

RESEARCH ARTICLE

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Binding of longer A β to transmembrane domain 1 of presenilin 1 impacts on A β 42 generation

Yu Ohki¹, Naoaki Shimada², Aya Tominaga¹, Satoko Osawa¹, Takuya Higo², Satoshi Yokoshima^{2,3}, Tohru Fukuyama^{2,3}, Taisuke Tomita^{1,4*} and Takeshi Iwatsubo^{1,4,5}

Abstract

Background: Amyloid- β peptide ending at 42nd residue (A β 42) is believed as a pathogenic peptide for Alzheimer disease. Although γ -secretase is a responsible protease to generate A β through a processive cleavage, the proteolytic mechanism of γ -secretase at molecular level is poorly understood.

Results: We found that the transmembrane domain (TMD) 1 of presenilin (PS) 1, a catalytic subunit for the γ -secretase, as a key modulatory domain for A β 42 production. A β 42-lowering and -raising γ -secretase modulators (GSMs) directly targeted TMD1 of PS1 and affected its structure. A point mutation in TMD1 caused an aberrant secretion of longer A β species including A β 45 that are the precursor of A β 42. We further found that the helical surface of TMD1 is involved in the binding of A β 45/48 and that the binding was altered by GSMs as well as TMD1 mutation.

Conclusions: Binding between PS1 TMD1 and longer A β is critical for A β 42 production.

Keywords: Presenilin, Secretases, Alzheimer disease, Intramembrane proteolysis, γ -Secretase modulator

Background

Several lines of evidence suggest that the accumulation of amyloid- β peptide (A β), a major component of senile plaques, is a common pathological feature in Alzheimer disease (AD) [1]. A β is generated through sequential cleavage by β - and γ -secretases of amyloid- β precursor protein (APP). γ -Secretase primarily cleaves APP to produce a C-terminal stub of APP (APP-CTF). Then, scission of APP-CTF by γ -secretase results in generation of various forms of A β with different C-terminal lengths. Especially, A β ending at the 42nd residue (A β 42), the most aggregable species, is initially and predominantly deposited in AD brains [2]. Moreover, familial AD-linked mutations in *Psen* (*Presenilin*; *PS*) 1, *Psen2* or *APP* genes cause an increase in A β 42 generation. Thus, A β 42 is considered as the most pathogenic species causative for AD [3].

γ -Secretase is an intramembrane-cleaving protease complex composed of four membrane spanning proteins:

PS, Nicastrin, Aph-1 and Pen-2 [4,5]. Extensive biochemical studies showed that the γ -secretase-mediated intramembrane cleavage of APP occurs in a processive manner [6]; APP-CTF is primarily cleaved at the ϵ -site located around the membrane-cytoplasm boundary to produce A β 49 or A β 48. Subsequently, these longer A β peptides are processed by stepwise cleavages to secrete shorter A β in two predominant production lines: A β 49 is processed to A β 43/40 via A β 46 (A β 40 production line), and A β 48 is processed to A β 42/38 via A β 45 (A β 42 production line). PS forms a channel-like catalytic pore structure within the membrane, and is endoproteolyzed to generate N- and C-terminal fragments (NTF and CTF, respectively) during the assembly of the protease-active complex [7,8].

Recently, small compounds that selectively regulate A β 42 production without affecting ϵ -cleavage emerged, which are termed γ -secretase modulators (GSMs) [9]. We have shown that a potent A β 42-lowering compound, GSM-1, directly targets the PS1 TMD1 [10]. Moreover, using substituted cysteine accessibility method (SCAM), we identified two different regions within TMD1 of PS1, i.e., a hydrophobic luminal region and a hydrophilic portion facing the catalytic site [11], that are differently

* Correspondence: taisuke@mol.f.u-tokyo.ac.jp

¹Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Full list of author information is available at the end of the article

involved in the action of GSM-1 [10]. However, the precise molecular mechanism whereby γ -secretase generates A β 42, as well as the role of TMD1 in A β 42 production, remains elusive. In this study, we identified TMD1 of PS1 as a regulatory domain for the processive cleavage of the A β 42 production line.

