Pen-2 is incorporated into the γ-secretase complex through binding to transmembrane domain 4 of presenilin 1

Naoto Watanabe1, Taisuke Tomita1*, Chihiro Sato1, Toshio Kitamura2, Yuichi Morohashi1, Takeshi Iwatsubo1*

From the 1Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences and 2Division of Cellular Therapy, Advanced Clinical Research Center, Institute of Medical Sciences, University of Tokyo, Tokyo, Japan

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Email: taisuke@mol.f.u-tokyo.ac.jp, iwatsubo@mol.f.u-tokyo.ac.jp

γ-Secretase is a multimeric membrane protein complex comprised of Presenilin (PS), Nicastrin (Nct), Aph-1 and Pen-2. It is a member of an atypical class of aspartic proteases that hydrolyzes peptide bonds within the membrane. During the biosynthetic process of the γ-secretase complex, Nct and Aph-1 form a heterodimeric intermediate complex and bind to the C-terminal region of PS, serving as a stabilizing scaffold for the complex. Pen-2 is then recruited into this trimeric complex and triggers endoproteolysis of PS, conferring γ-secretase activity. Although the Pen-2 accumulation depends on PS, the binding partner of Pen-2 within the γ-secretase complex remains unknown. We reconstituted PS1 in PS1/PS2 deficient cells by expressing a series of PS1 mutants in which one of the N-terminal six transmembrane domains (TMDs) was swapped with those of CD4 (a type I transmembrane protein) or CLAC-P (a type II transmembrane protein). We report that the proximal two-thirds of TMD4 of PS1, including the conserved Trp-Asn-Phe sequence, are required for its interaction with Pen-2. Using a chimeric CD4 molecule harboring PS1 TMD4, we further demonstrate that the PS1 TMD4 bears a direct binding motif to Pen-2. Pen-2 may contribute to the activation of the γ-secretase complex by directly binding to the TMD4 of PS1.

γ-Secretase is a cryptic aspartic protease that catalyzes the intramembrane proteolysis of scissile bonds within a transmembrane domain (TMD) of type I proteins (for review, see 1). Missense mutations in presenilin (PS) genes are linked to the early-onset familial Alzheimer’s disease (FAD). FAD mutations lead to altered cleavage of amyloid precursor protein (APP) and overproduction of amyloid β peptides (Aβ) ending at position 42 (Aβ42). Aβ42 peptides are more prone to formation of amyloid deposits (2). In contrast, ablation of PS genes causes a complete loss of γ-secretase activity and thus cleavage of a set of type I transmembrane proteins (e.g., APP, Notch, ErbB4, CD44). In addition to the beneficial loss of Aβ, failure to release Notch intracellular domain (NICD) causes lethality (3), perhaps masking the biological activities of other released intracellular domain. Thus, PS-dependent γ-secretase mediates a novel mode of proteolysis-dependent signal transduction (4). Genetic and biochemical analysis revealed that in addition to PS, γ-secretase is comprised of Nicastrin (Nct), Aph-1 and Pen-2. All four proteins define the minimal set of components required to reconstitute activity (5-8). PS, which contain the catalytic aspartate residues in adjacent TMDs, undergoes endoproteolysis to generate N- and C-terminal fragments (NTF and CTF, respectively) each containing one aspartyl residue (9, 10). The fragment form a stable heterodimer and are incorporated into a highly stabilized, high molecular weight (HMW) protein complex together with the other components (11-14). However, the stoichiometric composition and the mechanism of assembly of the γ-secretase complex have remained elusive. We have shown by a systematic mutational analysis that stabilization and HMW complex formation of PS is essential to the γ-secretase activity and requires preservation of the C terminus of PS (15-17). Recent studies suggest that the C terminus of PS directly interacts with Nct-Aph-1 subcomplex, the latter being initially formed in the biosynthetic pathway of the γ-secretase complex (18). Nct, a single pass transmembrane protein harboring a large extracellular region,
and Aph-1, a putative multipass transmembrane protein (19-21), form a subcomplex that is stable in the absence of PS, suggesting a direct interaction of these two components (22-26). Furthermore, overexpression of Aph-1 and Nct forms a relatively stable trimeric intermediate complex leading to accumulation of PS holoprotein, whereas genetic ablation of either Nct or Aph-1 caused a significant loss of PS stability (5, 8, 27-29). Thus, the Nct-Aph-1 subcomplex contributes to the stability of the γ-secretase complex.

