Transmembrane Domain 9 of Presenilin Determines the Dynamic Conformation of the Catalytic Site of γ-Secretase

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One of the most prominent drug targets for the treatment of Alzheimer disease is γ-secretase, a multi-protein complex responsible for the generation of the amyloid-β peptide. The catalytic core of the complex lies on presenilin, a multi-spanning membrane protease, the activity of which depends on two aspartate residues located in transmembrane domains 6 and 7. We have recently shown by cysteine-scanning mutagenesis that these aspartates are facing a water-filled cavity in the lipid bilayer, demonstrating how proteolytic cleavage of the substrates can be taking place within the membrane. Here, we demonstrate that transmembrane domain 9 and hydrophobic domain VII in the large cytoplasmic loop of presenilin are dynamic structural parts of this cavity. Hydrophobic domain VII is associated with transmembrane domain 7 in the membrane, probably facilitating the entrance of water molecules in the catalytic site. Transmembrane domain 9, on the other hand, exhibits a highly flexible structure, potentially involved in the transport of substrates to the catalytic site, as well as in the binding of γ-secretase inhibitors. The conserved proline-alanine-leucine motif at the cytoplasmic part of this domain is extremely close to the catalytic Asp and is crucial for conformational changes leading to the activation of the catalytic site. We, also, identify a unique mutant in this domain (I437C) that specifically blocks amyloid-β peptide production without affecting the processing of the physiologically indispensable Notch substrate. Our data are finally combined to propose a model for the architectural organization and activation of the catalytic site of presenilin.

Regulated intramembrane proteolysis is characterized by hydrolysis of peptide bonds within the hydrophobic environment of the membrane and is involved in a variety of physiological processes. So far, intramembrane-cleaving proteases have been found to represent three of the four mechanistic classes of proteases known: serine (rhomboids), aspartic (presenilin and signal peptide peptidase), and metalloproteases (site 2 protease) (1). Very recently, important insight into the mechanism underlying regulated intramembrane proteolysis came from the high resolution crystal structures of the GlpG rhomboid (2, 3) and S2P metalloprotease (4). A key observation derived from these structures was the spatial arrangement of the transmembrane helices in such a way that the catalytic residues are positioned in a water-containing cavity (rhomboid) or channel (metalloprotease) inside the membrane. The entrance of water in the membrane is facilitated by shorter membrane-crossing helices and re-entrant loops, and lateral gating was proposed as the mechanism for substrate entry into the otherwise sealed catalytic site. These observations suggest that the intramembrane-cleaving proteases, despite their obviously diverse catalytic activities, share common structural features that reflect adaptation to hydrolysis within the membrane.

Among the intramembrane-cleaving proteases, presenilin (PS) is unique, because its proteolytic activity is directly dependent on its association with three additional membrane proteins, nicastrin, APH-1, and PEN-2. All together they form the γ-secretase complex (5–10), which is responsible for the final production of the Aβ from the amyloid precursor protein (APP), a key event in the pathogenesis of Alzheimer disease (11).

Although γ-secretase is a prominent drug target for Alzheimer disease, its continuously growing list of substrates (12), including the developmentally indispensable Notch (13), as well as the identification of multiple differentially regulated cleavage sites within the transmembrane domain (TM) of the same substrate (14–18), have rendered inhibitor design an extra challenging task. At the same time, the highly hydrophobic nature of the complex makes structure-function approaches extremely difficult. In the absence of cell culture systems, mutations in APP, nicastrin and PEN-2 that disrupt γ-secretase activity have been used to identify changes in the spatial arrangement of the transmembrane helices critical for γ-secretase function (19–23).

The γ-secretase complex is composed of four membrane proteins, nicastrin, APH-1, PEN-2 and presenilin, with the tetrameric complex containing one presenilin, which is the catalytic component of the complex (24–26). γ-Secretase also requires cleavage of APP by the presenilin-related intracellular domain (ICD) to generate the transcriptionally active Notch intracellular domain (NICD) (27, 28). The majority of the γ-secretase activity is dependent on its association with three additional membrane proteins, nicastrin, APH-1, and PEN-2. All together they form the γ-secretase complex (29–31), which is responsible for the final production of the Aβ from the amyloid precursor protein (APP), a key event in the pathogenesis of Alzheimer disease (32).