Results

Fenofibrate directly targets the N-terminal fragment of presenilin 1

Fen-B [12] is a derivative of the A β 42-raising GSM, fenofibrate [13], coupled to a biotin moiety. Previous report showed that Fen-B directly targeted APP-CTF by photo-crosslinking using recombinant proteins or microsomes under overexpression conditions. However, the possibility of a nonspecific binding of fenofibrate to high concentrations of APP in an artificial condition was not excluded [14,15]. We performed a photoaffinity labeling experiment with Fen-B using microsomes prepared from brains of wild-type mice. We found that endogenous PS1 NTF, but neither of other γ -secretase components (i.e., PS1 CTF, Nicastrin, Aph-1 and Pen-2) nor APP-CTF, was specifically precipitated (Figure 1A). In addition, we detected a biotinylated band of ~30 kDa,

corresponding to the molecular weight of PS1 NTF, in the fraction incubated with Fen-B. To further confirm the specificity of labeling of PS1 NTF by Fen-B, membrane fractions of fibroblasts from *Psen1^{-/-}/Psen2^{-/-}* double knockout mice (DKO) [16] with or without overexpression of His-tagged PS1 [17] were subjected to PAL. Biotinylated PS1 NTF was specifically precipitated, indicating that PS1 NTF is targeted by Fen-B (Figure 1B). Finally, a specific binding of Fen-B to SPP, another aspartic intramembranous cleaving protease, which shared homology with PS [18], was also observed (Figure 1C). Taken together, we concluded that the *bona fide* molecular target of fenofibrate, in the context of modulation of intramembrane cleavage, are PS1 and SPP, i.e., the enzyme moieties.

To narrow down the fenofibrate binding site within PS1 NTF, we employed the limited digestion approach by inserting a thrombin substrate sequence into PS1 [10]. γ -Secretase containing PS1-Th1 mutant, in which thrombin cleavable sequence was inserted between D110 and G111 in the hydrophilic loop 1, harbored γ -secretase activity and was sensitive to fenofibrate (Figure 2A). Eight kDa N-terminal fragment of PS1 NTF generated by thrombin cleavage of PS1-Th1 after Fen-B

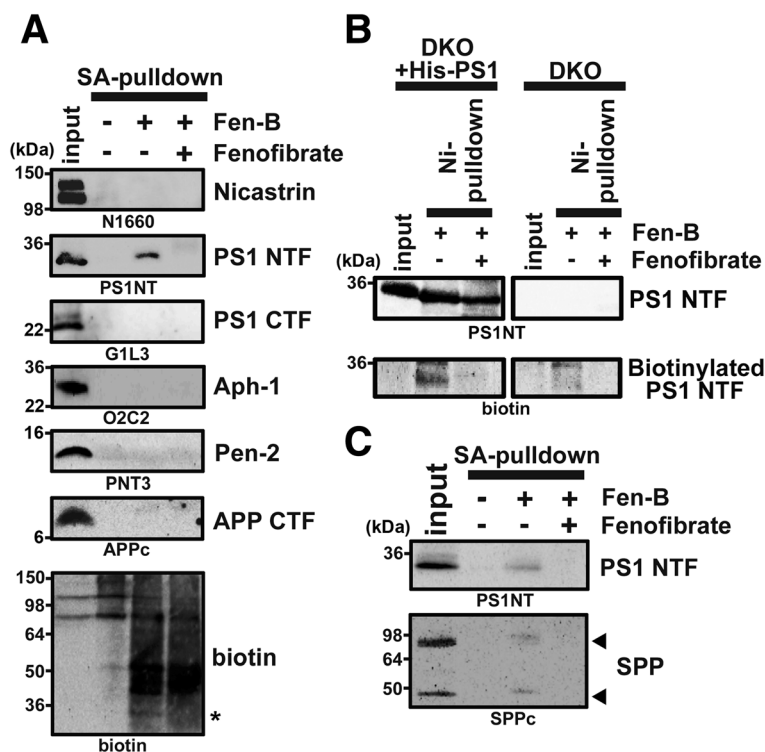


Figure 1 Fen-B directly targets PS1 and SPP. (A) PAL experiments using mouse brain membranes by Fen-B (10 μ M). This labeling was competed by treatment with fenofibrate (200 μ M). Note that only PS1 NTF was specifically labeled by Fen-B. Anti-biotin antibody also reacted with a band with ~30 kDa corresponding to PS1 NTF (an asterisk). **(B)** Fen-B labeling experiment for the DKO cells expressing His-tagged PS1. After PAL, His-tagged PS1 was purified with Ni²⁺-affinity column and analyzed by immunoblotting. **(C)** Fen-B also bound endogenous monomeric as well as dimeric SPP (arrows) in CHO cell membranes.