Pen-2 was originally identified by genetic screening in C. elegans as an enhancer of PS activity (21). Human pen-2 gene encodes a 101-amino-acid polypeptide that has a hairpin like topology with N- and C-termini being exposed to the lumen (30, 31). Subsequent studies indicated that, during the process of γ-secretase complex assembly, Pen-2 is incorporated into the PS-Nct-Aph-1 trimeric intermediate and provokes the endoproteolysis of PS and confers the proteolytic activity (5, 7, 8). It has also been shown that the length and the amino acid sequences of the C terminus of Pen-2 are crucial for the stability of PS fragments, suggesting a role of Pen-2 in the metabolism of the γ-secretase complex after its assembly (32-35). Recently, it was demonstrated that the generation of PS1 fragments as well as γ-secretase activity requires the proximal two-thirds of the first TMD of Pen-2, which mediate interactions with PS (33). Further report on the partial dissociation of the γ-secretase complex using a mild detergent (i.e., dodecyl β-D-maltoside) suggested the formation of a PS1 NTF-Pen-2 subcomplex (36). However, the identity of the functional and physiological binding partner of Pen-2 within the γ-secretase complex remains elusive. To address this issue, we reconstituted PS1 activity in PS1/PS2 deficient cells (37, 38) with chimera that harbor deletion or replacement TMD. We report that TMD4 of PS1 is the docking site for Pen-2 and is required to mediate its incorporation into the γ-secretase complex.

Materials and Methods

Plasmid construction, Cell culture, transfection and retroviral infection

cDNAs encoding PS1, Pen-2, APP carrying Swedish mutation (APP_NL), NotchΔE (NΔE) and CD4 were inserted into pMXs-puro or pLPCX (CLONTECH) (16, 18, 39-42). cDNAs encoding mutant PS1 or CD4 were generated by long PCR-based Quikchange™ (Stratagene) or SLIM protocol (43). Each TMD of PS1 was replaced with those of CD4 (Type I) or CLAC-P (Type II) according to its predicted topology (Fig. 1). All constructs were sequenced using Thermosequenase (Amersham Biosciences, Buckinghamshire, UK) on an automated sequencer (Li-Cor, Lincoln, NE). Maintenance of PS1/PS2-deficient fibroblasts (DKO cells) was done as previously described (16). Packaging cell line Plat-E was kept in DMEM containing 1 μg/ml puromycin (SIGMA) and 10 μg/ml blasticidin (44). Highly efficient retroviral infection system using pMXs-puro was applied as reported previously (45). Briefly, retroviral transfer plasmids were transfected by FuGENE 6 (Roche Biochemicals) into Plat-E cells. After 48 hr, conditioned media were filtered through 0.45 μm-size pore and used as a virus stock. For infection, DKO cells were cultured with a virus stock containing 5 μg/ml polybrene. After 24 hr, the virus stock was replaced with new media that did not contain virus and further analyzed. Stable infectants of APP_NL or NΔE in DKO cells were generated by infection of recombinant retrovirus and selection in DMEM containing puromycin. The infection efficacy was ~100% as estimated in a control experiment using pMXs-IG, a retroviral plasmid carrying GFP.

Antibodies and immunochemical analyses

Anti-G1Nr2 and PNT3 antibodies against GST-fused human PS1 N terminus or a synthetic peptide corresponding to the N-terminal 26 amino acids of human/mouse Pen-2, respectively, have been previously described (46, 47). Anti-SCAP monoclonal antibodies (#7601, #7606, #7623) were described previously (48). Other antibodies were purchased from BD transduction laboratories (anti-Flotillin-1, anti-GM130, anti-golgin-84), Chemicon (anti-PS1loop (MAB5232)), Cell Signaling Technology (anti-cleaved Notch1 (V1744)), Covance (anti-Aph-1aL (O2C2)), Diatec (anti-CD4 (EDU-2)) or Santa Cruz Biotechnology (anti-CD4 (H-370) and anti-Nct (N-19)). Immunoblot analysis, immunoprecipitation of CHAPSO-solubilized lysates, or quantitations of Aβ by two-site ELISAs using BNT77 as a capture antibody were performed as previously described (5, 15-17, 26, 39, 42, 46, 47, 49, 50).
Results

Chimeric mutant PS1 swapped at transmembrane domain 4 lacks the \( \gamma \)-secretase activity due to loss of interaction with Pen-2.

Several lines of evidence suggest that intramembranous helix-helix interactions play important roles in the assembly as well as activity of the \( \gamma \)-secretase complex (18, 24, 26, 33, 51-54). To identify functionally important transmembrane domains (TMDs) of PS1 without causing topological changes, we constructed PS1 mutants in which one of the six N-terminal TMDs was swapped with that of functionally irrelevant proteins (TMD-swap), i.e., CD4 (type I transmembrane protein; 18) or CLAC-P (type II transmembrane protein, 55). Especially, we focused on the N-terminal fragment (NTF) of PS1 for the following reasons. (i) The topological analyses of PS1 NTF have reached a genuine consensus, whereas the topology of the C-terminal fragment (CTF) is still controversial (56-61); (ii) PS1 NTF is believed to harbor functionally important domains: TMD1 and 2 are required for the proteolytic activity and implicated in substrate binding (62-64), and TMD6 bears a catalytic aspartate (8). (iii) Importantly, PS1 NTF-Pen-2 subcomplex has been detected by partial dissociation of the \( \gamma \)-secretase complex using dodecyl \( \beta \)-D-maltoside as a detergent (36). We systematically replaced TMDs of PS1 NTF according to their predicted topology (Fig. 1): TMDs exhibiting a type I orientation (i.e., TMD2, 4) were replaced with that of CD4 (TM2mt, TM4mt, respectively), and those with a type II orientation (i.e., TMD1, 3, 5) with that of CLAC-P (TM1mt, TM3mt, TM5mt, respectively). As aspartate residue at position 257 in TMD6 is predicted to be one of the catalytic residues for \( \gamma \)-secretase activity, we replaced amino acid sequences proximal to this aspartate (i.e., residues 244-255) with those in the corresponding position of CD4 TMD (TM6mt). We examined the effects of these PS1 mutants on the expression of and the assembly with other \( \gamma \)-secretase components in PS1/PS2 double knockout fibroblasts (DKO cells) by the recombinant retrovirus-mediated overexpression system. As reported, DKO cells lacked the expression of complex-type \( N \)-glycosylated (mature) Nct, as well as of Pen-2 (Fig. 2A) (65-67). Upon infection of a retrovirus encoding wt PS1, the levels of endoproteolysed forms of PS1 as well as of mature Nct recovered, and the expression of Pen-2 was also restored. To assess if a highly stable hetero-tetrameric complex (PS fragments-Nct-Aph-1-Pen-2) formed, we blocked translation by adding cycloheximide to the cells. Pen-2 polypeptides and PS1 fragments were highly stable (see Fig. 4), consistent with the formation of the HMW complex.