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of a high resolution crystal structure, a useful tool to probe the three-dimensional structure and dynamics of γ-secretase is cysteine-scanning mutagenesis (19–22). We (23) and others (24) have recently employed this technique to provide initial insight into the structural organization of the catalytic site of PS1, with emphasis on TMs 6 and 7 where the two catalytic aspartates reside (25). We showed that these two TMs form a water-containing cavity inside the membrane, with the catalytic aspartates very closely opposed to each other (23). This cavity is equivalent to the catalytic cavities found in the crystal structures of GlpG and S2P, allowing us for the first time to envisage how hydrolysis of the substrates can take place in the intramembrane catalytic site of PS1.

In the present work we investigated the potential contribution of TM9 to this hydrophilic cavity. This domain has been proposed to be important for the initial binding of the substrate to PS1 (26), but its topology has been extremely controversial, and only recently it was proven to actually cross the membrane (27, 28). Interestingly, it contains the proline-alanine-leucine or PAL motif, completely conserved in all presenilins and their homologues, the signal peptide peptidase family (29, 30). Substitution of the first proline of this motif with most other amino acids abolishes γ-secretase activity, as well as binding of a transition state inhibitor (31, 32) and the dipeptidic DAPT inhibitor (33) to PS1. Such experiments can be interpreted in two fundamentally different ways: either PAL is a direct part of the catalytic site, or it is distant from the active site and its mutation affects the conformation of the catalytic site itself. Intriguingly, so far no proof supporting either the former or the latter assumption has been provided.

Here we show that TM9 is a structural part of the catalytic cavity of PS1 directly interacting with the catalytic TM6 domain. We prove that this domain has a unique structure, characterized by high flexibility, potentially involved in the translocation of substrates from the initial binding site to the catalytic site of PS1. Extensive functional analysis identified a unique mutant (I437C), which can process Notch but not APP, indicating that there are structural elements responsible for differential substrate cleavage on PS1. Furthermore, we prove for the first time direct evidence supporting a dual role for the PAL motif in the function of PS1; on one hand, it initiates conformational changes leading to the activation of the catalytic site, and on the other hand, its extremely close proximity to the catalytic Asp257 implies a functional interaction with it. In addition, we show that hydrophobic domain HDVII, located in the loop connecting TMs 6 and 7, is embedded in the membrane, and it most probably facilitates the entrance of water in the catalytic cavity. Based on these observations, we finally propose a model for the architectural organization and activation of the catalytic site of PS1.

Labeling with Thiol-specific Reagents—Labeling with EZ-Link Biotin-HPDP (Pierce) was performed as before (23), but for 20 min at 4 °C. For cell surface specific labeling, cells in 100-mm plates were washed three times in cold phosphate-buffered saline and incubated with MTSET (Biotium) or Me3SO (control) for 30 min at 4 °C. The cells were then washed again, and MTSEA-Biotin (Biotium) was added for 20 min at 4 °C. After extensive washing to remove unbound reagent, the cells were lysed in buffer containing 1% Triton X-100, and 500 µg protein were precipitated with neutravidin beads and analyzed further in the same way as for Biotin-HPDP.

Reaction with γ-Secretase Inhibitors and Accessibility—Crude membrane aliquots were treated with inhibitor DAPT (10 µM) or X (1 µM) (Calbiochem) for 1 h on ice, after which Biotin-HPDP was added for 20 min. The reaction was terminated with 10 mM N-ethylmaleimide and 10 mM EDTA, and the membranes were pelleted by ultracentrifugation at 55,000 rpm for 1 h. Finally, 50 µg of membrane protein lysed in 1% Triton X-100 were precipitated overnight with 20 µl of neutravidin beads, and bound material was analyzed by Western blot, as above.

Analysis of APP and Notch Processing—Transduction of mouse embryonic fibroblasts with APP or NotchΔE adenovirus and in vitro assessment of Aβ or NICD production, respectively, have been described before (23). For statistical analysis, the data from six independent infection experiments were used for calculations (S.E. values are indicated), which were then subjected to one-way analysis of variance with a Bonferroni correction to determine their significance.

