Functional Analysis of the Transmembrane Domains of Presenilin 1

PARTICIPATION OF TRANSMEMBRANE DOMAINS 2 AND 6 IN THE FORMATION OF INITIAL SUBSTRATE-BINDING SITE OF γ-SECRETASE

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γ-Secretase is a multimeric membrane protein complex composed of presenilin (PS), nicastrin, Aph-1, and Pen-2, which mediates intramembrane proteolysis of a range of type I transmembrane proteins. We previously analyzed the functional roles of the N-terminal transmembrane domains (TMDs) 1–6 of PS1 in the assembly and proteolytic activity of the γ-secretase using a series of TMD-swap PS1 mutants. Here we applied the TMD-swap method to all the TMDs of PS1 for the structure-function analysis of the proteolytic mechanism of γ-secretase. We found that TMD2- or -6-swapped mutant PS1 failed to bind the helical peptide-based, substrate-mimic γ-secretase inhibitor. Cross-linking experiments revealed that both TMD2 and TMD6 of PS1 locate in proximity to the TMD9, the latter being implicated in the initial substrate binding. Taken together, our data suggest that TMD2 and the luminal side of TMD6 are involved in the formation of the initial substrate-binding site of the γ-secretase complex.

Mutations in PS genes are linked to the early onset familial Alzheimer disease. Familial Alzheimer disease-linked PS mutations influence the processing of APP, leading to an overproduction of amyloid peptide ending at the 42nd residue (Aβ42) that is more prone to forming amyloid deposits. Thus, PS-γ-secretase complex is a plausible therapeutic target for the treatment of Alzheimer disease (1, 2).

PS is a highly conserved polytopic membrane protein with nine transmembrane domains (TMDs), and two conserved aspartates in PS (i.e. Asp237 in TMD6 and Asp385 in TMD7) comprise the catalytic site of the γ-secretase (2). Chemical biological approaches using γ-secretase inhibitors (GSIs) revealed its unusual enzymatic characters; immobilized transition state analogue-type GSIs directly target PS NTF/CTF heterodimer and co-purify with γ-secretase substrates (4). In contrast, designed helical peptide-type GSI binds to PS irrespective of the presence or absence of transition state analogue-type GSIs that occupy the catalytic site (5, 6). These findings strongly suggested that γ-secretase has an “initial substrate-binding site” that is distinct from the catalytic site (2).

During the biosynthetic process of the γ-secretase complex, Nct and Aph-1 form a heterodimeric subcomplex and bind to PS (7, 8). Pen-2 then is assembled to the heterotrimeric complex and triggers the endoproteolysis of PS to generate N- and C-terminal fragments (NTF and CTF), which represent the catalytically active form (3). PS forms a catalytic pore structure in which catalytic aspartates face a hydrophilic environment, enabling the proteolysis of hydrophobic substrates within the lipid bilayer (9–11). However, information regarding the initial substrate-binding site within PS at the molecular level is still lacking. Using TMD-swap mutants of PS1 that are replaced at each one of the TMDs of the NTF with the TMDs of irrelevant proteins, we have shown that all the PS1 TMDs in NTF (i.e. TMDs 1–6), except for TMD3, are required for the acquisition of the γ-secretase activity (12). We further showed that PS1 TMD4 represents the direct binding region of Pen-2. Here we further systematically analyzed the TMD-swap mutants of PS1 and revealed the stepwise formation of the functional γ-secretase complex. Notably, we found that TMD2 and the luminal side of TMD6 of PS1 contribute to the formation of the initial substrate-binding site of the γ-secretase complex.
EXPERIMENTAL PROCEDURES

Plasmid Construction, Cell Culture, Transfection, and Retroviral Infection—cDNAs encoding PS1, APP carrying Swedish mutation (APP<sub>Sw</sub>), and NotchAE were inserted into pMXspuro (9, 11–13). cDNAs encoding mutant PS1 were generated by long PCR-based QuickChange<sup>TM</sup> strategy (Stratagene). All constructs were sequenced using Thermo Sequenase (USB Corp., Cleveland, OH) on an automated sequencer (Li-Cor, Lincoln, NE) (see Table 1). Maintenance of DKO cells, viral packaging in Plat-E cells, retroviral infection, and generation of stable infectant pools were done as described previously (9, 11–14).