Among the TMD-swap chimera, only TM3mt expression restored the maturation of Nct, Pen-2 level, and endoproteolysis (Fig. 2A). Co-immunoprecipitation analysis revealed that like wt PS1, TM3mt forms the HMW complex containing all four proteins, suggesting that replacement of TMD3 with that of CLAC-P did not interfere with the assembly of the \( \gamma \)-secretase complex (Fig. 2B).

All the six PS1 TMD-chimera were co-immunoprecipitated with Nct and Aph-1aL (Fig. 2B), in agreement with the recent reports that the Nct-Aph-1 subcomplex interacts with the C terminus of PS1 (18, 68). These TMD-swap PS1 mutants were not co-immunoprecipitated with membrane proteins irrelevant to the \( \gamma \)-secretase complex (Supplementary Fig. 1). With the exception of TM4mt, the remaining TMD-chimera restored accumulation of mature Nct and Pen-2 even though they failed to undergo endoproteolysis (Fig. 2A). However, the levels of mature Nct in DKO cells expressing TM1mt or TM5mt PS1 were noticeably lower than those in other mutants. In sharp contrast, TM4mt PS1 failed to support the maturation of Nct or the accumulation of Pen-2. Because Pen-2 accumulation was restored in the absence of TM1, 2, 3, 5 and 6 of PS1 TMDs, we hypothesized that TM4 was essential for the assembly of the \( \gamma \)-secretase complex, perhaps via recruitment of Pen-2. To examine if TM4mt interacted with Pen-2, we coinfected recombinant retrovirus encoding Pen-2 together with those encoding wt or TMD-chimera PS1 (Fig. 3). Overexpression of Pen-2 did not affect the generation of PS fragments nor Nct maturation (data not shown). Only the TM4mt chimera failed to interact with either endogenous or overexpressed Pen-2 polypeptides, suggesting that the TMD4 of PS1 directly contributes to the interaction of Pen-2 with PS1.

To rule out the possibility that replacement of PS1 TM4D with that of CD4 caused significant structural changes on PS1, we assessed its stability by cycloheximide treatment (Fig. 4A). As reported (11, 16), NTF of wt PS1 was stable at 12 hr of treatment, whereas PS1 holoprotein was mostly degraded during the chase of 4 hr. Consistent with the previous results that endoproteolysis-deficient functional PS (e.g., PS1/M292D (69)) as well as catalytic site
mutants (e.g., PS1/D385A (10)) are able to incorporate into a HMW complex, we confirmed that a subfraction of these uncleaved mutants persisted as stable holoprotein (16). These results are in agreement with the notion that the hetero-tetrameric interactions produce a highly stable complex (5). Our previous analysis suggested that the Nct-Aph-1 subcomplex stabilized PS (5, 26). TM4mt was also stabilized in the presence of Nct and Aph-1 proteins, indicating that TMD4 chimera folded properly and retained the proper PS1 conformation. However, only wt PS1 promoted the stability of Pen-2 (Fig. 4B). Taken together, these data confirm that TMD4 of PS1 plays an important role in the incorporation of Pen-2 into the γ-secretase complex and in the stabilization of Pen-2.

We next analyzed the γ-secretase activities of TMD-chimera in DKO cells coexpressing APPNL or NΔE. As reported, expression of wt PS1 restored proteolysis of these substrates to generate Aβ as well as NICD (Fig. 5). Consistent with the restoration of endoproteolysis, TM3mt exhibited a low, but obvious γ-secretase activity as revealed by the generation of Aβ42 and NICD. In contrast, despite their ability to participate in hetero-tetrameric complex formation, TM1mt, TM2mt, TM5mt and TM6mt did not display any γ-secretase activity to generate Aβ nor NICD. This may suggest that these TMDs play specific roles in the acquisition of proteolytic activity of the fully-assembled γ-secretase complex. In agreement with the result that TMD4 was required for the incorporation of Pen-2 into the γ-secretase complex, TM4mt showed no complementation of the proteolytic activity even when Pen-2 was coexpressed (data not shown). Thus, our results strongly suggest that TMD4 of PS1 plays a significant role in its interaction with Pen-2, as well as in the stabilization of Pen-2, the latter being a crucial molecule required for the γ-secretase activity.