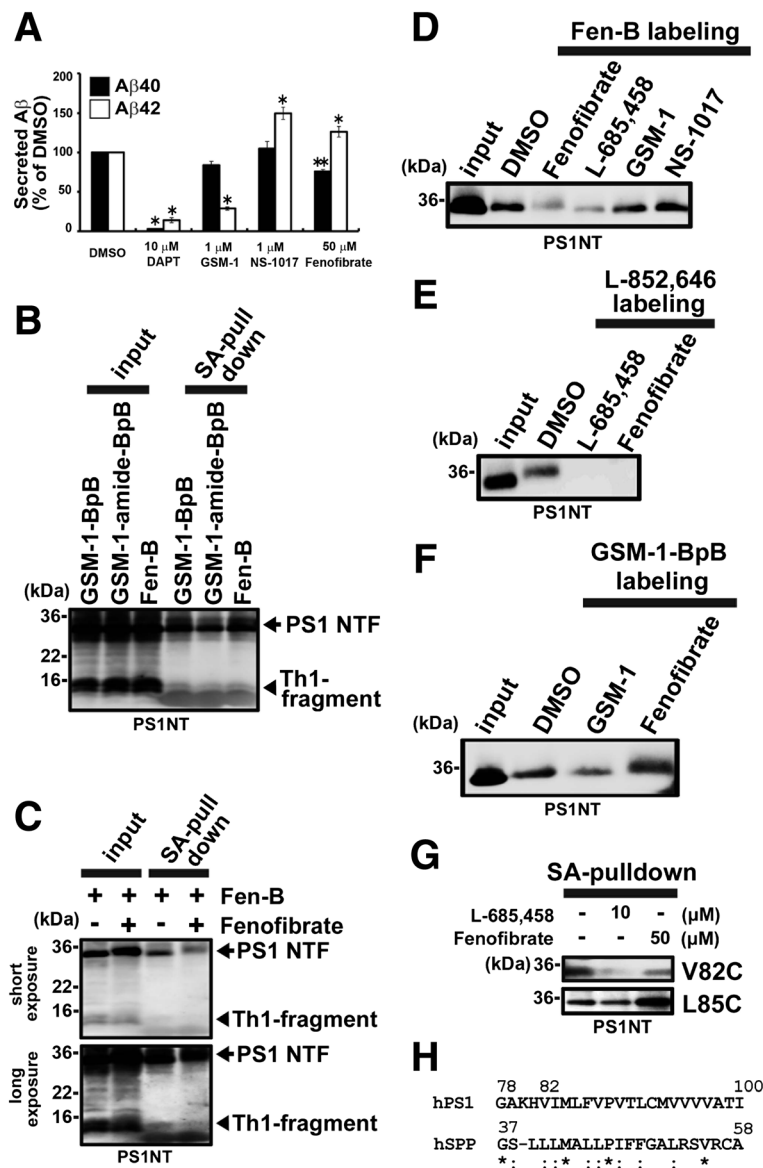


Figure 2 Cytosolic side of TMD1 forms a fenofibrate binding pocket. **(A)** Sensitivity of PS1-Th1 for GSMTs. Effect of DAPT (10 μM), GSM-1 (1 μM), NS-1017 (1 μM) and fenofibrate (50 μM) on secreted Aβ from wild-type PS1 containing γ-secretase using DKO cells stably expressing APPNL (n = 3, mean ± SD, *p < 0.01, **p < 0.05 at Student's t test). **(B)** Thrombin digestion experiments were performed after PAL by GSM-1-BpB (1 μM), GSM-1-amide-BpB (1 μM) and Fen-B (10 μM). Note that cleaved Th1-fragment (arrowhead) was precipitated and detected by anti-PS1 NTF antibody. **(C)** Labeling competition analysis of Fen-B (10 μM) in the presence of fenofibrate (100 μM) using PS1-Th1 microsomes. Upper and lower panels show short and long exposures, respectively. **(D)** Labeling competition analyses were performed with fenofibrate (100 μM), L-685,458 (10 μM), GSM-1 (100 μM) and NS-1017 (100 μM) for the labeling of PS1 NTF by Fen-B (10 μM). **(E)** Labeling competition experiment with L-685,458 (10 μM) and fenofibrate (100 μM) for the labeling of PS1 NTF by L-852,646 (100 nM). **(F)** Labeling competition analysis by GSM-1-BpB (1 μM) in the presence of GSM-1 (100 μM) or fenofibrate (100 μM) using CHO cell microsomes. **(G)** SCAM analyses of microsomes from DKO cells expressing single-Cys mt PS1 containing one Cys at 82 or 85 positions in the presence or absence of indicated compounds. Note that the labeling of V82C was decreased and of L85C was increased by preincubation with fenofibrate. **(H)** Alignment of amino acid residues of PS1 TMD1 (78th to 100th residues) and 37th to 58th residues of SPP, which includes predicted TMD1 (32nd to 54th residues [19]). Asterisks and colons indicate conserved and similar amino acids, respectively.

crosslinking was specifically precipitated in a similar fashion to that by phenylpiperidine-type photoprobes, suggesting that Fen-B also targets to the most N-terminal region of PS1, including TMD1 (Figure 2B and

