Mutagenesis Mapping of the Presenilin 1 Calcium Leak Conductance Pore*

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Missense mutations in presenilin 1 (PS1) and presenilin 2 (PS2) proteins are a major cause of familial Alzheimer disease. Presenilins are proteins with nine transmembrane (TM) domains that function as catalytic subunits of the γ-secretase complex responsible for the cleavage of the amyloid precursor protein and other type I transmembrane proteins. The water-filled cavity within presenilin is necessary to mediate the intramembrane proteolysis reaction. Consistent with this idea, cysteine-scanning mutagenesis and NMR studies revealed a number of water-accessible residues within TM7 and TM9 of mouse PS1. In addition to γ-secretase function, presenilins also demonstrate a low conductance endoplasmic reticulum Ca2+ leak function, and many familial Alzheimer disease presenilin mutations impair this function. To map the potential Ca2+ conductance pore in PS1, we systematically evaluated endoplasmic reticulum Ca2+ leak activity supported by a series of cysteine point mutants in TM6, TM7, and TM9 of mouse PS1. The results indicate that TM7 and TM9, but not TM6, could play an important role in forming the conductance pore of PS1. These results are consistent with previous cysteine-scanning mutagenesis and NMR analyses of PS1 and provide further support for our hypothesis that the hydrophilic catalytic cavity of presenilins may also constitute a Ca2+ conductance pore.

Presenilins belong to the family of aspartic proteases, and they are involved in regulated intramembrane proteolysis, a mechanism that is used to cleave peptide bonds within the lipid bilayer (1–3). Presenilins are 50-kDa proteins that contain nine transmembrane (TM) domains (4, 5) and reside primarily in the endoplasmic reticulum (ER) membrane (6), with the N terminus oriented toward the cytosol and the C terminus toward the ER lumen. The complex of presenilins with nicastrin, APH-1, and PEN-2 subunits is transported to the cell surface and endosomal structures and functions as γ-secretase, which cleaves the amyloid precursor protein and releases the amyloid β-peptide, the principal constituent of the amyloid plaques in the brains of Alzheimer disease patients (7). Following assembly, the presenilin 1 (PS1) and presenilin 2 (PS2) holoproteins undergo endoproteolysis in the cytosolic loop between TM6 and TM7, resulting in the generation of a 35-kDa N-terminal fragment and an 18–20-kDa C-terminal fragment, which remain associated with each other in the “mature” γ-secretase complex (8–15). Mutation of the catalytic residue Asp-257 or Asp-385 in TM6 and TM7, respectively, abolishes the catalytic activity of presenilins as well as binding to transition state inhibitors of γ-secretase (15–17). Consistent with the role of presenilins as the catalytic subunits of γ-secretase (7, 15), genetic inactivation of the PS genes or familial Alzheimer disease (FAD) PS mutations affect amyloid precursor protein processing (18).

There is increasing evidence that presenilins also have functions outside of γ-secretase (3, 19). One of these functions is related to Ca2+ signaling. Many FAD mutations in presenilins result in deranged Ca2+ signaling (reviewed in Ref. 20). Although the connection between FAD mutations in presenilins and abnormal Ca2+ signaling has been known for over a decade (21), the mechanistic explanation to these findings has been controversial (20). Recently, we proposed that presenilin function as passive low conductance ER Ca2+ leak channels (22). This hypothesis was based on a number of experimental observations. We showed that wild type (WT) PS1 and PS2, but not FAD mutants, reconstituted into planar lipid bilayers were able to form low conductance divalent cation-permeable ion channels (22). We were not able to resolve single channel openings in these experiments and used noise analysis to estimate the unitary currents mediated by presenilins in bilayers (22). When cesium ions were used as a current carrier, the amplitude of the noise currents was increased by 4-fold, but the single channel openings still could not be resolved in the bilayers (22).

The abbreviations used are: TM, transmembrane; ER, endoplasmic reticulum; PS1, presenilin 1; PS2, presenilin 2; FAD, familial Alzheimer disease; mPS1, mouse PS1; SCAM, cysteine-scanning mutagenesis; MEF, mouse embryonic fibroblast; DKO, double knock-out; HCSS, HEPES-controlled salt solution; IO, ibomycin; HDVII, hydrophobic domain VII; ANOVA, analysis of variance.

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From these experiments, we concluded that WT PS1 and PS2, but not FAD mutants, are able to support low conductance leak channels. We further demonstrated that presenilins account for a majority of passive ER Ca\(^{2+}\) leak activity in mouse embryonic fibroblasts (MEFs) deficient in PS1/PS2 and that the resulting deregulated intracellular Ca\(^{2+}\) signaling can be rescued by the expression of WT presenilins, but not FAD mutants (22). In additional studies, we discovered that many, but not all, FAD mutations in PS1 and PS2 impair their ER Ca\(^{2+}\) leak function (22–24). These observations suggest that presenilins play a pivotal role in deranged Ca\(^{2+}\) signaling in Alzheimer disease, and they also provided further support for the contribution of disturbed Ca\(^{2+}\) homeostasis to Alzheimer disease pathogenesis (20, 25, 26).

Our proposal that presenilins support ER Ca\(^{2+}\) leak activity has been challenged by another group (27, 28). In particular, it has been argued that presenilins lack an ion conductance pore and are therefore not able to function as ion channels (27, 28). The crystal structure of PS is not available, and the presence of an ion conductance pore cannot be confirmed or refuted based on structural analysis. To address this question in the absence of the crystal structure of presenilins, we performed a systematic analysis of the ER Ca\(^{2+}\) leak function of a series of point mutations in TM6, TM7, and TM9 of PS1. These mutations were used in previous structure-function studies of \(\gamma\)-secretase to identify a water-containing cavity in PS1 that would allow for the hydrolysis required for proteolytic cleavage by \(\gamma\)-secretase (29, 30). Our hypothesis is that water-accessible residues in PS1 could also form an ion conductance pore. Here, we took advantage of this mouse PS1 (mPS1) mutant series to map the ion conductance pore of PS1. The results suggest that TM7 and TM9, but not TM6, are likely to be involved in forming the ion conductance pore of PS1. This conclusion is consistent with previous mapping of water-accessible residues in the PS1 sequence by the cysteine-scanning mutagenesis (SCAM) approach (29–32) and with recent NMR studies of the PS1 C-terminal fragment (33). Our results provide further mechanistic support for the hypothesis that presenilins may have ER Ca\(^{2+}\) leak function.