The proximal two-thirds of PS1 TMD4 including the conserved WNF motif is crucial for the direct interaction with and the stabilization of Pen-2

To narrow down the critical region within the TMD4 of PS1 required for the incorporation of Pen-2, we systematically replaced each third of PS1 TMD4 with the corresponding subregions of CD4 TMD (Fig. 6A). A chimeric protein in which the C-terminal one-third of the PS1 TMD4 is replaced with the corresponding TMD sequences of CD4 (PPC) was endoproteolyzed to generate PS1 fragments and was able to rescue the expression of mature Nct as well as of Pen-2 in a similar fashion to wt PS1 (Fig. 6B). In contrast, mutants swapping either the N-terminal or central regions of TMD4 (CPP, PCP, respectively) were not cleaved and did not rescue the expression of mature Nct and Pen-2 (Fig. 6B). To determine which of the subregions (i.e., proximal or central) in TMD4 is involved in the interaction with Pen-2, we further analyzed chimeric mutants in which two-thirds of TMD4 were replaced with those of CD4 (i.e., PCC, CPC, CCP), and found that none of these mutants complemented the PS1/2-null phenotypes (Fig. 6B). This suggests that both the N-terminal (proximal) and central regions of TMD4 are required for Pen-2-dependent PS functions.

We next analyzed the interaction of the PPC, PCC, CPC or CCP chimeric proteins with overexpressed Pen-2 in DKO cells (Fig. 6C). Consistent with the results of the rescue experiments, only PPC mutant PS1 bound to Pen-2 to a similar level as wt PS1. CCP mutant failed to interact with Pen-2, although a small amount of Pen-2 was coprecipitated with the PCC and CPC mutants. Combined with the ability of these mutants to impact Pen-2 levels (Fig. 6B), these results suggest that both the proximal and central regions of TMD4 are cooperatively involved in the stable interaction of PS1 with Pen-2.

Alignment of amino acid sequences of PS family proteins reveals that the TMD4 sequences contain an “NxxxxDYxTxxxxxWNFGxVGxxxI” consensus motif (x denotes unconserved residues; see Fig. 10, upper panel). Within the N-terminal two-thirds of TMD4, we focused on the sequence “WNF” (Fig. 7A), because PCC mutant completely lacked the accumulation of and interaction with Pen-2, whereas PPC mutant behaved normally as wt PS1 (see Fig. 6). Replacement of the WNF sequence with alanine residues (WNF_AAA) completely abolished the accumulation of Pen-2, maturation of Nct (Fig. 7B) and its interaction with Pen-2, whereas WNF_AAA bound to Nct and Aph-1αL (Fig. 7C, and data not shown). Since CPP mutant PS1 did not rescue Pen-2, we surmised that among the N-terminal two-thirds of PS1 TMD4, the WNF sequence located within the central region, along with the more N-terminal region, are cooperatively involved in the PS1-Pen-2 interaction, and thus contributing to the
stabilization of Pen-2 and its incorporation into the γ-secretase complex.

The amino acid sequences within the TMD4 of PS1 harbor an intrinsic and direct binding motif to Pen-2.

These chimeric studies identified TMD4 of PS1 as the intrinsic binding domain of Pen-2. Genetic and functional studies indicate that the binding of Nct-Aph-1 subcomplex to PS is the primary step in the maturation of the γ-secretase complex, which is followed by the incorporation of Pen-2 (5). To examine whether preassembly of Nct-Aph-1-PS1 subcomplex is required for the recruitment of Pen-2, we analyzed DKO cells expressing two types of endoproteolysis-deficient PS1 mutants: PS1/M292D that forms a functionally active complex, and PS1/455stop that is unable to bind to Nct and fails to support its maturation (similar to TM4mt (18, 68-71; Fig. 8A)). Interestingly, PS1/455stop bound to and rescued the level of Pen-2 similarly to wt PS1 or PS1/M292D, suggesting that the acquisition of stability of Pen-2 takes place independently of the interaction with the Nct-Aph-1 subcomplex. Because a PS1 NTF-Pen-2 subcomplex has been detected in partially-disrupted γ-secretase complexes solubilized by dodecyl β-D-maltoside (36), we overexpressed NTF of wt or TM4mt PS1 (NTF/TM4mt) in DKO cells and analyzed their interactions with Pen-2 (Fig. 8B). Importantly, PS1 NTF overexpressed in DKO cells bound Pen-2, indicating this interaction did not require preassembly of PS1-Nct-Aph-1 subcomplexes. As expected, NTF/TM4mt failed to interact with Pen-2 even in the presence of MG132 (an inhibitor of proteasome-mediated degradation of NTF/TM4mt and Pen-2). Further, PS1 CTF did bind to the Nct-Aph-1 subcomplex, but did not associate with Pen-2 (67, and data not shown). These results supported our view that an intrinsic binding site for Pen-2, located within the PS1 NTF, can mediate PS1-Pen-2 interaction independent of the interaction with the Nct-Aph-1 subcomplex.