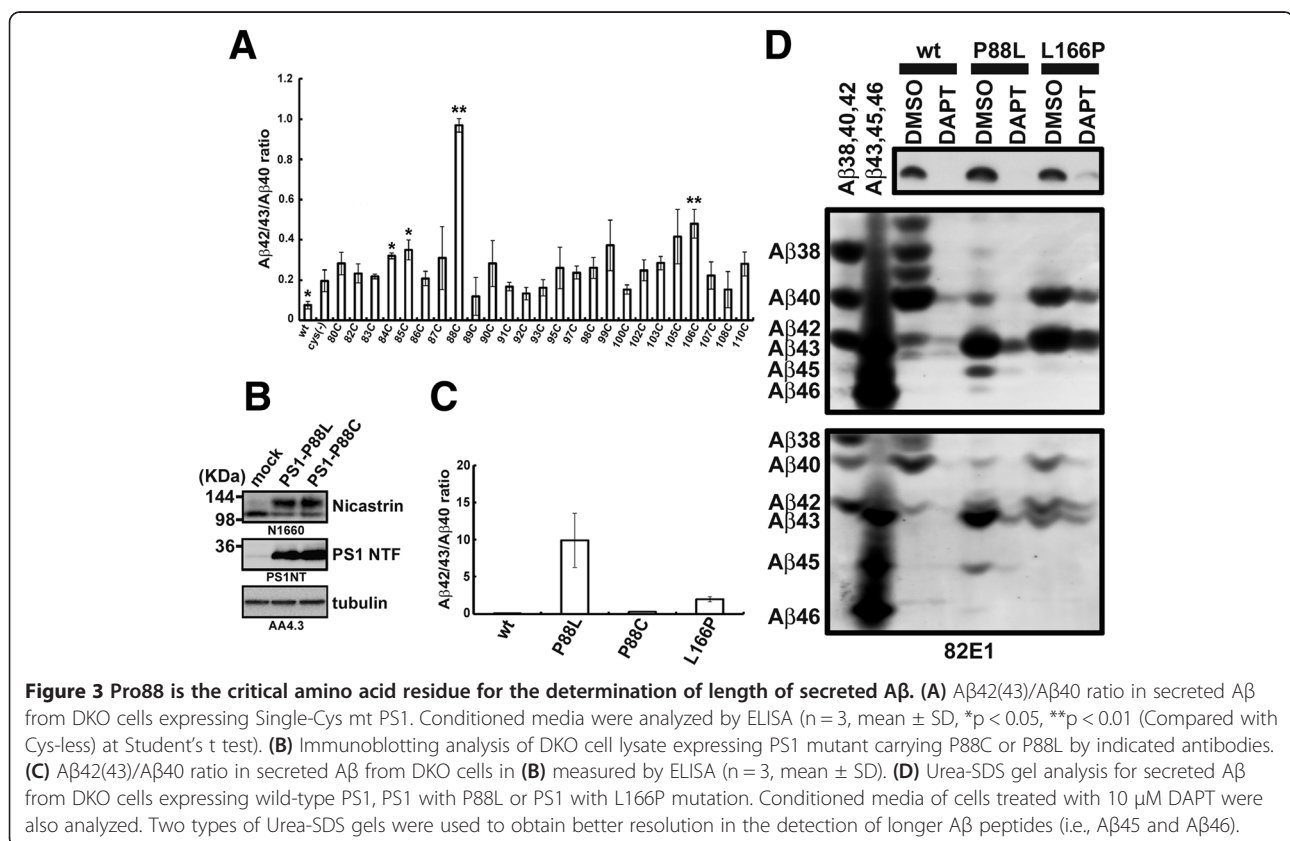
C). We showed that the cytosolic side of TMD1 participates in the catalytic hydrophilic pore [7,11]. To analyze the relationship between the fenofibrate binding site and the catalytic site within TMD1, we employed the cross-

competition analysis in Fen-B labeling using different classes of compounds. Labeling of PS1 NTF by Fen-B was diminished by L-685,458, that directly targets the cytosolic side of TMD1 (Figure 2D) [11]. Consistent with this, labeling of PS1 NTF by L-852,646, an L-685,458-based photoprobe, was inhibited by fenofibrate (Figure 2E). In contrast, neither GSM-1 nor NS-1017, which targets the luminal region of TMD1, affected the binding of Fen-B (Figure 2D). Moreover, biotinylation of PS1 NTF by GSM-1-BpB was hardly affected by fenofibrate (Figure 2F), suggesting that the binding site of fenofibrate is distinct from that of GSM-1 within TMD1. We then performed a labeling competition experiment in SCAM [7,11], the latter being a biochemical method to deduce the structure of the membrane protein by position-specific biotinylation and to identify the targeting site of the compound of interest. Preincubation of fenofibrate decreased the biotinylation at Val82, supporting the notion that fenofibrate targets the catalytic site. In contrast, labeling of Leu85 was increased, indicating that fenofibrate evokes a conformational change of the catalytic site in TMD1 (Figure 2G). Intriguingly, Gly37 to Ala58 of SPP, which encompassed the predicted SPP TMD1 (Ile32 to Ser54) [19], showed a substantial homology to primary sequence of N-terminal region of PS1 TMD1 (i.e., Gly78 to Ile100) (Figure 2H), suggesting the

possibility that fenofibrate targets to the predicted SPP TMD1. Taken together with the results of chemical biological experiments, the binding site of fenofibrate was estimated to locate around Val82 in TMD1, leading to the conformational change of the catalytic site of γ -secretase.