**MATERIALS AND METHODS**

*Expression Constructs and Generation of Stable Cell Lines*—mPS1 and Cys-less mPS1 constructs were described previously (29, 30). Briefly, all mPS1 mutants were constructed using a multisite-directed mutagenesis kit (Stratagene). Immortalized mouse embryonic fibroblasts (MEFs) from PS1/PS2 double knock-out (DKO) mice were cultured in Dulbecco’s modified Eagle’s/F-12 medium containing 10% fetal bovine serum (Sigma). At 30–40% confluency, the MEFs were transduced using a replication-defective recombinant retroviral expression system (Clontech) with either WT or mutant PS1. Stable cell lines were selected based on their acquired resistance to 5 \(\mu\)g/ml puromycin (29, 30). Wild-type, DKO, and rescued MEFs were cultured as described above and previously (22–24).

*Ca\(^{2+}\) Imaging Experiments*—Ca\(^{2+}\) imaging experiments were performed as described previously (22–24). Briefly, the cells were cultured on poly-d-lysine (Sigma)-coated 12-mm round glass coverslips in 24-well plates and loaded with 5 \(\mu\)M Fura-2/AM (Molecular Probes) in HEPS-controlled salt solution (HCSS; 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl\(_2\), 2 mM CaCl\(_2\), 15 mM glucose, and 20 mM HEPS, pH 7.3) for 45 min at 37 °C. For Ca\(^{2+}\) imaging experiments, the coverslips were mounted onto a recording/perfusion chamber (RC-26G, Warner Instrument), positioned on a movable stage of an Olympus IX-70 inverted microscope, and washed with HCSS/Ca\(^{2+}\)-deficient buffer (buffered with EGTA to 50 nm Ca\(^{2+}\)). A field that contained 6–10 cells with similar Fura-2/AM loading (signal intensity) was selected for each experiment. In Ca\(^{2+}\) imaging experiments, the cells were intermittently excited by 340 and 380 nm UV light (DeltaRam illuminator, Photon Technology International) using a Fura-2 dichromatic filter cube (Chroma Technologies) and imaged using a 60× UV-grade oil-immersed objective (Olympus). The emitted light was collected by an IC-300 camera (Photon Technology International), and the images were digitized by ImageMaster Pro software (Photon Technology International). Ionomycin (IO; Sigma) was dissolved in HCSS/Ca\(^{2+}\)-deficient buffer (5 \(\mu\)M) prior to application to the cells. Images at 340- and 380-nm excitation wavelengths were captured every 2 s and shown as 340/380 nm image ratios at time points as indicated. Background fluorescence was determined according to the recommendations of Photon Technology International and subtracted. The absolute values of free cytosolic Ca\(^{2+}\) concentrations in these experiments were determined from the equation (34) \([\text{Ca}^{2+}]_\text{cyt} = K_\text{d}([R - R_{\text{min}}]/(R_{\text{max}} - R)\times(S_{380/340}/S_{380}))\), where \(K_\text{d} = 140\) nm is the affinity of Fura-2 for Ca\(^{2+}\), \(R\) is the experimentally determined 340/380 nm ratio, \(R_{\text{max}}\) is the 340/380 nm ratio for Fura-2 saturated with Ca\(^{2+}\) (determined by application of 20 \(\mu\)M Ca\(^{2+}\) and 10 \(\mu\)M IO at the end of the experiment), \(R_{\text{min}}\) is the 340/380 nm ratio for Ca\(^{2+}\)-free Fura-2 (determined by addition of 10 mM EGTA following \(R_{\text{max}}\) determination), and \(S_{380/340}/S_{380}\) is the ratio of the fluorescence intensity of the Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms of Fura-2 at 380 nm (\(S_{380}/S_{380}\) = 2 in our experiments). To rule out artifacts in imaging experiments, one coverslip from each cell type or PS1 mutant was imaged from three to four different fields, and each field selected had 6–10 cells. A minimum of three coverslips from a 24-well plate were imaged for each cell type and PS1 mutant.

*ER Ca\(^{2+}\) Measurements*—The ER Ca\(^{2+}\) levels in MEF cells were measured using Mag-Fura-2 (35) as described previously (22–24) using the Photon Technology International Ca\(^{2+}\) imaging setup described above. Briefly, the cells were first loaded with 2 \(\mu\)M Mag-Fura-2/AM (Molecular Probes) in HCSS for 30 min at 37 °C and permeabilized by application of 10 \(\mu\)M digitonin in intracellular buffer (125 mM KCl, 25 mM NaCl, 10 mM HEPS, and 0.1 mM MgCl\(_2\), pH 7.3) containing 170 nm free Ca\(^{2+}\) (clamped by 5 mM EGTA) and 3 mM ATP. Mag-Fura-2 signals were collected as 340/380 nm ratios for the duration of the experiment, the ER membrane was permeabilized with 5 \(\mu\)M IO, and the cells were washed with ATP- and Ca\(^{2+}\)-free calibration buffer (125 mM KCl, 25 mM NaCl, and 10 mM HEPS, pH 7.3) containing 0.8 mM EGTA. The Mag-Fura-2 signals were calibrated in the presence 10 \(\mu\)M IO using a series of calibration buffers with free Ca\(^{2+}\) clamped to defined concentrations by 1 mM nitrotriacetic acid as described previously (22). Based on these calibration results, the Mag-Fura-2 340/
Compared with DKO MEFs, the size of the IO-releasable Ca$^{2+}$ pool is significantly smaller (***, p < 0.05 by analysis of variance (ANOVA)) in Cys-less mPS1 and WT mPS1. C, the average ER Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_{ER}$) are shown for DKO MEFs and DKO MEFs stably expressing WT mPS1 and Cys-less mPS1 constructs as the mean ± S.D. (n = number of cells analyzed). Compared with DKO MEFs, the [Ca$^{2+}$]$_{ER}$ level is significantly smaller (***, p < 0.05 by ANOVA) in Cys-less mPS1 and WT mPS1.