To unequivocally demonstrate that the Pen-2 binding region resides within the TMD4 of PS1, we constructed chimeric CD4 proteins in which the single TMD was swapped with the TMD4 of PS1 (CD4_TM4) or with that of PS1 replaced at the WNF sequence with three alanine residues (CD4_AAA). Strikingly, overexpression of CD4_TM4, but not of wt CD4 or CD4_AAA, in DKO cells increased the level of Pen-2 (Fig. 9A). CD4_TM4 formed a complex with endogenous as well as exogenous Pen-2 in PS null background, whereas neither wt CD4, CD4_AAA nor irrelevant membrane proteins pulled down exogenously overexpressed Pen-2 (Fig. 9B, Supplementary Fig. 2). Cycloheximide treatment showed that the expression of CD4_TM4 increased the stability of Pen-2 in a similar fashion to wt PS1 (Fig. 9C, compare with Fig. 4C). As all CD4 chimeras were relatively stable polypeptides, the ability to stabilize Pen-2 reflects its interaction with the CD4_TM4 chimera. Collectively, these results indicate that TMD4 of PS1 is required and sufficient to mediate a direct interaction with Pen-2.

Discussion

A series of genetic and biochemical studies have revealed the mechanisms underlying the sequential assembly of the γ-secretase complex components (1). To obtain further information about the relationships between PS1 (especially its TMDs) and other γ-secretase components, we constructed and expressed a series of “TMD-swap” chimeric PS1 proteins in which one of the N-terminal six TMDs were replaced with those of irrelevant transmembrane proteins (i.e., CD4 and CLAC-P), and examined their binding to other components, as well as their ability to complement the levels of individual subunits and the γ-secretase activity in PS null background. Swap of most of the TMDs, besides that of TMD3, abolished the γ-secretase activity. However, most of the TMD mutants formed the tetrameric γ-secretase complex, except for the TM4mt that lacked the interaction with Pen-2. Further chimeric analysis suggested that the N-terminal two-thirds of the TMD4 of PS1 is the direct binding site of Pen-2. This binding stabilizes Pen-2, and converts the “proenzymatic” PS1-Aph-1-Nct ternary complex into a fully activatable γ-secretase. Importantly, the TMD4 sequence of PS1 introduced into an irrelevant transmembrane protein, CD4, retained the capacity to bind to Pen-2, underscoring the specificity of the sequence in this interaction. Recently, Kim and Sisodia have also found that TMD4 of PS1 is the binding site of Pen-2 by swapping the TMDs of PS1 with those of SREBP cleavage activating protein (personal communication), which strongly corroborates our findings.

Previous studies have suggested that the intramembrane interactions through TMDs may serve as the major mechanism underlying the assembly of the γ-secretase complex components (18, 24, 26, 33, 51-54). However, introduction of a deletion or point mutation into PS1 occasionally caused significant topological
changes of the TMD, rendering PS1 polypeptide unstable and making the functional and interaction studies difficult (11, 15, 16). Therefore, we employed the “TMD-swap” method for the analysis of the individual roles of TMDs. The finding that all TMD mutants within the PS1 NTF were able to form the PS1-Nct-Aph-1 trimeric complex, whereas PS1 harboring a small deletion at the C terminus (i.e., PS1/455stop) failed to form this complex, was consistent with the previous report that the C terminus of PS1 is the interaction domain with the Nct-Aph-1 subcomplex (18, 68). Swap of TMD1, 2 or 6 caused a complete loss of the γ-secretase activity, despite the formation of the hetero-tetrameric complex. This may suggest that these TMDs are directly involved in the enzymatic machinery, or serve as the interaction sites with as yet unknown factor(s) required for the proteolytic activity. Of note, PS1 lacking TMDs 1 and 2 (ATM12) has been shown to exhibit a dominant negative phenotype in the presence of endogenous PS, implicating these N-terminal TMDs in the binding with substrates as well as in the endoproteolysis of PS (62-65, 71). Another interesting finding was that TMD3mt showed the least defects in the complex formation as well as the proteolytic activity. Moreover, TM3mt-containing γ-secretase predominantly produced Aβ42. It is intriguing that a number of FAD-linked mutations, some of which result in overproduction of Aβ42 in culture and a very early-onset clinical manifestation of Alzheimer’s disease, map to TMD3 (e.g., Leu166Pro) (72). Notably, amino acid residues linked to FAD mutations in TMD3 align along the helical face (73). Thus, it is tempting to speculate that the helical face of TMD3 of PS1 harbors an allosteric domain that modulates the activity and the cleavage site specificity of γ-secretase. In contrast, loss of TMD5 abolished the maturation of Nct as well as the γ-secretase activity, but the formation of a hetero-tetrameric complex was not affected. After assembly, which takes place in the early secretory compartment (i.e., ER, ERGIC) (74, 75), the assembled γ-secretase complex is transported to the post-Golgi compartments including the plasma membrane where mature Nct can be found in lipid rafts (76, 77). It should further be examined whether the loss of Nct maturation in the γ-secretase complex harboring TM5mt PS1 is due to an abnormal trafficking of the γ-secretase complex, which may indicate involvement of TMD5 in this process.