Intermediate longer A was secreted by TMD1 mutant PS1

This finding prompted us to hypothesize that TMD1 is potentially involved in the regulation of the processivity of γ -secretase to generate A β 42. To address this issue, we screened single cysteine (single-Cys) mutants of TMD1 in cysteine-less PS1 used in SCAM. While cysteine-less PS1 increased A β 42(43)/A β 40 ratio compared to that of wild-type PS1-expressing cells, some single-Cys mutants showed further augmentation in A β 42(43)/A β 40 ratio (Figure 3A). Among these mutants, P88C mutant showed a dramatic increase in the A β 42(43)/A β 40 ratio. To further analyze the functional significance of Pro88 in the processive cleavage, we analyzed two PS1 mutants, P88C and P88L. Surprisingly, P88L mutation caused an increase in the A β 42(43)/A β 40 ratio, to a level higher than that with L166P mutant PS1 (Figure 3C and D), one of the most potent pathogenic PS1 mutations [20]. In addition, P88L mutant elicited secretion of the intermediate, longer form of A β , i.e. A β 45

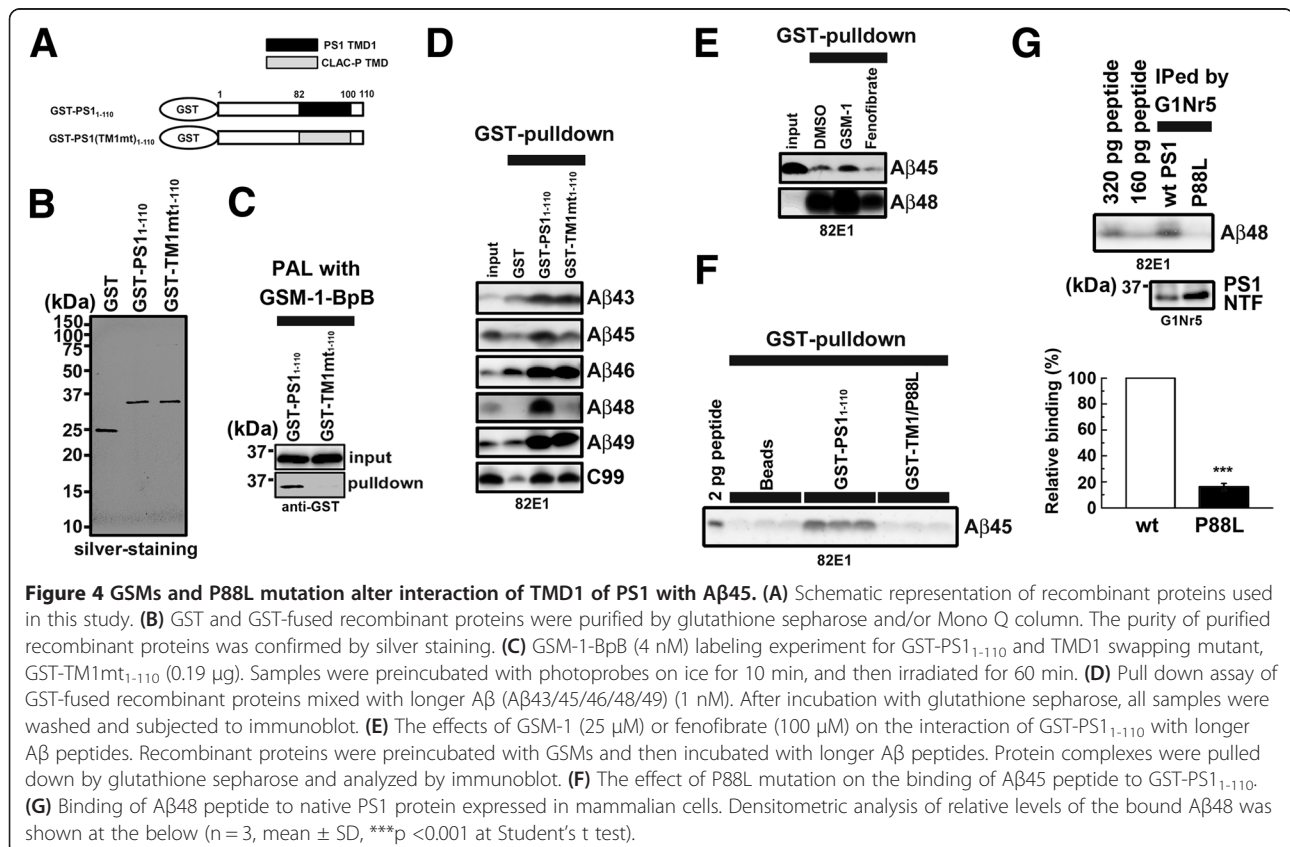


and A β 46, but neither in the wild-type nor L166P mutant PS1 (Figure 3D). These data strongly implicated TMD1 in the regulation of the C-terminal length of A β .