Antibodies and Immunochromatographic Analysis—Anti-G1N<sub>2</sub>, G1L3, and PNT3 antibodies against glutathione S-transferase-fused human PS1 N terminus, glutathione S-transferase-fused human PS1 loop, or a synthetic peptide corresponding to the N-terminal 26 amino acids of human/mouse Pen-2, respectively, have been previously described (3, 15, 16). Anti-PS1<sub>NT</sub>, anti-PS-C<sub>3</sub>, and anti-SPP<sub>ct</sub> antibodies were kindly provided by Drs. G. Thinakaran (University of Chicago, Chicago, IL), A. Takashima (RIKEN, Saitama, Japan), and T. E. Golde (Mayo Clinic, Jacksonville, FL), respectively (17–19). The monoclonal antibody anti-α-tubulin AA4.3 developed by Dr. C. Walsh was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by The University of Iowa, Department of Biology, Iowa City, IA. Other antibodies were purchased from Chemicon (anti-PS1 loop (MAB5232)), Cell Signaling Technology (anti-cleaved Notch1 (V1744)), Covance (anti-APh-1aL (O2C2)), Santa Cruz Biotechnology (anti-Nct (O2C2)), and Bio-Rad (anti-α-tubulin). Reduced glutathione and bovine serum albumin were purchased from Sigma-Aldrich. Other chemicals were from Fisher Scientific.

Membrane Fractionation, Immunoblot Analysis, Immunoprecipitation of CHAPSO-solubilized lysates, in vitro γ-secretase assay, cycloheximide treatment, trypsin digestion, or quantitation of Aβ by two-site ELISAs using BNT77 as a capture antibody were performed as described previously (3, 8, 9, 11–16, 20).

Blue Native-PAGE (BN-PAGE) Analysis—BN-PAGE was performed according to the manufacturer's protocol (Invitrogen). Briefly, membrane fractions were suspended in Native PAGE<sup>™</sup> sample buffer containing 1% digitonin (Wako Biochemicals). The mixtures were centrifuged for 10 min at 15,000 × g and Coomassie Brilliant Blue was added to the supernatant to give a final concentration of 0.25% w/v. NativeMark<sup>™</sup> unstained protein standard (Invitrogen) was used as a molecular weight standard. After electrophoresis, the gel was transferred to polyvinylidene difluoride membranes. The membranes were destained briefly in methanol before being incubated with specific antibodies.

Compounds, Photoaffinity Labeling, Substituted Cysteine-scanning Method (SCAM), and Cross-linking Experiments—L-685,458, peptide 11 (pep.11) and pep.11-Bt were purchased from Bachem (Torrance, CA), Ito Life Sciences (Moriya, Japan), and BEX (Tokyo, Japan), respectively. L-852,646 was kindly provided by Dr. Yueming Li (21). All methanethiosulfonate (MTS) reagents (Toronto Research Chemicals) were dissolved in dimethyl sulfoxide (DMSO) at 200 μM prior to use or stored at −80 degree until use. Photoaffinity labeling, SCAM, and cross-linking experiments were performed as described previously (6, 9, 11, 22, 23).