RESULTS

**Cys-less mPS1 Retains ER Ca$^{2+}$ Leak Channel Function**—In previous SCAM studies of γ-secretase, most residues in TM6, TM7, and TM9 of mPS1 were mutated to cysteine (29, 30). Here, we aimed to take advantage of this mPS1 mutant series to map the ion conductance pore of PS1. To facilitate SCAM experiments, a Cys-less mPS1 construct was generated by mutating the five endogenous cysteines to alanines in the mPS1 sequence (29). It was previously shown that the resulting Cys-less mPS1 was able to support γ-secretase function in stably transfected DKO MEF cells, similar to WT mPS1 (29). Is the ER Ca$^{2+}$ leak function also preserved in Cys-less mPS1? To answer this question, we evaluated the ability of Cys-less mPS1 to rescue the ER Ca$^{2+}$ leak pathway deficiency in PS1/PS2 DKO cells. Consistent with our previous results (22–24), application of 5 μM IO resulted in high amplitude and long-lasting Ca$^{2+}$ signals in DKO cells (Fig. 1A, blue trace), indicating that ER Ca$^{2+}$ stores are overfilled in these cells. Stable transfection of DKO cells with a WT mPS1 construct resulted in attenuated responses to IO (Fig. 1A, black trace). Therefore, ER Ca$^{2+}$ leak channel function is conserved between mouse and human PS1. The IO-induced Ca$^{2+}$ signals in the DKO cells stably transfected with Cys-less mPS1 (Fig. 1A, red trace) were similar to those in mPS1-transfected cells. To quantitatively compare the size of IO-sensitive Ca$^{2+}$ pools measured in different experiments, we calculated the area under the Fura-2 signal curve in each experiment. On average, the areas under the IO-induced Ca$^{2+}$ signals were equal to 56 ± 11 μM s$^{-1}$ (n = 19) in DKO cells, 22 ± 5 μM s$^{-1}$ (n = 53) in mPS1 rescue cells, and 26 ± 8 μM s$^{-1}$ (n = 47) in Cys-less mPS1 rescue cells (Fig. 1B). From these data, we conclude that expression of Cys-less mPS1 rescues the ER Ca$^{2+}$ leak pathway deficiency in DKO cells and restores the Ca$^{2+}$ content of ER stores to WT levels. These observations were further supported by direct measurements of ER Ca$^{2+}$ concentrations using Mag-Fura-2 (22, 23). We found that the [Ca$^{2+}$]$_{ER}$ concentration was equal to 183 ± 42 μM (n = 32) in DKO cells, 107 ± 23 μM (n = 39) in mPS1 rescue cells, and 91 ± 17 μM (n = 42) in Cys-less mPS1 rescue cells (Fig. 1C). From these rescue experiments, we concluded that Cys-less mPS1 protein retains ER Ca$^{2+}$ leak function.

**Effects of Cysteine Mutations in TM6, TM7, and TM9 of mPS1 on ER Ca$^{2+}$ Leak Function**—The preservation of ER Ca$^{2+}$ leak function in the Cys-less mPS1 mutant (Fig. 1) enabled us to study the conservation of ER Ca$^{2+}$ leak function in a series of cysteine mutants generated in previous SCAM studies (29, 30). In each experiment, the size of the IO-sensitive Ca$^{2+}$ pool was measured in DKO MEF cells stably transfected with mPS1 Cys point mutants in TM6, TM7, and TM9 (29, 30). The size of the IO-sensitive Ca$^{2+}$ pool for each cell line was calculated by integrating an area under the Fura-2 signal curve as described above (22–24). When a series of cysteine mutants in TM6 were analyzed, we discovered that the application of 5 μM IO resulted in significantly greater Ca$^{2+}$ responses in the T245C, S254C, and A260C rescue lines than in the WT mPS1 rescue line (data not shown). On average, the area under the IO-induced Ca$^{2+}$ curves was two times higher in the T245C, S254C and A260C lines than in the mPS1 line (Fig. 2A). In contrast, the areas under the IO-induced Ca$^{2+}$ curves were not significantly increased in the W247C, L250C, V255C, D257C, L258C, V259C, V261C, L262C, and P264C rescue lines compared with the mPS1 rescue line (Fig. 2A). From these results, we concluded that Thr-245, Ser-254, and Ala-260 in TM6 are critical for channel function of PS1 and that mutation of these residues to cysteine impairs the ability of PS1 to conduct Ca$^{2+}$ ions. In contrast to these three residues, nine other residues in TM6 can be mutated to cysteine without loss of channel activity. These data are consistent with our previous analysis of the D257A and ΔE8 mutants in TM6 of human PS1, both of which retained ER Ca$^{2+}$ leak function (22, 24). The rescue lines with cysteine mutations at positions 246, 248, 249, 251, 252, 253, 256, and 263 of TM6 were not available to us, and these mutations in TM6 have not been tested in a Ca$^{2+}$ assay.