GSMs and P88L mutation affected the interaction between TMD1 and longer A β species

Our unexpected observation of an abnormal secretion of longer A β 45 from cells expressing P88L mutant PS1 prompted us to further investigate the functional role of TMD1 during the enzymatic process, especially the processive cleavage. Notably, previous reports indicated that a region between Val82 to Ser132 encompassing TMD1 directly participates in the interaction with γ -secretase substrates (i.e., APP-CTF) *in vitro* [21-23]. Moreover, A β 46 has been shown to accumulate by DAPT, which inhibits processive cleavage, and to be coimmunoprecipitated with PS1 [23,24]. These results implied the possibility that TMD1 directly recognizes the longer A β species during the processive cleavage of γ -secretase. To test this idea, we performed *in vitro* binding assays of various longer A β species with purified GST, GST-PS1₁₋₁₁₀ and GST-TM1mt₁₋₁₁₀, the latter harboring the TMD1 sequence (i.e., Val82 to Ile100) replaced with a TMD of an unrelated membrane protein, CLAC-P [10,25] (Figure 4A and B). Replacement of TMD1 of PS1 with CLAC-P sequence abolished the γ -secretase activity [17]

as well as binding of GSM-1-BpB *in vitro* [10] (Figure 4C), indicating chimeric PS1 with CLAC sequence has a distinct property. Then synthetic A β peptides were coincubated with recombinant proteins and pulled down by glutathione sepharose (Figure 4D). In this condition, we detected specific binding of recombinant C99-FLAG to GST-PS1₁₋₁₁₀, as previously reported by Annaert et al [21]. In addition, we detected binding of all synthetic longer A β peptides (A β 49, A β 48, A β 46, A β 45, A β 43) with GST-PS1₁₋₁₁₀, suggesting that TMD1 of PS1 directly binds to longer A β peptides, and that the cytoplasmic domain of APP is not involved in this binding. Unexpectedly, GST-TM1mt₁₋₁₁₀ was capable of interacting with C99-FLAG as well as with peptides belonging to the A β 40 production line (A β 49, A β 46 and A β 43). However, the binding of A β species of the A β 42 production line (i.e., A β 48 and A β 45) was significantly reduced by swapping the TMD1 sequence (Figure 4D). We further analyzed the effect of TMD1-targeting GSMs as well as P88L mutation on the binding of A β 45 and A β 48 to TMD1. Intriguingly, GSM-1 augmented the interaction of TMD1 with A β 45 as well as A β 48, whereas it was reduced by fenofibrate (Figure 4E). In addition, introduction of P88L mutation in GST PS1₁₋₁₁₀ decreased the pull down of A β 45 (Figure 4F). Finally, we observed a specific binding of A β 48 with native PS1



protein expressed in the mammalian cells, and this interaction was almost diminished by the P88L mutation (Figure 4G). We did not observe specific binding of A β 45 with PS1 holoprotein expressed in mammalian cells, presumably due to weak binding of A β 45 to PS1 protein. Nevertheless, these data suggest that the processivity of the γ -secretase for A β 42 production is defined by the tenacity of interaction between TMD1 and longer A β , which may determine the retention of the substrate in the catalytic site. Collectively, we uncovered the significant function of TMD1 of PS1 as a binding site for the longer A β species, especially A β 45 and A β 48, during the processive cleavage of the A β 42 production line, and the effects of GSMs on A β 42 production by changing the affinity between TMD1 and the longer A β peptides.

Discussion

Understanding the molecular mechanism of the processive cleavage by γ -secretase is critical to the development of effective GSMs. We previously reported that phenylpiperidine-type GSMs are bound to TMD1 of PS1 [10]. Here, we further showed that fenofibrate, an A β 42-raising GSM, also directly targets TMD1, while Fen-B was reported as APP-targeting photoprobe [12]. Recently, some papers reported that large amount of A β 42 or C99 forms aggregates that cause non-specific binding to GSMs [14,15]. Therefore, we have used brain microsomes obtained from wild-type mouse for the photocrosslinking experiment.