RESULTS

Transmembrane Domains 8 and 9 of Presenilin 1 Are Required for the γ-Secretase Activity—We have previously analyzed the functional role(s) of the PS TMDs using TMD-swap mutants (12). We applied this strategy to PS1 CTF and constructed cDNAs encoding PS1 mutants (i.e. TM8mt, TM9mt) in which TMD8 (408–428 amino acids) or TMD9 (434–453 amino acids) was replaced with TMDs of functionally unrelated transmembrane proteins, i.e. CD4 (type I transmembrane protein) or CLAC-P (type II transmembrane protein), respectively (Table 1). As reported previously, overexpression of wild-type (WT) PS1 in DKO cells resulted in the generation of PS fragments and recovered the levels of mature Nct and the accumulation of Pen-2 (Fig. 1A) (12). Both TM8mt and TM9mt also rescued the accumulation of mature Nct and Pen-2, although the level of mature Nct in DKO cells expressing TM8mt was lower than that in cells expressing WT PS1. However, endoproteolysis of TM8mt or TM9mt PS1 was not observed. We next analyzed the γ-secretase activities of the TMD-swap mutants in DKO cells. Unexpectedly, neither TM8mt nor TM9mt recovered the γ-secretase activity to generate NICD or Aβ in cell-based assays (Fig. 1, B and C). These TMD-swap mutants also displayed no proteolytic activity in vitro assays (data not shown).

To address whether replacement of the PS1 TMDs interferes with the formation of γ-secretase complex, we analyzed the CHAPSO lysates of DKO cells expressing WT or mutant PS1 by co-immunoprecipitation (Fig. 1D). Upon overexpression of WT PS1, all other γ-secretase components (i.e. Nct, Aph-1aL, and Pen-2) were co-precipitated with WT PS1. In contrast, TM4mt and the C-terminal deletion mutant PS1 that lacks the C-terminal 12 amino acids (i.e. PS1/455stop) failed to bind the Pen-2 or Nct-Aph-1 subcomplex, respectively, in accordance with previous reports (12, 24, 25). Notably, all TMD-swap mutants including TM8mt and TM9mt interacted with Nct, Aph-1aL, and Pen-2, indicating that the primary amino acid sequences of TMD8 or -9 are dispensable to the interaction with γ-secretase components. Taken together, these results indicate that the primary structures of PS1 TMD8 and -9 con-

### TABLE 1

| TM1mt | TM2mt | TM3mt | TM4mt | TM5mt | TM6mt | TM8mt | TM9mt |
|-------|-------|-------|-------|-------|-------|-------|-------|
| 408IACFVAILIGLCLTLLLLAIF428 | 408MALIVLGGVAGLLLFIGLGIF428 | 408MALIVLGGVAGLLLFIGLGIF428 | 408MALIVLGGVAGLLLFIGLGIF428 | 408MALIVLGGVAGLLLFIGLGIF428 | 408MALIVLGGVAGLLLFIGLGIF428 | 408MALIVLGGVAGLLLFIGLGIF428 | 408MALIVLGGVAGLLLFIGLGIF428 |
| PS1 TMD1 | CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P |
| CLAC-P | PS1 TMD2 | CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P |
| CLAC-P | CLAC-P | PS1 TMD3 | CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P |
| CLAC-P | CLAC-P | CLAC-P | PS1 TMD4 | CLAC-P | CLAC-P | CLAC-P | CLAC-P |
| CLAC-P | CLAC-P | CLAC-P | CLAC-P | PS1 TMD5 | CLAC-P | CLAC-P | CLAC-P |
| CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P |
| CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P | CLAC-P |

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*Initial Substrate-binding Site of γ-Secretase*
Intramolecular Complementation Assay by Co-expression of NTF and CTF Individually in DKO Cells

—Several lines of evidence suggest that the biologically active form of PS is the NTF/CTF heterodimer, although the functional significance of endoproteolysis remains to be elucidated. To ask whether ineffective endoproteolysis inhibited the proteolytic activity of the TMD-swap PS mutant, we further examined the effects of TMD-swaps in an intramolecular complementation analysis by co-expression of NTF and CTF in DKO cells (Fig. 2A). The level of protein expression of TM4mt NTF was relatively low, supporting the notion that the interaction of PS1 with Pen-2 is involved in the stabilization of the γ-secretase complex (12, 26) (see below). As reported, expression of WT NTF and WT CTF from separate vectors was able to restore the γ-secretase activity (25) (Fig. 2B). Interestingly, co-expression of TM3mt NTF with WT CTF improved the total Aβ-generating as well as the NICD-generating activities of γ-secretase more than those in cells expressing the TM3mt holoprotein (12). In addition, TM3mt showed a significant decrease in the production of Aβ40 and a reciprocal increase in that of Aβ42, respectively. Of note, the same trend in the generation of Aβ40 and Aβ42 had been observed in holoprotein-based TM3mt PS1 (12). In fact, the total levels of Aβ and NICD were almost similar to those in cells expressing WT NTF/WT CTF (n = 3, p = 0.12 (for Aβ) and p = 0.30 (for NICD) by Student’s t test). This result suggests that the TMD3 of PS1 harbors as yet unknown function to promote the γ-secretase activity after endoproteolysis regardless of the substrates. Further analysis on the role of TMD3 on substrates other than APP and Notch would be required.