In contrast to TM6, many cysteine mutations in TM7 impaired the ER Ca$^{2+}$ leak function of mPS1. On average, the area under the IO-induced Ca$^{2+}$ curve was significantly higher in the E376C, G382C, G384C, D385C, I387C, and Y389C rescue lines than in the mPS1 rescue line (Fig. 2B). These results indicate that these six positions within TM7 are critical for the ER Ca$^{2+}$ leak function.
Ca\(^{2+}\) leak function of PS1. This is consistent with our previous finding that the D385A mutation in human PS1 causes loss of ER Ca\(^{2+}\) leak function (36). The other nine positions in TM7 were dispensable for ER Ca\(^{2+}\) leak activity; the sizes of the IO-sensitive Ca\(^{2+}\) pools in the G378C, K380C, L381C, L383C, F388C, S390C, L392C, G394C, and K395C rescue lines were not different from the size in the mPS1 rescue line (Fig. 2B). The rescue lines with a cysteine mutation at positions 377, 379, 386, 391, and 393 of TM7 were not available, and these mutations have not been tested in a Ca\(^{2+}\) assay.

**FIGURE 2. Summary of the IO-releasable Ca\(^{2+}\) ER pool in mutants of TM6, TM7, and TM9 mPS1.** A, the average size of the IO-releasable Ca\(^{2+}\) pool is shown for DKO cells stably expressing Cys mutants in TM6 as the mean ± S.D. (n = number of cells analyzed). Compared with cells stably transfected with Cys-less mPS1 and WT mPS1, the size of the IO-releasable Ca\(^{2+}\) pool is significantly larger (***, p < 0.05 by ANOVA) in T245C, S254, and A260C of TM6, whereas the other TM six residues did not show any significant difference. B, the average size of the IO-releasable Ca\(^{2+}\) pool is shown for DKO cells stably expressing Cys mutants in TM7 as the mean ± S.D. (n = number of cells analyzed). Compared with cells stably transfected with Cys-less mPS1 and WT mPS1, the size of the IO-releasable Ca\(^{2+}\) pool is significantly larger (***, p < 0.05 by ANOVA) in E376C, G382C, G384C, D385C, I387C, and Y389C of TM7, whereas the other TM six residues did not show any significant difference. C, the average size of the IO-releasable Ca\(^{2+}\) pool is shown for DKO cells stably expressing Cys mutants in TM9 as the mean ± S.D. (n = number of cells analyzed). Compared with cells stably transfected with Cys-less mPS1 and WT mPS1, the size of the IO-releasable Ca\(^{2+}\) pool is significantly larger (***, p < 0.05 by ANOVA) in L435C, P436C, I439C, F441C, F455C, Y446C, F447C, and T449C of TM9, whereas the other TM six residues did not show any significant difference.
The analysis of mutations in TM9 offers results similar to those obtained with TM7. On average, the size of the IO-sensitive Ca\(^{2+}\) pool was increased by 2-fold in the L435C, P436C, I437C, S438C, T440C, G442C, V444C, L452C, V253C, Q454C, and P455C rescue lines compared with the mPS1 rescue line (Fig. 2C). The results indicate that these eight positions in TM9 are critical for the ER Ca\(^{2+}\) leak function of PS1. The cysteine mutations at 12 other positions in TM9 did not affect PS1 channel function, as the size of the IO-sensitive Ca\(^{2+}\) pool was not significantly increased in the P433C, A434C, I437C, S438C, T440C, G442C, V444C, Y451C, L452C, V253C, Q454C, and P455C rescue lines compared with the mPS1 rescue line (Fig. 2C). The rescue lines with mutations at positions 443, 448, and 450 of TM9 were not available, and these mutations have not been tested in a Ca\(^{2+}\) assay. The results of these experiments indicate that many residues within TM7 and TM9, but only a few residues within TM6, are important for the ER Ca\(^{2+}\) leak function of PS1, as point mutations of these residues to cysteine cause disruption of ER Ca\(^{2+}\) leak activity.

Role of Hydrophobic Domain VII (HDVII) and the PAL Motif in ER Ca\(^{2+}\) Leak Channel Function—In previous studies, we found that FAD mutants PS1-ΔE8 (deletion of exon 8) and PS1-ΔE9 (deletion of exon 9) are functional as ER Ca\(^{2+}\) leak channels (22, 24). Both PS1-ΔE8 and PS1-ΔE9 result in partial deletion of HDVII, which is thought to regulate water entry into the water cavity between TM6 and TM7 during the catalytic cycle of γ-secretase (30). To test whether further deletion of the hydrophilic loop between TM6 and TM7 affects ER Ca\(^{2+}\) leaks, we used MEFs stably expressing mPS1-Δloop (Asp-302–Glu-372). Compared with WT MEFs, IO induced high amplitude and long-lasting Ca\(^{2+}\) signals in MEFs expressing mPS1-Δ (Asp-302–Glu-372). On average, the areas under the IO-induced Ca\(^{2+}\) signals were equal to 56 ± 11 \(\mu M \cdot s^{-1}\) (\(n = 45\)) in DKO cells, 20 ± 5 \(\mu M \cdot s^{-1}\) (\(n = 41\)) in mPS1 rescue cells, and 55 ± 12 \(\mu M \cdot s^{-1}\) (\(n = 57\)) in mPS1-Δloop rescue cells (Fig. 3A). From these data, we concluded that deletion of the Asp-302–Glu-372 region causes loss of mPS1 ER Ca\(^{2+}\) leak activity. These results suggest that the appropriate length of the hydrophilic loop between TM6 and TM7 is important for the channel function of PS1.