Scissile bonds for processive cleavage by γ -secretase have hypothetically been mapped on different surfaces in the α -helical model of APP TMD [26]. This raises the possibility that the distinct processive cleavages by γ -secretase, i.e., those leading to production of A β 49-46-43-40 or A β 48-45-42-38, are determined by the recognition of one or the other of the specific helical surfaces. However, the domain on γ -secretase that recognizes the helical surface on the substrate is yet to be identified. It has previously been suggested that TMD1 of PS1 is involved in the binding of APP-CTF, a direct substrate of γ -secretase [21,22]. Here we found that longer A β peptides that are generated as intermediate products in the A β 42 production line (i.e., A β 45 and A β 48), which also are direct substrates for the processive cleavage, retain the capacity to interact with TMD1 of PS1. It is highly likely that the "gripping tenacity" of the substrate binding site facing the catalytic pore would determine the processivity of A β 48 and A β 45 on the A β 42 production line, which can be modulated by small compounds. Consistently, Okochi et al. have recently reported that A β 42 is bound to the γ -secretase complex [27] and the binding was modulated by GSMs, although they have not identified the binding site of A β within the enzyme complex. Thus, we propose that TMD1 of PS1 functions as a

binding site of longer A β species for γ -secretase during the processive cleavage, which specifically determines the efficiency of the processive cleavage of the A β 42 production line. Structural analyses suggested that the catalytic cavities of rhomboid protease [28], another intramembrane-cleaving enzyme, or those of FlaK [29] and PSH [30], archaeal GxGD proteases, are unable to accommodate all the amino acid residues of the transmembrane sequence of the substrates. This suggests that a major part of the TMD of substrates remains within the membrane and is gripped by enzymes to incorporate the cleavage site into the intramembrane catalytic site during proteolysis. While the precise structure of human PS1 still remains unclear, our SCAM results on PS1 [11], as well as the recently reported x-ray crystal structure of PSH [30], the latter being composed of 9-transmembrane domains similarly to human PS1, altogether suggested that TMD1 locates in proximity to the catalytic aspartate in TMD7. The results of these structural analyses also support our notion that TMD1 functions as a substrate binding domain during the processive cleavage by γ -secretase.

TMD1 of PS1 bound not only to longer A β peptides of the A β 42 production line, but to those of A β 40 line (Figure 4D). P88L mutation in TMD1 of PS1 caused an increased secretion of not only A β 45, but also A β 43 and A β 46 (Figure 3F), suggesting that the interaction between TMD1 and longer A β species is also critical for the processivity in the A β 40 production line. Intriguingly, swapping TMD1 sequence of PS1 with that of CLAC-P, an unrelated membrane protein, did not affect the binding of A β 43, A β 46 and A β 49 peptides. In the helical net diagram, similar side chains at the luminal side of PS1 TMD1 and CLAC-P TMD comprised an interface on the α -helical model only in one side (Figure 5A and B) [31]. Thus, one helical surface of TMD1 is involved in the binding of longer A β species in the A β 40 production line, whereas the other surface specifically interacts with those in the A β 42 production line in the PSH-based PS1 model (Figure 5C). Pharmacological and chemical biological studies suggest that the substrate enters the catalytic site via the initial substrate binding site, in which TMD2, 6 and 9 are involved [17]. Especially, TMD6 and 9 have been implicated in the lateral entry of the substrate from the crystal structure of PSH [30]. However, helical peptide-type GSIs that target the initial substrate binding site equally inhibited the production of A β 40 or A β 42 [32,33]. Thus, we hypothesize that C99 or longer A β is gripped by TMD1 after the lateral entry (Figure 5D). Structural model also suggested that residues related to the A β 42 production line in the luminal side of TMD1 are located on the surface of PS1 polypeptide, which might be targeted by GSM-1 [10]. In fact, several side chain interactions have been identified in

Methods

Compounds, peptides and antibodies

GSM-1, GSM-1-BpB, NS-1017, GSM-1-amide-BpB, Fen-B and DAPT were synthesized as described [10,12,36]. L-685,458 and fenofibrate were purchased from Bachem and SIGMA, respectively. L-852,646 [37] was kindly provided from Dr. Y. Li (Sloan-Kettering Cancer Center). Synthetic longer A β peptides (i.e., β -amyloid (1-43, #23573), (1-45, #61956-01), (1-46, #62076-01), (1-48, #61965-01), (1-49, #61963-01) were purchased from Anaspec. A β (1-40) (#4307-v) and A β (1-42) (#4349-v) peptides were purchased from Peptide institute. The rabbit polyclonal antibodies anti-PS1 NTF (G1Nr5), anti-PS1 CTF (G1L3) and anti-Pen-2 (PNT3) were raised as described [38-40]. Anti-PS1 NTF (PS1NT) [41] and anti-SPP (SPPc) [42] were kindly gifted from Drs. G. Thinakaran (The University of Chicago) and T. Golde (University of Florida). Anti-nicestrin N1660 (SIGMA), anti-APP CTF (Immuno-Biological Laboratories), anti-Aph-1aL O2C2 (Covance), anti-human A β 82E1 (Immuno-Biological Laboratories) and anti-biotin (Bethyl) were purchased from indicated vendors. The monoclonal antibody anti- α -tubulin AA4.3 developed by Dr. C. Walsh was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by The University of Iowa, Department of Biology, Iowa City, IA.