For in vitro generation of AICD, 3 µg of microsomal membranes prepared in buffer B (50 mM PIPES, pH 7, 0.25 M sucrose, 1 mM EGTA, 1% CHAPS) were incubated with 0.2 µM substrate (C99-3x FLAG purified from overexpressing COS cells), 0.0125% phosphatidylethanolamine, 0.1% phosphatidylcholine, and 2.5% Me3SO at 37 °C for 3 h. The reaction was terminated with Nu-Page sample buffer, the proteins were separated on a 12% Bis-Tris gel, and AICD production was visualized with anti-FLAG monoclonal antibody (Sigma).

RESULTS

Amino Acid Residues in TM9 Are Exposed to a Water-containing Cavity in the Membrane—We performed a systematic mutagenesis scan of TM9 by individually replacing most of its amino acid residues with cysteine (Fig. 1A, orange). We stably expressed the mutants in fibroblasts lacking both PS1 and PS2 and found that they were all, apart from G442C, able to restore PS1 endoproteolysis, as well as nicastrin maturation and PEN-2 stabilization, which indicates that they can rescue formation of the γ-secretase complex in the knock-out cells (Fig. 1B).

We then probed the microenvironment of the introduced cysteines using different sulfhydryl-specific reagents. These reagents react with a cysteine only when its side chain is an

EXPERIMENTAL PROCEDURES

Construction of Cell Lines Stably Expressing PS1 Mutants, Preparation of Cell Lysates, and Immunoblotting—Construction of cell lines stably expressing PS1 mutants, preparation of cell lysates, and immunoblotting were performed as described previously (23).
ionized thiolate; this depends on the presence of water, therefore cysteines embedded in the lipid bilayer cannot bind to the reagents. In this context, treatment of intact cells expressing the different cysteine mutants with the membrane-permeable EZ-link Biotin-HPDP reagent resulted in labeling of all cysteines throughout TM9 (Fig. 2A). Some of these cysteines showed quite weak reactivity (i.e. Y446C and F441C) that was still above the background signals of the negative controls (CL PS1, L392C, and wild type PS1). We tend to consider this labeling as positive, and we take it into account in our overall interpretation of the data, although some caution with regard to these weak signals is appropriate. Our results indicate that all of the cysteines in TM9 are exposed to hydrophilic environment, implying that either TM9 does not cross the membrane or the different residues in the region become to variable extents exposed to water within the membrane. The latter is only possible if TM9 adopts distinct conformations within the cell. The fact that some residues are strongly modified by Biotin-HPDP and others not is indeed compatible with such a hypothesis.

To investigate further the two possibilities, we performed a labeling experiment with two additional sulfhydryl-specific reagents, MTSEA-Biotin and MTSET (the chemical structure of the reagents is shown in supplemental Fig. S1A). In contrast to Biotin-HPDP, which labels any ionized thiolate in the cell...
intracellular, extracellular, or in a water cavity inside the membrane), MTSEA-Biotin cannot cross the plasma membrane, and as a result, in intact cells it binds exclusively to cysteines that are extracellular or located in a water cavity open to the extracellular space. To discriminate between these two positions, bulky charged reagents, such as MTSET, can be used prior to the reaction with MTSEA-Biotin. Because of the charge, these reagents cannot enter deeply into the membrane; therefore a cysteine can react with them only if it is located in open hydrophilic space (such as an extracellular loop or at the borders of a transmembrane domain), and that reaction would prevent subsequent binding to MTSEA-Biotin.