The primary amino acid sequences of Pen-2 are highly conserved in metazoans (21). Substitutions of evolutionarily conserved residues of Pen-2 cause a loss-of-function phenotype (33-35). It stands to reason that the molecular interactions of Pen-2 within the γ-secretase complex would also be highly conserved. In fact, we have shown that overexpression of human Pen-2 can rescue the knockdown phenotype of Drosophila Pen-2 in S2 cells (5). Genes encoding PS-like proteins are present not only in Bilateria (e.g., mammals, fish, insects) and Magnoliophyta (e.g., A. thaliana, O. sativa), but also in Entamoebidae (e.g., E. histolytica), Mycetozoa (e.g., D. sterium), Parabasalidea (e.g., T. vaginalis) or Euglenozoa (e.g. T. brucei, T. cruzi). All these PS-like proteins contain the “GxGD” (x denotes unconserved residues) and “PALP” motifs, suggesting that they belong to the PS-type intramembrane aspartic protease family (16, 78, 79). Alignment of amino acid sequences of these PS family proteins reveals that the TMD4 sequences are also highly conserved and contain an ”NxxxDYxTxxxxxWNGFxVGxxI” consensus motif (Fig. 10A). Our results indicate that the WNF sequence that locates at the center of TMD4 is necessary for the interaction of PS1 with Pen-2. Helical alignment of the TMD4 sequences further suggests that a conserved motif comprised of amino acid residues N-D-T-x-N may form an interface located on one side of the TMD4, that might be involved in its interaction with Pen-2 (Fig. 10B, underlined residues in consensus). It is noteworthy that the asparagine (N) residue of the WNF motif locates on this interface, and is the most highly conserved residue within PS TMD4 beyond species, supporting the view that this residue might play a critical role. Kim and Sisodia reported that amino acid sequences at the N-terminal two-thirds of TMD1 of Pen-2 comprise a functionally important domain mediating its interaction with PS1 (31). Considering that both the TMD4 of PS1 and TMD1 of Pen-2 show a type I orientation, it is possible that these two hydrophobic domains interact with each other at their N-terminal two-third regions by a helix-helix interaction through specific amino acids, leading to the formation of a functional γ-secretase complex. Notably, TMD4 of PS1 has an unusual characteristic in that FAD-linked amino acid substitutions are rare and their location is highly biased: all three mutation sites are located within the C-terminal one-third of the TMD4 sequence (http://www.molgen.ua.ac.be/ADMutations/default.cfm, 69, 70; Fig. 9 asterisks). These findings
are also consistent with the proposed molecular mechanism whereas the N-terminal region of TMD4 is critical for the γ-secretase activity.

In contrast to the exchangeable relationships between *Drosophila* and mammalian γ-secretase components (5, 17), it is noteworthy that human Pen-2 was unable to rescue the phenotype *pen-2* mutant in *C. elegans*, whereas the overexpression of all four human γ-secretase components rescued the Egl phenotype (21). However, the overexpression of human PS1 can rescue the Egl phenotype in sel-12 mutant *C. elegans* (80), suggesting that human PS1 has a capacity to interact with *C. elegans* PEN-2 but SEL-12 protein is unable to bind to human Pen-2. In fact, the amino acid sequences of the N-terminal two-thirds of *C. elegans* PS (i.e., SEL-12, HOP-1) TMD4 are largely divergent from those of mammalian as well as *Drosophila* PS, including those comprising the N-D-T-x-N consensus motif (Fig. 10A). In contrast, the amino acid sequences of the N-terminal region of Pen-2 TMD1 are less divergent between mammals and *C. elegans* (21). It is possible that the mechanism of interaction between PS and Pen-2 is somewhat different between *C. elegans* and mammals or insects.

The mechanistic impact of the binding of Pen-2 to PS1 TMD4, in relation to the functional and structural changes in the γ-secretase complex, remains unknown. Pen-2 is the smallest subunit of the γ-secretase and incorporated into the complex at the final stage of maturation. It is required for the endoproteolysis of PS and N-glycosyl maturation of Nct (5, 7, 8, 22, 34, 66). It is possible that the binding of Pen-2 causes a structural change in PS in a way to directly elicit its proteolytic activity, or alternatively, to convert the holoprotein in a manner that makes it susceptible to endoproteolysis which in turn exposes or masks a specific trafficking signal required for the activation of the γ-secretase complex. It has been shown that the requirement of the sequence specificity of Pen-2 TMD2 for the formation of the γ-secretase complex and its proteolytic activity is not so stringent compared to that of TMD1 (33). Moreover, insertion of GFP into the cytoplasmic loop of Pen-2 did not affect the activation of γ-secretase in *C. elegans* (21). However, recent studies indicate that the C terminus of Pen-2 plays a significant role in the stability of endoproteolyzed PS1 (32-35), although the mechanism of the degradation of the γ-secretase complex remains unclear. Thus, it is plausible that the two-dimensional topological mapping of the TMD4 of PS and the TMD1 and C terminus of Pen-2 determines the metabolic fate (i.e., activation, stabilization and degradation) of the γ-secretase complex.