Intriguingly, some residues on TMD3 have been implicated in the determination of PS1/PS2 specificity of a subset of the γ-secretase inhibitors (26). Nevertheless, these data support the idea that TMD3 is involved in the acquisition of proper cleavage activity of the γ-secretase after the assembly of the complex.

However, none of the other TMD-swap mutants expressed in an endoproteolyzed form rescued any γ-secretase activity, suggesting that the structures of each of these TMDs of PS1, other than TMD4, are indispensable to the acquisition of γ-secretase activity, independently from their roles in PS endoproteolysis.

TMD1, -5, -8, and -9 Are Required for the Stability, but Not the Formation, of the HMW Complex

—During the assembly of the functional γ-secretase complex, Nct acquires resistance against trypsin and undergoes the complex-type N-glycosylation (27). These posttranslational modifications represent conformational maturation of the extracellular domain of Nct and hence are necessary for functional activity of the γ-secretase. It was recently reported that the TMDs of PS1 are required for the formation of the HMW complex (28). However, our data suggest that the TMDs of PS1 are crucial for the stabilization of the γ-secretase complex, but not for its assembly. This is consistent with the results of previous studies where only the TMDs of PS1 but not those of PS2 were required for the formation of the HMW complex (29). The reasons why these TMDs are involved in the formation of the HMW complex remain to be elucidated. Nevertheless, the TMDs of PS1 play a key role in the stabilization of the γ-secretase complex, suggesting that they are involved in the acquisition of proper conformation of γ-secretase after the assembly of the complex.
the proper trafficking of the γ-secretase complex (28–31). Mature Nct proteins were detected in all TMD-swap-expressing cell lysates, except for TM4mt, suggesting that the trafficking of the γ-secretase complex was not significantly altered by the swapping of TMDs (Fig. 3). Moreover, the matured form of Nct proteins expressed with TMD-swap PS1 acquired the resistance against trypsin treatment independent of the PS1 genotype. However, the levels of mature Nct were significantly lower in cells expressing TM1mt, TM5mt, TM8mt, and TM9mt PS1 when compared with WT PS1. Altogether, the maturation of Nct was unaffected by TMD-swap mutation in PS1 TMDs, except for the TM4mt.

The formation of stabilized HMW complex containing PS is tightly related to its enzymatic function (15, 18). Upon BN-PAGE analysis, all components of the γ-secretase complex were detected at the position of the HMW complex with an apparent molecular mass of 440 kDa (Fig. 4A). We used the endogenous signal peptide peptidase (SPP) as a loading control, which was mainly detected as a 200-kDa complex irrespective of the overexpression of PS1.4 Mutant PS1 carrying M292D, D257A, or D385A mutation was also detected as the ~440-kDa HMW complex, suggesting that the HMW complex formation was independent from the endoproteolysis or the catalytic activity of PS1 (Fig. 4B). Consistent with the results of the immunoprecipitation assay, all TMD-swap PS1 mutants were also detected as the HMW complex together with other γ-secretase components. Notably, TM4mt was detected as an ~440-kDa complex, too, indicating that Pen-2 is dispensable for the HMW complex formation. In good agreement with our previous result that TM4mt was unstable (12), the amount of the TM4mt-containing HMW complex was smaller than that of WT PS1. In addition, the steady state levels of TM1mt-, TM5mt-, TM8mt-, or TM9mt-containing HMW complex were significantly lower than that of the HMW complex containing WT PS1. To further analyze the effects of swap of TMDs on the γ-secretase complex, we examined the stability of the HMW complex by cycloheximide treatment. Complex containing WT PS1 was highly stable at the chase of 12 h (Fig. 5A). In contrast, TM1mt-, TM4mt-, TM5mt-, TM8mt-, or TM9mt-containing HMW complexes were rapidly degraded (Fig. 5B), whereas those containing TM2mt, TM3mt, or TM6mt were highly stabilized similarly to that containing WT PS1. We then examined the major conformational change of TMD-swap PS1 mutants by trypsin digestion (Fig. 6). Unexpectedly, the patterns of digested bands of the