We verified this principle by analyzing two mutants we have previously characterized (28), K430C and L460C, located in the loop domains flanking TM9 at the amino-terminal and carboxy-terminal ends, respectively. Because K430C is intracellular, it does not react with MTSEA-Biotin (supplemental Fig. S1B). On the contrary, the extracellular L460C not only gets labeled by the reagent, but this labeling is partially blocked when cells are preincubated with MTSET (supplemental Fig. S1), which verifies that indeed L460C is located in open hydrophilic space. We then proceeded with assessing the accessibility of all cysteines in TM9 to MTSEA-Biotin, with and without preblocking with MTSET. The results depicted in Fig. 2B show that at the cell membrane TM9 is exposed to water all the way up to the PAL motif at the cytoplasmic end of the domain. In agreement with TM9 crossing the membrane, MTSEA modification of only three mutants at the most extracellular part of the domain (Y451C, Q454C, and P455C) is completely blocked by MTSET pretreatment, suggesting that the rest of the domain is located in a closed hydrophilic environment, inside the membrane, restricted to charge. Interestingly, a more quantitative evaluation of the results with all of the reagents (Fig. 2C) revealed positions that show differential accessibility at the cell membrane (MTSEA-Biotin) versus the whole cell (Biotin-HPDP). Overall, it is clear that the residues in TM9 are accessible to water to a variable extent, pointing strongly toward the existence of distinct conformations of the domain.

Both TM9 and HDVII Contribute Structurally to the Catalytic Site of PS1 — We next investigated whether TM9 is exposed to the same water-containing cavity we previously identified for catalytic domains 6 and 7 (23). We used PS1 bearing a mutation in TM9 (P433C) in combination with either D257C (TM6) or D385C (TM7) and performed cross-linking experiments on
isolated membranes with the homobifunctional cross-linker M2M (Fig. 3A). We observed a specific cross-linking product in the case of the P433C/D257C double mutant, which proves that P433C is located in the same hydrophilic cavity as D257C, with a maximum distance of 5.2 Å between the two sulfhydryl groups. Surprisingly, no cross-linking was observed between TMs 7 and 9 (P433C/D385C), using M2M or the much longer M17M cross-linker (spacer arm length 24.7 Å) (Fig. 3A).

To further elucidate the position of TM9 relatively to the catalytic site, we constructed an additional series of double mutants, with either D257C or D385C on one side and a cysteine residue in TM9 on the other side, scanning like this the whole length of TM9. All of the cross-linking results with D257C verified the close proximity of TM6 and TM9, with a maximum distance of 5.2 Å between the two sulfhydryl groups. Surprisingly, no cross-linking was observed between TMs 7 and 9 (P433C/D385C), using M2M or the much longer M17M cross-linker (spacer arm length 24.7 Å) (Fig. 3A).

FIGURE 3. TM9 is part of the catalytic site cavity, closely opposed to Asp257 but not to Asp385. A, specific cross-linking of TM9 with TM6 but not with TM7. Membrane fractions from cell lines expressing the indicated double cysteine mutants were treated with cross-linker (M2M or M17M) with and without preblocking the free sulfhydryls with N-ethylmaleimide (NEM). Protein extracts were separated in SDS-PAGE under nonreducing conditions and visualized in Western blot with antibodies toward both PS1 NTF and CTF. A representative experiment using M2M is shown. B, cross-linking of several cysteines in TM9 with D257C. Note that in the A434C/D257C and L435C/D257C double mutants there is an additional band in the control lanes, sensitive to reduction with β-mercaptoethanol (C), which indicates that it represents a spontaneous disulfide bridge between the sulfhydryls. D, overview of all positions in TM9 that could be cross-linked to D257C. The color code corresponds to the intensity of the cross-linked band versus the full-length protein. Dark purple represents >80% of cross-linking, light purple represents 50 – 80%, and pink represents <50% of cross-linking. The degree of cross-linking is gradually lower as we move from the intracellular to the extracellular part of the domain, suggesting sliding of this domain. No cross-linking is observed between any cysteine tested in TM9 and D385C in TM7.

To further elucidate the position of TM9 relatively to the catalytic site, we constructed an additional series of double mutants, with either D257C or D385C on one side and a cysteine residue in TM9 on the other side, scanning like this the whole length of TM9. All of the cross-linking results with D257C verified the close proximity of TM6 and TM9, with a maximum distance of 5.2 Å between the two sulfhydryl groups. Surprisingly, no cross-linking was observed between TMs 7 and 9 (P433C/D385C), using M2M or the much longer M17M cross-linker (spacer arm length 24.7 Å) (Fig. 3A).