The highly conserved \(^{335}\)PAL\(^{335}\) motif located within TM9 is important for the γ-secretase function of PS1. It has been proposed that the PAL motif participates in conformational changes of PS1 during proteolysis (30). It has been shown that Pro-433 of the PAL motif is pivotal for the structural flexibility of TM9 and, as a result, the versatility of the catalytic site of the γ-secretase (30). Mutation of Pro-433 to either alanine or leucine abolishes γ-secretase cleavage of substrates, and it has been proposed to favor PS1 to be locked in a more open conformation (30, 37). Does the PAL motif also regulate the ion conductance function of PS1? Our data show that mutations L435C and P436C led to overloaded ER Ca\(^{2+}\) and increased response to IO, whereas mutations P433C and A434C did not (Fig. 2C). To further investigate the importance of the PAL motif, we evaluated Ca\(^{2+}\) signals in the P433A and P433L stable DKO rescue lines. We found that, on average, the areas under the IO-induced Ca\(^{2+}\) curves were equal to 56 ± 11 \(\mu M \cdot s^{-1}\) (\(n = 45\)) in DKO cells, 20 ± 5 \(\mu M \cdot s^{-1}\) (\(n = 41\)) in mPS1 rescue cells,

25 ± 6 \(\mu M \cdot s^{-1}\) (\(n = 39\)) in P433A rescue cells, and 19 ± 6 \(\mu M \cdot s^{-1}\) (\(n = 44\)) in P433L rescue cells (Fig. 3B). From these data, we concluded that both the P433A and P433L PS1 mutants are functional in the ER Ca\(^{2+}\) leak assay. These results are consistent with the hypothesis that the P433A and P433L mutations lock mPS1 in an open conformation that may allow free Ca\(^{2+}\) ion passage via its conductance pore.

**DISCUSSION**

In this study, we used Ca\(^{2+}\) imaging experiments to investigate the effect of Cys substitutions in TM6, TM7, and TM9 of mPS1 (Fig. 4A) on ER Ca\(^{2+}\) leak function. We discovered that many mutations in TM7 and TM9 and only a few mutations in TM6 resulted in loss of ER Ca\(^{2+}\) leak function of mPS1 (Fig. 4B). It is of interest to compare our results with previous structure-function studies of PS1. Using the SCAM approach, the water accessibility of the TM6, TM7, and TM9 residues was evaluated in previous biochemical experiments (29–32). The C-terminal portion of human PS1 that includes TM7–TM9 was recently incorporated into SDS micelles and studied by NMR (33). A comparison of these studies is outlined in Table 1. To enable this comparison, we classified each residue within the predicted TM6, TM7, and TM9 into one of several groups, determined by their water accessibility, based on the analysis by Tolia et al. (29, 30) and their ER Ca\(^{2+}\) leak function (this study). Group 1 contains the residues that are not water-exposed and mutations of which had no effect on the ER Ca\(^{2+}\) leak function of mPS1 (Fig. 4B, white circles). Group 2 contains the residues that are not water-exposed and mutations of which resulted in loss of the ER Ca\(^{2+}\) leak function of mPS1 (Fig. 4B, gray circles). Group 3 contains the water-exposed residues and mutations of which had no effect on the ER Ca\(^{2+}\) leak
FIGURE 4. Results of structure-function analysis of TM6, TM7, and TM9 of mPS1. A, vertical representation of PS1 TM1–TM9. The conformation of the TM7–TM9 region of PS1 is based on NMR studies (33). B, structure-function analysis of TM6, TM7, and TM9 of mPS1. Amino acids are represented by the single letter code. White residues are “buried” in SCAM experiments (29, 30) and do not cause loss of Ca\(^{2+}\) leak activity when mutated to cysteine (Group 1). Gray residues are buried in SCAM experiments and cause loss of Ca\(^{2+}\) leak activity when mutated to cysteine (Group 2). Light blue residues are exposed to water in SCAM experiments and do not cause loss of Ca\(^{2+}\) leak activity when mutated to cysteine (Group 3). Dark blue residues are exposed to water and cause loss of Ca\(^{2+}\) leak activity when mutated to cysteine (Group 4). Black residues were not tested in the Ca\(^{2+}\) assay (Group 5). C, comparison of PS1 TM6, TM7, and TM9 arrangement in the proposed Ca\(^{2+}\) channel or γ-secretase complex. Views from the ER lumen (for Ca\(^{2+}\) channel) and from the extracellular space (for γ-secretase) are shown.
function of mPS1 (Fig. 4B, light blue circles). Group 4 contains the water-exposed residues and mutations of which resulted in loss of the ER Ca\textsuperscript{2+} leak function of mPS1 (Fig. 4B, dark blue circles). Group 5 contains the residues that have not been tested in ER Ca\textsuperscript{2+} leak experiments (Fig. 4B, black circles).

It is apparent from this comparison (Fig. 4B and Table 1) that there is a good correlation between the water accessibility of individual residues in SCAM experiments and the importance of these residues for the ER Ca\textsuperscript{2+} leak activity of PS1. Only three residues in TM6 (Thr-245, Ser-254, and Ala-260) were water-accessible in the SCAM experiments conducted by Tolia et al. (29). Mutations at each of these three residues to cysteine resulted in loss of ER Ca\textsuperscript{2+} leak function (Fig. 4B and Table 1, Group 4). Nine other TM6 residues tested in our experiments were not water-accessible by SCAM (29) and had no effect on ER Ca\textsuperscript{2+} leak function when mutated to cysteine (Fig. 4B and Table 1, Group 1). These results suggest that most of TM6 is buried in the hydrophobic part of the membrane and that TM6 residues have limited participation in forming the ion conductance pore of PS, with only three residues (Thr-245, Ser-254, and Ala-260) facing the pore. Interestingly, the catalytic residue Asp-257 within TM6 appears to be buried in the membrane. The only exception is Gly-382; this residue is buried and plays a role in the ER Ca\textsuperscript{2+} leak activity of PS1 based on previous (22, 36) and current (Fig. 2A) experiments.

