6), and one might speculate that these alter associations of PS1 with molecules in the cytoplasm. Since little of the polypeptide chain protrudes into the ER lumen, a direct interaction between PS1 and luminal proteins seems unlikely, although associations with other ER membrane proteins are possible. If PS1 binds to APP, a possibility suggested by enhanced production of the Aβ 42 fragment in cells expressing mutant PS1 (16–19), this interaction would most likely occur transiently during transit of APP through the ER and Golgi and would probably involve either the transmembrane region or short cytoplasmic tail of APP.

The topology model proposed here makes it unlikely that PS1 functions as a conventional G-protein-coupled receptor, but is consistent with a role for PS1 in processes such as vesicle docking and budding, membrane-cytoskeletal interactions, or ion and solute transport. The recently described endoproteolytic cleavage of PS1 (13), which is predicted to occur following HR6, would be expected to generate a C-terminal fragment that lacks any transmembrane segments. Whether this fragment is soluble would depend on how tightly HR7–HR10 are associated with the lipid bilayer, but if it were diffusible it could serve to transmit a signal to other regions of the cell. Knowing the membrane topology of PS1 will now facilitate attempts to isolate interacting molecules and provide a starting point for further investigations of the biological activity of normal and mutant forms of the protein.

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Membrane Topology of Presenilin 1

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