With β-mercaptoethanol (Fig. 3C), which means that it represents spontaneous cross-linking of the two cysteines. This implies very close opposition of these particular residues (distance ~2 Å between the two sulfhydryl groups) (34, 35) and suggests the close interaction of the functionally important PAL motif with the catalytic Asp257. In addition to that, if we map all the positions in TM9 that can be cross-linked to Asp257, it is obvious that the degree of cross-linking is quantitatively different as we move from the cytoplasmic toward the extracellular part of TM9, suggesting again different intermediary conformations of this domain in the membrane. In fact, the consecutive cross-linking of the individual residues suggests the possibility that TM9 “slides” over Asp257 (Fig. 3D).

Remarkably, no cross-linking with D385C was observed with any cysteine in TM9 tested (Fig. 3E, supplemental Fig. S2B, and data not shown). This is completely unexpected, because we have previously shown that Asp257 and Asp385 face each other in the same catalytic cavity (23), and it can only be explained if we postulate that another domain of PS1, exposed to the cavity, is located between TM9 and TM7.
A plausible candidate would be TM8, and to test this we introduced cysteines in the cytoplasmic part of the domain and tested their accessibility to Biotin-HPDP (Fig. 4, A and E). Only a few positions, all at the border of the TM with the cytoplasmic side of the membrane, were found to be accessible and thus exposed to water (Fig. 4A). Thus, TM8 is largely embedded in the lipid bilayer, as expected from a classical transmembrane domain. When we tested whether the water-accessible cysteines in TM8 were close to the catalytic aspartates, we observed that they could all be cross-linked to D257C, which means that they are located close to TM6 but again not to D385C (Fig. 4B).

We then checked HDVII, located in the loop connecting TMs 6 and 7, which has previously been hypothesized to be partially embedded in the membrane (36). This domain contains the site of “presenilinase” cleavage (37), which results in the formation of PS1 NTF and CTF fragments, the biologically active form of the protein. All of the cysteines introduced in HDVII were found to be accessible to water, because they could be modified with Biotin-HPDP (Fig. 4, C and E). Interestingly, modification was much more prominent for full-length PS1 compared with the cleaved NTF and CTF fragments, which suggests a difference in the local environment of HDVII before and after presenilinase cleavage. We chose V296C in HDVII to further test whether it could be cross-linked to the catalytic aspartates. This time, V296C could be cross-linked to D385C but not to D257C (Fig. 4D). This observation indicates that HDVII, at least in one conformation, is located very close to TM7 within the catalytic cavity, probably concealing it from TMs 8 and 9.

\textit{γ}-Secretase Activity of TM9 Cysteine Mutants and Binding to Specific Inhibitors—The cross-linking experiments above clearly show that TM9 is structurally associated to the catalytic cavity of PS1, most likely in a dynamic fashion. The question was raised therefore whether this domain also contributes to the catalytic function of presenilin per se. We first investigated whether the processing of APP was altered by any of the cysteine substitutions in TM9. Aβ-specific enzyme-linked immunosorbent assay (ELISA) (Fig. 5A) revealed that the I437C mutant had the most severe loss-of-function phenotype, followed by G442C and mutants in the PAL motif; P433C reduced the levels of both Aβ40 and 42, whereas A434C (a reported Alzheimer disease clinical mutation) and P436C showed a significant decrease in Aβ40 with a simultaneous increase in Aβ42. Particularly interesting were substitutions I439C and F441C that caused a remarkable 3–4-fold...
increase only in the production of Aβ42. The F441C mutant, together with P433C and G442C, also affected the cleavage of APP at the e-site, as assessed by in vitro generation of AICD from FLAG-tagged C99 (Fig. 5B). Finally, three of these four mutants (G442C, P433C, and F441C), as well as P436C, showed severe impairment of NICD production in
significantly to the regulation of the different cleavage sites in both APP and Notch substrates. These results provide direct evidence that TM9 contributes to the formation of the catalytic site of PS1.