In sum, we have demonstrated that the TMD4 of PS1, especially its N-terminal two-thirds, was required (loss of Pen-2 binding in TM4mt PS1) and sufficient (positive interaction of CD4_TM4 with Pen-2) for PS1-Pen-2 interactions and therefore it constitutes the intrinsic docking site for Pen-2. Further biochemical and cell biological studies focusing on the TMDs will facilitate our understanding about the structure-function relationships of the γ-secretase complex, especially the mechanism of its activation and the role of Pen-2.

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Footnotes
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The abbreviations used are: AD, Alzheimer's disease; Aβ, amyloid β peptide; APP, β-amyloid precursor protein; CCP, CD4-CD4-PS1 chimera; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHX, cycloheximide; CPC, CD4-PS1-CD4 chimera; CPP, CD4-PS1-PS1 chimera; CTF, carboxyl-terminal fragment; DKO, PS1/PS2 double knockout fibroblasts; ELISA, enzyme-linked immunosorbent assay; FL, full-length; Nct, Nicastrin; NICD, Notch intracellular domain; NTF, amino-terminal fragment; mt, mutant; PCC, PS1-CD4-CD4 chimera; PFP, PS1-CD4-PS1 chimera; PPC, PS1-PS1-CD4 chimera; PS, presenilin; TMD, transmembrane domain; wt, wild-type.

Figure legends

Figure 1 Schematic depiction of TMD-swap PS1 mutants.
The names of the PS1 mutants are indicated at the left of each sequence. Original TMDs and TMDs replaced with those of CLAC-P or CD4 are indicated as white, shaded and black boxes, respectively. Alignments of amino acid sequences of the TMDs of PS1 and CD4 or CLAC-P are shown at the right. Amino acid sequences remained as those of PS1 in TM6mt are underlined.

Figure 2 Complementation of maturation of Nct and accumulation of Pen-2 by expression of TMD-swap PS1 mutants in DKO cells.
A. Immunoblot analysis of DKO cells transiently infected with wt or mutant PS1 (as indicated above
the panel). Cell lysates were analyzed by immunoblotting with each antibody (as indicated below the panel). B. Co-immunoprecipitation analysis of 1% CHAPSO-solubilized fractions from DKO cells. Soluble fractions were precipitated by G1Nr2 or MAB5232 antibody and then analyzed by immunoblotting with each antibody (as indicated below the panel). mNct: mature Nicastrin; imNct: immature Nicastrin. Note that all TMD-swap PS1 mutants formed a hetero-tetrameric complex, except for TM4mt that lacked binding to Pen-2.

Figure 3 Interactions of TMD-swap PS1 mutants with overexpressed Pen-2 in DKO cells.

Co-immunoprecipitation analysis of 1% CHAPSO-solubilized fractions from DKO cells transiently and doubly infected with wt or mutant PS1 (as indicated above the panel) and Pen-2. Fractions containing equal amounts of Pen-2 ("input" at the upper panel shows the steady-state level of overexpressed Pen-2 in DKO cells) were precipitated by G1Nr2 antibody as in Fig. 2 and then analyzed by immunoblotting with each antibody (as indicated below the panel).

Figure 4 Stability of TMD-swap PS1 mutants and Pen-2 overexpressed in DKO cells.

A. Analysis of half-lives of wt or mutant PS1 (indicated at the left of the panel) in DKO cells incubated in culture media containing CHX (30 μg/ml). Lysates prepared after various incubation periods (0-12 hr) were analyzed by immunoblotting with G1Nr2 antibody. Note that TM4mt showed higher stability compared with wt PS1 holoprotein during the CHX chase period in a similar manner to M292D or D385A mutants. B. Stability of overexpressed Pen-2 in DKO cells. Total lysates from CHX-treated DKO cells transiently coexpressing wt or TM4mt PS1 (indicated at the left of the panel) and Pen-2 were analyzed using PNT3 antibody. Numbers shown above each lane denote the incubation period.

Figure 5 γ-Secretase activities in DKO cells transiently expressing TMD-swap PS1 mutants.

Sandwich ELISA analysis of secreted Aβ from APPNL-stable DKO cells (A) and immunoblot analysis of the generation of NICD from NαE-stable DKO cells (B) transiently expressing TMD-swap PS1 mutants. The levels of Aβ40 and Aβ42 are shown by white and black bar, respectively. Only TM3mt exhibited a significant γ-secretase activity in the complementation analyses (*: p<0.001, **: statistically significant (p=0.053) by Dunnet test).

Figure 6 Functional analysis of chimeric mutant PS1 substituted at one- or two-thirds of the TMD4.