When TM7 was analyzed in SCAM experiments, it was determined that the stretch of residues (Gly-384, Asp-385, Ile-387, and Tyr-389) surrounding the catalytic residue Asp-385 is water-accessible (29). Mutations at each of these four residues abolished the ER Ca\textsuperscript{2+} leak function of mPS1 (Fig. 4 and Table 1, Group 4). These results indicate that the portion of TM7 surrounding the Asp-385 catalytic residue lines the ion conductance pore of PS and likely forms part of the selectivity filter of the potential ion conductance channel (Fig. 4B). Glu-376 at the beginning of TM7 was also water-accessible and critical for ER Ca\textsuperscript{2+} leak function (Fig. 4B and Table 1, Group 4), indicating that this residue likely forms part of the entrance to the pore of the potential Ca\textsuperscript{2+} channel, most likely in conjunction with Ala-260 from TM6 (Fig. 4B). Nine other residues within TM7 were buried in the SCAM experiments of Tolia et al. (29) and had no effect on ER Ca\textsuperscript{2+} leak function when mutated to cysteine (Fig. 4B and Table 1, Group 1). It is likely that these residues form the hydrophobic face of the TM7 helix facing the membrane. The only exception is Gly-382; this residue is buried based on experiments by Tolia et al. (29), but a mutation of this residue caused loss of ER Ca\textsuperscript{2+} leak function (Fig. 4B and Table 1, Group 2). It is likely that mutation of this residue affects the conformation of the 384-387 GDIY region and induces pore constriction. The presence of such critical glycine residues within a pore loop is a typical feature of most ion channels (38).

Our data reveal that TM9 had the most mutations that affected ER Ca\textsuperscript{2+} leak function (Fig. 4B and Table 1). Eight mutations in TM9 (Fig. 4B and Table 1, Group 4) abolished ER Ca\textsuperscript{2+} leak activity, and the mutations were spread across the TM domain. These observations correlate with the fact that TM9 is flexible, modulates the dynamics of the catalytic site of the γ-secretase complex, and is highly water-accessible (30). One can infer that the loss of function of the Ca\textsuperscript{2+} leak by cysteine mutations may inhibit conformational changes that would allow pore opening by affecting key residues that are involved in inter/intramolecular interactions that are essential for allowing ions to pass through the potential conductance pore of PS1. Interestingly, the highly hydrophobic F44FYFAT449 cysteine mutations may inhibit conformational changes

The table below provides a comparison of structure-function studies of TM6, TM7, and TM9 of PS1:

**Table 1**

| Residue | SCAM water accessibility | NMR secondary structure | SCAM water accessibility | ER Ca\textsuperscript{2+} leak channel |
|---------|-------------------------|------------------------|-------------------------|---------------------------------------|
| TM6     |                         |                        |                         |                                       |
| Thr-245 | Exposed                 | ND                     | ND                      | LOF 4                                 |
| Ala-246 | ND                      | ND                     | ND                      | F 1                                   |
| Trp-247 | Buried                  | Buried                 | ND                      | F 1                                   |
| Leu-248 | ND                      | ND                     | ND                      | F 1                                   |
| Ile-249 | ND                      | ND                     | ND                      | F 1                                   |
| Leu-250 | Buried                  | Exposed                | ND                      | LOF 4                                 |
| Ala-251 | ND                      | ND                     | ND                      | F 1                                   |
| Val-252 | ND                      | ND                     | ND                      | F 1                                   |
| Ile-253 | ND                      | ND                     | ND                      | F 1                                   |
| Ser-254 | Exposed                 | Buried                 | ND                      | LOF 4                                 |
| Val-255 | Buried                  | Buried                 | ND                      | F 1                                   |
| Tyr-256 | ND                      | ND                     | ND                      | F 1                                   |
| Asp-257 | Buried                  | Buried                 | ND                      | F 1                                   |
| Leu-258 | Buried                  | Buried                 | ND                      | F 1                                   |
| Val-259 | ND                      | ND                     | ND                      | F 1                                   |
| Ala-260 | Exposed                 | Exposed                | ND                      | LOF 4                                 |
| Val-261 | Buried                  | Buried                 | ND                      | F 1                                   |
| Leu-262 | Buried                  | Buried                 | ND                      | F 1                                   |
| Cys-263 | ND                      | ND                     | ND                      | F 1                                   |
| Pro-264 | Buried                  | Buried                 | ND                      | F 1                                   |
| TM7     |                         |                        |                         |                                       |
| Glu-376 | Exposed                 | ND                     | Loop                   | LOF 4                                 |
| Arg-377 | ND                      | ND                     | Loop                   | ND 5                                  |
| Gly-378 | Buried                  | Exposed, not in pore   | Loop                   | ND 5                                  |
| Val-379 | ND                      | ND                     | Loop                   | ND 5                                  |
| Lys-380 | Exposed                 | Exposed                | Loop                   | F 1                                   |
| Leu-381 | Buried                  | Exposed                | Loop                   | F 1                                   |
| Gly-382 | Buried                  | ND                     | Loop                   | LOF 2                                 |
| Leu-383 | Buried                  | Exposed                | ND                     | F 1                                   |
| Gly-384 | Exposed                 | Helix 7                | Helix 7                | LOF 4                                 |
| Asp-385 | Exposed                 | Helix 7                | Helix 7                | LOF 4                                 |
| Phe-386 | ND                      | ND                     | Helix 7                | ND 5                                  |
| Ile-387 | Exposed                 | Helix 7                | Helix 7                | LOF 4                                 |
| Phe-388 | Buried                  | Buried                 | Helix 7                | F 1                                   |
| Tyr-389 | Exposed                 | Buried                 | Helix 7                | LOF 4                                 |
| Ser-390 | Buried                  | Buried                 | Helix 7                | F 1                                   |
| Val-391 | ND                      | ND                     | Helix 7                | ND 5                                  |
| Leu-392 | Buried                  | Buried                 | Helix 7                | F 1                                   |
| Val-393 | ND                      | ND                     | Helix 7                | ND 5                                  |
| Gly-394 | Buried                  | Buried                 | Helix 7                | ND 5                                  |
| Lys-395 | Buried                  | Exposed, not in pore   | Helix 7                | F 1                                   |
| TM9     |                         |                        |                         |                                       |
| Pro-433 | Exposed                 | ND                     | Loop                   | F 3                                   |
| Ala-434 | Exposed                 | Exposed                | Loop                   | F 3                                   |
| Leu-435 | Exposed                 | Exposed                | Loop                   | LOF 4                                 |
| Pro-436 | Exposed                 | ND                     | Loop                   | LOF 4                                 |
| Ile-437 | Exposed, not in pore    | Exposed                | Loop                   | F 3                                   |
| Ser-438 | Exposed                 | Exposed                | Loop                   | F 3                                   |
| Ile-439 | Exposed                 | Exposed                | Loop                   | LOF 4                                 |
| Thr-440 | Exposed                 | Exposed                | Loop                   | F 3                                   |
| Phe-441 | Exposed                 | Buried                 | Helix 9a               | LOF 4                                 |
| Gly-442 | Exposed                 | Buried                 | Helix 9a               | F 3                                   |
| Leu-443 | ND                      | ND                     | Helix 9a               | ND 5                                  |
| Val-444 | Exposed                 | Buried                 | Helix 9a               | F 3                                   |
| Phe-445 | Exposed                 | Exposed                | Helix 9a               | LOF 4                                 |
| Tyr-446 | Exposed                 | Exposed                | Helix 9a               | LOF 4                                 |
| Phe-447 | Exposed                 | Buried                 | Helix 9a               | LOF 4                                 |
| Ala-448 | ND                      | ND                     | Helix 9a               | ND 5                                  |
| Thr-449 | Exposed                 | Buried                 | Helix 9a               | LOF 4                                 |
| Asp-450 | ND                      | ND                     | Helix 9a               | ND 5                                  |
| Tyr-451 | Exposed                 | Exposed, not in pore   | Loop                   | F 3                                   |
| Leu-452 | Exposed                 | Buried                 | Loop                   | F 3                                   |
| Val-453 | Exposed                 | Buried                 | Loop                   | F 3                                   |
| Gln-454 | Exposed, not in pore    | Exposed                | Loop                   | F 3                                   |
| Pro-455 | Exposed                 | Buried                 | Loop                   | F 3                                   |
Mutagenesis Mapping of the PS1 Ca²⁺ Leak Conductance Pore