The Role of the PAL Motif—Providing evidence that TM9 of PS1 is directly involved in the formation of the catalytic site raises new questions concerning the specific role of the highly conserved PAL motif at the cytoplasmic part of the domain, and especially the first proline (Pro433). Previous work has shown that amino acid substitutions at this position affect activity dramatically, but the formation of the transition state inhibitor DAPT blocked only modification of Y446C, T449C, Y451C, L452C, and V453C, whereas the non-transition state inhibitor DAPT blocked only modification of F441C, Y451C, and V453C (Fig. 5C). Interestingly, although these amino acids are located along the whole TM, they are mostly concentrated on one face of the putative helix (Fig. 5E). This experiment indicates that the affected residues are part of the binding site of the tested inhibitors or, alternatively, that the binding of the inhibitors causes such a conformational change in the catalytic site that the accessibility of several positions in TM9 to Biotin-HPDP becomes hampered. Both possibilities suggest the importance of TM9 for its interaction with TM6. Indeed, as shown in Fig. 6A, the P433L mutation abolishes the cross-linking of V444C with TM9, indicating a change in the relative position of the two TMs within the cavity.

The Role of Pro433—To investigate the contribution of TM9 to the function of PS1, we used cysteine-scanning mutagenesis to probe the role of the conserved Pro433 in the PAL motif. Surprisingly, when Pro433 was substituted by alanine, the 0% cross-linking product either with the 5.2 Å M2M or the 16.9 Å 3,6,9-trioxo-1,11-diyldibismethanethiosulfonate cross-linker. Introducing the P433L mutation results in almost complete cross-linking of D257C with D385C. C, the active P433A substitution has the same effect as P433L in the cross-linking between the catalytic aspartates in TMs 6 and 7. NEM, N-ethylmaleimide; CL, cysteine-less; FL, full-length.

**DISCUSSION**

In the present study we used cysteine-scanning mutagenesis to investigate the contribution of TM9 to the function of PS1. Several positions within this TM were found accessible to water inside the lipid bilayer and could be specifically cross-linked to TM6, proving that this domain is structurally part of the intramembrane water-filled cavity we previously identified for the catalytic TMs 6 and 7 (23). Unexpectedly we found that cross-linking between TM9 and TM7 was not possible, most likely because of the hydrophobic HDVII domain interposing between them. HDVII is part of the

**FIGURE 6. Conformational rearrangements upon mutation of Pro433 in the PAL motif.** A, the P433L mutation abolishes cross-linking between D257C (TM6) and V444C (TM9), indicating a change in the relative position of the two TMs within the cavity. B, cross-linking of D257C (TM6) and D385C (TM7) yields about 50% cross-linked product either with the 5.2 Å M2M or the 16.9 Å 3,6,9-trioxo-1,11-diyldibismethanethiosulfonate cross-linker. Introducing the P433L mutation results in almost complete cross-linking of D257C with D385C. C, the active P433A substitution has the same effect as P433L in the cross-linking between the catalytic aspartates in TMs 6 and 7. NEM, N-ethylmaleimide; CL, cysteine-less; FL, full-length.
large cytoplasmic loop between TM6 and TM7, and it contains the site of PS1 endoproteolysis by presenilinase (37). The pattern of accessibility of several cysteines in the domain to Biotin-HPDP indicates that HDVII is located in an open hydrophilic environment in full-length PS1. After presenilinase cleavage, this domain becomes more embedded in the membrane, interacting closely with TM7 (distance less than 5.2 Å) within the catalytic cavity. This observation provides for the first time a structural base for the reported involvement of this domain in substrate (36) and inhibitor binding (39), as well as in PS1 activity (40).

All together, our cross-linking results suggest a possible arrangement of the TM6–7–9 domain forming the catalytic cavity as shown in Fig. 7A. TM6 and TM7 are opposed to each other in the cavity, with the aspartates facing each other (at least in the active conformation); TM9 is located very close to TM6 and HDVII is embedded in the cavity in such a way that it conceals TM7 from cross-linking to TM8. Of course, the exact three-dimensional positioning of these domains with respect to the membrane cannot be detailed because of the lack of a crystal structure and the possible contribution of other not yet tested TMs. Nevertheless, we can deduce how the cytoplasmic parts of TM6–7–9 are spatially organized relatively to each other to create a hydrophilic cavity within the membrane, similar to that of the rhomboids (2, 3). In contrast to the rhomboids, which are open to the extracellular environment, the presenilin cavity is apparently more exposed to the cytoplasm. In addition, our results suggest that HDVII alternates between positions in and out of the membrane, and it is easy to envisage how such a membrane re-entrant loop can facilitate the entrance of water molecules in the intramembrane catalytic site.