4 N. Watanabe, T. Iwatsubo, and T. Tomita, unpublished data.
TMD-swap PS1 mutants were almost similar to those of WT PS1, suggesting that the TMD-swap mutations did not cause significant defects in the structural integrity of the PS1 polypeptides. Taken together, these results indicate that the TMD1, -5, -8, and -9 of PS1 are critical regions for the stabilization of the γ-secretase subsequent to the formation of the HMW complex.

Photoaffinity Labeling Experiments Using TMD-swap PS1 Mutants—
The biochemical characters of the HMW complex containing the TM2mt or TM6mt PS1 were almost similar to that with WT PS1. To gain further insights into the molecular mechanisms underlying the enzymatic defects of these mutants, we took the photoaffinity labeling approach. The helical peptide-type GSI-based photoprobe pep.11-Bt and the transition state analogue-type GSI-based photoprobe L-852,646 are directly targeted to the initial substrate-binding site and the catalytic site, respectively (5, 6, 21). These probes specifically labeled PS1 NTF as well as PS1/M292D holoprotein, whereas WT PS1 holoprotein was never positively labeled, supporting the notion that the PS1 fragments forming the stabilized HMW complex are the proteolytically active form and that these two distinct functional sites are formed independently of the endoproteolysis (Fig. 7A). Moreover, pep.11-Bt bound to the catalytic site mutants (i.e. PS1/D257A and PS1/D385A), whereas L-852,646 did not specifically label these catalytic site mutants. Thus, formation of the initial substrate-binding site of γ-secretase appears to take place independently of that of the catalytic site, in agreement with the previous prediction (4). In contrast, TM2mt and TM6mt PS1 were labeled neither by the pep.11-Bt-binding site nor by the L-852,646 catalytic site photoprobes. Photoaffinity labeling experiments combined with intramolecular complementation (i.e. co-expression of TM2mt or TM6mt NTF and WT CTF) also resulted in no specific binding of the probes (Fig. 7B). These results suggest that TMD2 and the luminal side of TMD6 have critical role(s) in the formation of the initial substrate-binding site and the catalytic site.

TMD2 and -6 Locate in Proximity to TMD9—We have previously reported the functional role of the extracellular/luminal side of PS1 TMD9 in the initial substrate binding of the γ-secretase by SCAM using MTS reagents (9, 11). Of note, a sulfhydryl-based cross-linking experiment revealed that TMD9 locates in proximity to the residue Leu250 in TMD6. To test whether TMD2 also locates in proximity to TMD6, we generated mutant PS1 that harbors a single cysteine substituted at residue Ser132, which is placed at the juxtamembrane region of TMD2 on a Cys-less PS1 basis (PS1Cys/Ser132C). Amino acid substitution of Ser132 to cysteine did not affect the γ-secretase activity (Fig. 8A). SCAM labeling using N-biotinylaminoethyl methanethiosulfonate and competition experiments by charged MTS reagents (i.e. negatively charged 2-sulphonato-
ethyl methanethiosulfonate (MTSES) and the bulkier, positively charged 2-(trimethylammonium)-ethyl methanethiosulfonate (MTSET) revealed that Ser132 faces an open hydrophilic environment at the extracellular/luminal side (Fig. 8B). Next we tested whether S132C was cross-linked with residues Asp450 and Gln454 replaced with Cys, which are located at TMD9 and are the most C terminal, respectively (Fig. 8C). We utilized MTS cross-linkers MTS-4-MTS (M4M) and MTS-14-MTS (M14M) with spacer moieties of 7.8 and 20.8 Å long, respectively. We found that the N- and C-terminal fragments of PS1Cys(−)/S132C/D450C double mutant are cross-linked. In contrast, no cross-linked product of the fragments of PS1Cys(−)/S132C/Q454C was detected. These data imply that TMD2 is also located in proximity to TMD9 similarly to TMD6. Taken together, we concluded that TMD2, -6, and -9 of PS1 collaborate to form the initial substrate-binding site of the γ-secretase complex.