patch of TM9 is water-accessible, and mutations in this region affected ER Ca²⁺ leak function (Fig. 4B and Table 1, Group 4), but not γ-secretase activity (30), suggesting a significant role for this region in the proposed ion conductance channel. NMR studies demonstrated recently that TM9 is made up of two small α-helices, helices 9a and 9b, which are connected by a proline kink (33). Although residues in helix 9b (residues 456–461) are accessible to the membrane surface, those in helix 9a (residues 440–449) show little or no such accessibility (33). These results are in agreement with our findings that the residues affecting Ca²⁺ leak activity in TM9 may line the pore of the proposed ion conductance channel. However, even though most mutated residues in TM9 were found to be exposed to water (Fig. 4B and Table 1, Group 3) (30), not all of them affected Ca²⁺ leak function, which verifies the proposed hypothesis that different subsets of these residues are exposed to the γ-secretase catalytic cavity in different PS conformations (30). Clearly, there is only partial overlap regarding the γ-secretase and ER Ca²⁺ leak function of particular residues in TM9, which may be attributed to the conformational flexibility observed in this domain.

The PAL motif is imperative for the structural flexibility of TM9 and, as a result, the versatility of the catalytic site of γ-secretase (30). It has been demonstrated that P433L abolishes the γ-secretase activity of PS1, most likely by locking the catalytic site of PS1 in an inactive open conformation of the catalytic site (30, 37). We found that the P433L mutant maintained Ca²⁺ leak activity (Fig. 3B). These results suggest that conformational changes needed for γ-secretase processing of a substrate are not needed for ER Ca²⁺ leak function. These data also indicate that the “open inactive” γ-secretase conformation locked by the Pro-433 mutation is able to function as an ion channel. On the basis of these results, we reason that both P433A and P433L permit the conductance pore of PS1 to be open by allowing HDVII to exit the conductance pore, which in turn allows the passage of ions across the channel more readily. However, cysteine mutations at Leu-435 and Pro-436 abolished Ca²⁺ leak function (Fig. 2C), indicating that these residues likely form the entrance to the pore of the proposed ion channel, most likely in conjunction with Ala-260 from TM6 and Glu-376 from TM7 (Fig. 4B).

Deletion of the hydrophilic loop between TM6 and TM7, mPS1-Δ(Asp-302–Glu-372), caused loss of ER Ca²⁺ leak function (Fig. 3A), whereas partial deletion in the PS1-ΔE8 and PS1-ΔE9 mutants did not (22, 24). These data suggest an important role for HDVII in ion conductance, which correlates with its function as a membrane re-entrant loop that controls the access of water to the cavity in the case of γ-secretase (30). Importantly, the mPS1-Δ(Asp-302–Glu-372) deletion still contains the HDVII domain intact (amino acids 280–300), but in this case, the surrounding loop is much shorter, which would potentially hinder the movement of HDVII in and out of the pore, blocking ion/water entry. Alternatively, the region deleted in mPS1-Δ(Asp-302–Glu-372) includes helix β (Glu-356–Ile-368), an α-helical region in the assumed-to-be unstructured long N-terminal identified by the NMR studies (33). It is possible that this region could play an important role in channel gating.