Within this cavity a valuable role is assigned to TM9. We have previously demonstrated that the carboxyl-terminal tail of PS1, including this domain, can bind directly to APP-C99, suggesting that this is part of the substrate-binding site on the protease (26). Here we show that TM9 affects strongly /H9253-secretase activity and inhibitor binding, indicating an additional role for this domain in the catalytic function per se, which is well in agreement with the proposed close proximity of substrate-binding and catalytic site on PS1 (41). Interestingly, activity measurements identified mutants with a remarkable increase in the production of Aβ (i.e. I439C and F441C), accompanied by no effect (I439C) or even the loss of AICD and NICD production (F441C). Particularly revealing was the mutant I437C, where the severe loss of APP cleavage (shown in both Aβ and AICD

FIGURE 7. Proposed role of TM9 in the catalytic function of PS1. A, view of TMs 6–9 from the intracellular side, arranged around the water cavity, as deduced from cross-linking results (this study and Ref. 23). Swiss-PDB Viewer was used for the construction of the helices, and the proposed distances between residues, based on the length of the cross-linkers used, are represented by dotted yellow lines. Note that this is a representation of the relevant position and interactions between the helices and not an established model of their three-dimensional structure, which is impossible because of a lack of relevant structural information. B, simplified model of proposed activation-related motions of TM9 within the catalytic site. TMs 6 and 7 are shown in green, with catalytic Asp257 and Asp385 represented by a yellow asterisk. TM9 (highlighted in pink) is located within cross-linking distance to Asp257 but not Asp385, because of HDVII (purple) protruding in the cavity. An activation step, which could be initiated by the substrate binding in the vicinity of TM9, is the signal for PAL-dependent molecular motion of the flexible TM9. The exact type of motion is not clear yet, but it could involve translation, rotation, pivotal movement (for examples, see Ref. 52), or a combination of the above and is expected to be accompanied by relative movements of the surrounding region, i.e., HDVII, resulting in activation of the catalytic site for substrate cleavage.
production) was not mirrored in the processing of Notch, as there was absolutely no effect on NICD generation. The impact of such a finding is exceptional, because it demonstrates the principle that certain structural changes can be implemented on presenilin so that Aβ production is specifically blocked, without affecting the processing of other physiologically indispensable substrates. In this regard, we propose that TM9 plays a regulatory role in catalysis, contributing to cleavage or even substrate specificity, which renders it a valuable target for further drug development efforts.

Overall, our results suggest that TM9 is involved not only in the initial binding of the substrate but also in its subsequent transport and handling in the catalytic site. Of course, such a gating function would require a great amount of flexibility from a transmembrane domain, and our experimental observations are strongly supportive for such a model. Indeed, all of the cysteines we introduced in TM9 were found to different extents accessible to water, reminiscent of established moving helices of other membrane proteins, when tested in a mixture of all native conformations (22, 42, 43). This extensive but differential accessibility pattern verifies that TM9 is highly mobile, and it exists in distinct conformations within the cell, a unique characteristic compared with all other investigated domains in γ-secretase.

An important role in the observed flexibility of TM9 can be attributed to the highly conserved PAL motif at the cytoplasmic end of the domain. It is widely accepted that proline induces flexible kinks in transmembrane helices (38, 44), and we show here that any mutation of Pro333 of the PAL motif is accompanied by remarkable structural rearrangements within the catalytic site. The position of TM9 changes relatively to TM6, and the result is that PS1 becomes fixed in one conformation with the catalytic aspartates in TMs 6 and 7 closely opposed to each other. Thus, Pro333 seems indispensable for molecular motions of TM9 related to the activation of the catalytic site. In addition, the PAL motif seems to contribute also to the catalytic function of PS1 in a more direct way. The spontaneous cross-linking of A434C and L435C to D257C in TM6 is very unusual for two of PS1 in a more direct way. The spontaneous cross-linking of A434C and L435C to D257C in TM6 is very unusual for two

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