A. Amino acid sequences of TMDs of PS1, CD4 and various chimeras in which one-third (CPP, PCP, PPC) or two-thirds (PCC, CPC, CCP) of PS1 TMD4 was replaced with the corresponding sequences of CD4 TMD. Unchanged amino acid sequences of PS1 in each chimeric mutant are underlined. Asterisks indicate the locations of amino acid substitutions linked to FAD. B. Immunoblot analysis of DKO cells transiently expressing the chimeric PS1. Cell lysates were analyzed by immunoblotting with each antibody (as indicated below the panel). C. Co-immunoprecipitation analysis of 1% CHAPSO-solubilized fractions from DKO cells transiently coexpressing wt or mutant PS1 (as indicated above the panel) and Pen-2.

Figure 7 WNF sequence is essential for the interaction of PS1 with Pen-2

A. Amino acid sequences of TMD4 of wt PS1, TM4mt and WNF_AAA. Unchanged amino acid sequences of PS1 in each chimeric mutant are underlined. Asterisks indicate the locations of amino acid substitutions linked to FAD. B. Immunoblot analysis of DKO cells transiently expressing PS1 mutants. Cell lysates were analyzed by immunoblotting with each antibody (as indicated below the panel). C. Co-immunoprecipitation analysis of 1% CHAPSO-solubilized fractions from DKO cells transiently coexpressing wt or mutant PS1 (as indicated above the panel) and Pen-2. Soluble fractions were precipitated by MAB5232 antibody and then analyzed by immunoblotting with each antibody (as indicated below the panel).

Figure 8 Pen-2 interacts with PS1 independently of the incorporation of Nct into the complex.

A. 1% CHAPSO-solubilized cell lysates (input, left) or immunoprecipitated fractions by G1Nr2
were analyzed by immunoblotting with each antibody (as indicated below the panel). Note that PS1/455stop was able to bind to and increase Pen-2, whereas it failed to support Nct maturation. B. Co-immunoprecipitation analysis of MG132-treated DKO cells stably expressing wt or TM4mt PS1 NTF (as indicated above the panel).

Figure 9 Direct binding of Pen-2 to PS1 TMD4 chimerically expressed as TMD of CD4.
A. Immunoblot analysis of DKO cells expressing CD4 or its chimeric mutant. Cell lysates were analyzed by immunoblotting with each antibody (as indicated below the panel). B. Co-immunoprecipitation analysis of 2% CHAPSO-solubilized fractions from DKO cells expressing CD4 chimera (as indicated above the panel) coinfecte with or without Pen-2 (right and left, respectively). Soluble fractions were precipitated by EDU-2 antibody and then analyzed by immunoblotting with each antibody (as indicated below the panel). C. Analysis of half-lives of overexpressed CD4 and Pen-2 (indicated at the right of the panel) in DKO cells incubated in culture media containing CHX (30 µg/ml). Lysates prepared after various incubation periods (0-12 hr) were analyzed by immunoblotting with each antibody. Numbers shown above each lane denote the incubation period.

Figure 10 Schematic representation of TMD4 of PS.
A. Alignment of amino acid sequences of TMD4 of PS1 and its homologues. Letters refer to the single amino acid code. Amino acids identical among multiple organisms are shown in black. Asterisks denote the locations of amino acid substitutions linked to FAD. Amino acid residues that locate on the same interface within the consensus sequence based on the helical wheel prediction (in B) are underlined. B. α-Helical wheel alignment consisting of 18 amino acids of TMD4 of human PS1 starting at the N-terminal asparagine as the 1st residue. The locations of amino acids after the 18th residue (valine) are shown in brackets. Black circles indicate the locations of the consensus amino acid residues. Asterisks and underlines are as defined in A.

Supplementary Figure 1 No interaction of TMD-swap PS1 mutants with irrelevant membrane proteins.
Co-immunoprecipitation analysis of 1% CHAPSO-solubilized fractions from DKO cells expressing TMD-swap PS1 mutants. Soluble fractions were precipitated by MAB5232 antibody (A) or antibodies against irrelevant membrane proteins (i.e., anti-GM130, anti-golgin-84, anti-SCAP (#7601, #7606, #7623), EDU-2) (B) and then analyzed by immunoblotting with each antibody (as indicated below the panel).

Supplementary Figure 2 No interaction of CD4 chimera with irrelevant membrane proteins.
Co-immunoprecipitation analysis of 2% CHAPSO-solubilized fractions from DKO cells expressing CD4 chimera. Soluble fractions were precipitated by EDU-2 antibody (A) or antibodies against irrelevant membrane proteins (i.e., anti-GM130, anti-golgin-84, anti-SCAP (#7601, #7606, #7623) (B) and then analyzed by immunoblotting with each antibody (as indicated below the panel).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A

B

Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10
Supplementary Figure 1
Supplementary Figure 2
Pen-2 is incorporated into the γ-secretase complex through binding to transmembrane domain 4 of presenilin 1
Naoto Watanabe, Taisuke Tomita, Chihiro Sato, Toshio Kitamura, Yuichi Morohashi and Takeshi Iwatsubo

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