Taken together, our observations support our hypothesis that exposure to water greatly increases the chance that cysteine mutant residues in TM7 and TM9 of PS1 will affect ER Ca²⁺ leak activity, and this is strong evidence that the respective TM domains are important for forming the ion conductance pore. Our results also demonstrate that the catalytic cavity and ion conductance pore of mPS1 overlap, at least partially. It is important to remember that Ca²⁺ leak function is supported by the holoprotein of PS1 in the ER and that γ-secretase function is supported by cleaved PS1 in the plasma membrane. Therefore, these two functions are mutually exclusive and never performed by the same PS1 molecule simultaneously. Nevertheless, we propose a model that can potentially reconcile both ion conductance and γ-secretase activities of PS1 based on the relative rearrangement of TM6, TM7, and TM9 in the membrane (Fig. 4C). According to this model, the catalytic residue Asp-385 of TM7 permanently lines the conductance pore, based on our results and others, whereas Asp-257 of TM6 is largely inaccessible. Gly-384, Asp-385, Ile-387, and Tyr-389 surround Asp-385 and face the conductance pore, forming part of the selectivity filter. The structurally flexible TM9 constitutes the other essential portion of the conductance pore, where the water-accessible 465FYFAT469 patch would be facing the pore region. Upon γ-secretase activation, there is a change in conformation in TM9 that would allow Asp-257 of TM6 and Asp-385 of TM7 to align and cleave substrates while maintaining water entry into the pore (Fig. 4C). The PAL motif plays an important role in this conformational rearrangement. This later conformational change is important for the γ-secretase activity of PS1, but not for Ca²⁺ leak activity. This conclusion is supported by analysis of the P433L mutant, which abolishes the γ-secretase function of PS1, but not Ca²⁺ leak function, and is consistent with our results that Asp-257 and most other residues in TM6 are dispensable for the ER Ca²⁺ leak activity of PS1. Further experiments utilizing mutations of other TM domains that could be involved in the formation of the conductance pore of presenilins should also be tested. For example, recent application of the SCAM approach demonstrated that TM1 of mPS1 is water-exposed and faces the catalytic pore of γ-secretase (39). Eventually, solving the crystal structure of PS will provide definitive information about the location of the Ca²⁺ conductance pore within the PS1 molecule.

In summary, we have provided experimental evidence that cysteine point mutations in TM7 and TM9 and, to a lesser extent, TM6 lead to loss of the ER Ca²⁺ leak function of PS1. The data from this study provide initial insight into the location and composition of the ion conductance pore of PS. Interestingly, using fluorescence lifetime imaging microscopy and differentially labeled PS1 constructs, it was discovered that PS1 forms homodimers in intact mammalian cells (40). Although PS1 dimers have been studied regarding γ-secretase activity and function in the past (41–43), how this configuration relates to the ion conductance function of PS1 is unknown. In previous studies, we demonstrated that FAD mutant PS1 exerts a dominant-negative effect on the ER Ca²⁺ leak function of wild-type PS1 (22). These results provide indirect support for the hypothesis that ER Ca²⁺ leak activity may be mediated by dimers or oligomers of PS holoproteins. Interestingly, it was revealed that
PS1 holoproteins with a D257A mutation are also able to form dimers, suggesting that cleavage within the loop is not necessary for dimerization (40). These data align with our finding that mutation of Asp-257 did not affect the ER Ca²⁺ leak function of PS1 and could potentially support a possible homodimer configuration of PS1 for the hypothesized ion channel. Further biochemical and structure-function studies will be needed to determine the stoichiometry of the PS-formed conductance pore more precisely.

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REFERENCES

1. Urban, S., Schlieper, D., and Freeman, M. (2002) Curr. Biol. 12, 1507–1512
2. Brown, M. S., Ye, J., Rawson, R. B., and Goldstein, J. L. (2000) Cell 100, 391–398
3. Hass, M. R., Sato, C., Kopan, R., and Zhao, G. (2009) Semin. Cell Dev. Biol. 20, 201–210
4. Audouin, H., Hansson, E. M., Melén, K., Bergman, A., Farmery, M. R., Winblad, B., Lendahl, U., von Heijne, G., and Näslund, J. (2005) J. Biol. Chem. 280, 35352–35360
5. Spasov, D., Tolia, A., Dillen, K., Baert, V., De Strooper, B., Vrijens, S., and Annaert, W. (2006) J. Biol. Chem. 281, 26569–26577
6. Annaert, W., Levesque, L., Craessaerts, K., Dierinck, I., Snellings, G., Westaway, D., George-Hyslop, P. S., Cordell, B., Fraser, P., and De Strooper, B. (1999) J. Cell Biol. 147, 277–294
7. De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., Von Figura, K., and Van Leuven, F. (1998) Nature 391, 387–390
8. Thirup, G., Borclett, D. R., Lee, M. K., Slunt, H. H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, I., Levey, A. I., Gandy, S. E., Jenkins, N. A., Copeland, N. G., Price, D. L., and Na¨slund, J. (2005) J. Neurosci. 25, 1230–1239
9. Takagi, S., Tominaga, A., Sato, C., Tomita, T., and Iwatsubo, T. (2010) Proc. Natl. Acad. Sci. U.S.A. 107, 9644–9649
10. Bryniki, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
11. Hofer, A. M. (1999) Methods Mol. Biol. 114, 249–265
12. Zhang, H., Sun, S., Herreman, A., De Strooper, B., and Bezprozvanny, I. (2007) J. Neurosci. 27, 12081–12088
13. Sato, C., Tomina, Y., Tominaga, A., Watanabe, N., Sato, C., Natsugari, H., Fukuyama, T., Iwatsubo, T., and Tomita, T. (2006) J. Biol. Chem. 281, 14670–14676
14. McPherson, J. J. (2000) J. Biol. Chem. 275, 689–694
15. Hebert, S. S., Godin, C., Tomiyama, T., Mori, H., and Lévesque, G. (2003) Biochem. Biophys. Res. Commun. 304, 668–674
16. Cervantes, S., González-Duarte, R., and Marfany, G. (2001) FEBS Lett. 505, 81–86
17. Hsiao, T. Y., Nair, S. P., Christiansen, T. N., and Garzoni, E. (2006) Biochem. Biophys. Res. Commun. 336, 675–680
18. Cervantes, S., Saura, C. A., Pomares, E., González-Duarte, R., and Marfany, G. (2004) J. Biol. Chem. 279, 36519–36529