DISCUSSION

A number of studies have revealed the seminal roles of the TMDs of the γ-secretase components in the assembly of the active complex and acquisition of its proteolytic activity (12, 15, 17, 18, 24, 25, 32, 33). Previously we and others found the requirement of TMD4 in the binding with Pen-2 (12, 33). Here we furthered the extensive analysis of a series of TMD-swap PS1 proteins and examined their ability to form functional γ-secretase complex in PS null background. The replacement of TMD1, -2, -5, -6, -8, or -9 with irrelevant polypeptide abolished the ability to generate Aβ or NICD, whereas these mutants formed the HMW complex with other γ-secretase components (i.e. Nct, Aph-1, and Pen-2). Among the mutants, swap of TMD1, -5, -8, or -9 affected the stability of HMW complex, suggesting that the acquisition of stability of the complex is required for the proteolytic activity. Notably, using the photoaffinity labeling approach, we found that the TMD2 and luminal side of TMD6 of PS1 contribute to the formation of the initial substrate-binding site and the catalytic site. Moreover, an intramolecular complementation assay suggested the role of TMD3 in the acquisition of proper γ-secretase activity after endoproteolysis. The predicted scheme of the relationship between the

FIGURE 5. Stability of the HMW complex harboring TMD-swap PS1 mutants. A, analysis of stability of the HMW complex containing WT PS1 in DKO cells incubated in culture medium containing cycloheximide (CHX) (30 mg/ml). DKO cells after various incubation periods (0–12 h) were solubilized in BN-PAGE lysis buffer and analyzed by BN-PAGE analysis with each antibody (as indicated below the panel). In immunoblot using anti-Nct antibody, numbers shown above or below each lane denote the incubation period and the relative band intensity, respectively. Note that all components were stable over 12 h. 8, analysis of stability of the HMW complex harboring TMD-swap PS1 mutant probed by anti-Nct antibody.

FIGURE 6. Trypsin digestion of TMD-swap PS1 polypeptides in DKO cells. 1% CHAPSO-solubilized lysates prepared from DKO cells transiently transfected with WT PS1 (A) or TMD-swap PS1 mutants (B) were divided into four samples and incubated with trypsin at the indicated concentrations above the panels and analyzed by immunoblotting using the anti-PS1 C terminus antibody (anti-PS-C3). Full-length PS1 (PS1 FL) and CTF are shown by black arrowheads and arrows, respectively. Note that the digestion patterns of mutant PS1 are almost similar, whereas the stability of HMW complex is different (see Fig. 5B).
assembly of the γ-secretase and the function of PS1 TMDs is shown in Fig. 9.

Active γ-secretase is known to form a highly stable protein complex (34). Systematic mutational studies of PS support our previous view that the formation of stabilized HMW complex is tightly related to its proteolytic activity (15, 18, 34–36). Nct-Aph-1 subcomplex has been considered as the stabilization factor for the γ-secretase complex (7, 8). In fact, we have recently shown that single chain variable fragment against Nct diminished the stability and activity of the γ-secretase complex (31). However, TM1mt, TM5mt, TM8mt, and TM9mt PS1 failed to stabilize despite their capability to bind Nct-Aph-1 subcomplex and form the HMW complex. These PS1 mutants recovered complex-type N-glycosylated Nct polypeptides that acquired trypsin resistance, indicating that conformational maturation of Nct and the trafficking of the γ-secretase complex occur independently of the stability of PS1. Thus, it is reasonable to conclude that the interaction of PS1 with the Nct-Aph-1 subcomplex is not sufficient for the stability of PS1. Instead, these TMDs might contribute to the stabilization as well as structural integrity of PS1 within the functional γ-secretase architecture, which is critical to the enzymatic activity.

Both D257A/PS1 and D385A/PS1 were labeled by pep.11-Bt, but not L-852,646, in the photoaffinity labeling experiments. This is consistent with the previous result that transition state analogue-type and helical peptide-type GSIs target spatially different regions in PS1 and indicates that the formation of the initial substrate-binding site occurs independently of the catalytically active conformation of the γ-secretase complex (4–6). Thus, the result that TM2mt and TM6mt failed to interact with both types of photoprobe suggests that TMD2 and the luminal side of TMD6 are responsible for the formation of the initial substrate-binding site after or during stabilization. In addition, we have found that both TMD2 (this study) and TMD6 (9, 11) are located in proximity to TMD9 within the PS1 CTF. Thus, the former two TMDs might be responsible for the formation of initial substrate-binding site in PS1. However, we cannot fully exclude the possibility that other portions of PS1 are also involved in the substrate binding, and the integrity of these portions was disrupted by conformational changes caused by TMD-swap mutagenesis.

Molecular mechanisms whereby the intramembrane-cleaving enzymes hydrolyze membrane-embedded substrates still remain enigmatic. However, x-ray crystallographic and subsequent biochemical analyses of rhomboid and site-2 proteases, the serine-type and metalloprotease-type intramembrane-cleaving enzymes, respectively, provided some clues to the atypical proteolytic process; initial substrate–enzyme interaction
Initial Substrate-binding Site of γ-Secretase

should occur via helix–helix interface in a hydrophobic environment (37). Then the substrate is transferred into the hydrophilic catalytic pore through the lateral gate. TMD9 of PS1 is implicated in the substrate binding and the gating mechanism, whereas structural information regarding PS1 at higher resolution has been lacking. Of note, using recombinant protein-based binding assay, a region containing PS1 TMD2 is implicated in the direct substrate binding (38). In regard to TMD6, we have shown that the luminal side of TMD6 is exposed to a hydrophilic environment along with the catalytic aspartate residue, Asp257, by SCAM (9, 10). Pharmacological and chemical biological analyses have indicated that the initial substrate binding and the catalytic sites are located in proximity (5, 6).

Thus, the luminal side of TMD6 might be one of the structural constituents not only of the catalytic site but also of the initial substrate-binding site. The luminal half of TMD6 has been shown to comprise an α-helical structure, of which one plane formed by residues Ala246, Leu250, and Asp257 is exposed to the hydrophilic milieu of the catalytic pore (9). We propose that the reverse side of this segment that faces the lipid bilayer might comprise the initial substrate-binding interface. Binding of the substrate to the initial binding site composed of TMD2, -6, and -9 may induce a conformational change of PS1 and facilitate the access of the substrate to the catalytic site. A similar substrate-binding and catalytic site relationship has been reported in the rhomboid (39).

In sum, we have revealed that PS1 TMD1, -5, -8, and -9 are required for the stabilization of the HMW complex and that TMD2 and the luminal side of TMD6 contribute to the formation of the initial substrate-binding site of the γ-secretase. However, we cannot fully exclude the possibility that the TMD swapping might alter the overall conformation of the presenilin polypeptide in a way that could alter its function. Additional mutagenesis studies will be needed to further identify the critical domains in PS1 for the substrate recognition. X-ray crystallographic analyses would eventually reveal the mechanism by which the γ-secretase complex recognizes the substrate and the substrate enters the active site.

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