Plasmid construction, cell culture manipulation and cell based assay

cDNAs encoding PS1 and APP carrying Swedish mutation (APPNL) were inserted into pMXs-puro [43]. cDNAs encoding mutant PS1 were generated by long PCR-based QuikChangeTM strategy (Stratagene). To produce recombinant proteins, cDNAs encoding PS1 were cloned into pGEX-6P-1 vector (GE healthcare) [10]. Maintenance of cultured cells, transfection, retroviral infection, two-site enzyme-linked immunosorbent assay (ELISA), or immunoblotting using Urea/SDS-PAGE gel system as described [10,39,44,45].

Photoaffinity labeling and SCAM experiments

Preparation of samples for photoaffinity labeling experiments [46] was performed as follows. Brains of C57J/B6 mouse (3-5 month age) or cultured cells were homogenized with homogenize buffer (20 mM HEPES (pH 7.0), 140 mM KCl, 250 mM sucrose, 0.5 mM diisopropyl fluorophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 1 μ g/ml tosyllysine chloromethyl ketone, 1 μ g/ml anti-pain, 1 μ g/ml leupeptin, 10 μ g/ml phosphoramidon, 5 mM EDTA, 1 mM EGTA) using Potter-Elvehjem Tissue Grinder (Wheaton), and membrane fractions were collected by ultracentrifugation at 100,000 \times g

(Beckman) [10]. PAL experiments utilizing avidin-biotin catch principle [47] and thrombin digestion experiments after PAL were performed as previously described [10]. Briefly, after resuspension of the microsome in the homogenize buffer by 25G needle with syringe, protein content was measured by BCA assay (Thermo Fisher Scientific). 1 mL of microsome-containing solution (1 mg/ml protein) was preincubated with compounds for 30 min on ice. Then photoprobes were added and incubated for 10 min on ice under the dark condition. UV irradiation (352 nm) was performed on ice for 1 hr with a UV lamp (Model XX-15BLB, UVP). The approximate distance from UV lamp to the samples was 10 cm. The biotinylated proteins were precipitated by streptavidin sepharose (GE healthcare) in 1% SDS containing homogenization buffer. For SCAM, all methanethiosulfonate reagents (Toronto Research Chemicals) were dissolved in dimethyl sulfoxide at 200 mM prior to use or stored at 80 degree until use. The methods for SCAM and competition experiments using biotinylaminoethyl methanethiosulfonate have been described in detail before [10,11]. Briefly, stable DKO cells expressing cysteine mutant PS1 were grown on two 15-cm dishes per single analysis. Cells were scraped in PBS and resuspended in 2 ml of SCAM homogenization buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, Complete protease inhibitor cocktail (Roche Biochemicals)). Cells were disrupted by a Polytron homogenizer (Hitachi), and nuclei and large cell debris were pelleted by centrifugation at 1,500 \times g for 10 min. The postnuclear supernatants were centrifuged, and the resultant microsomal pellets were resuspended in 0.2 ml of PBS in a syringe, and 0.1 mM biotinylaminoethyl methanethiosulfonate was added to this fraction. After 30 min incubation at 4 degree, microsomes were centrifuged twice to wash out. The resultant pellets resuspended in 1% SDS/PBS were incubated with the streptavidin sepharose overnight and analyzed as in the intact cell biotinylation experiment. In PAL or SCAM experiments, we loaded 1.5 and 20% of samples as "input" and "bound", respectively, in all immunoblot analyses.

Protein purification and binding assay

GST-fusion recombinant proteins were expressed in *E. Coli* (BL21 DE3) (Novagen) and purified by two step procedures using glutathione sepharose and mono Q columns (GE Healthcare) as manufacturer's instruction. All recombinant proteins were finally diluted with recombinant protein preparation buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 0.25% CHAPSO). C99-FLAG was purified from Sf9 cells infected with recombinant baculovirus encoding C99-FLAG and diluted at 1 μ g/ml in recombinant protein preparation buffer. To perform binding assay, 0.5 μ g of GST-fusion recombinant

proteins were mixed at 1 μ g of C99-FLAG, 1 (for A β 43, A β 45, A β 46, A β 48 and A β 49) or 10 (for A β 40 and A β 42) nM of synthetic A β in 1 ml of recombinant protein preparation buffer, and incubated at 4 degree overnight. After addition of glutathione sepharose, samples were then washed with the buffer and precipitates were eluted by boiling in sample buffer. For binding assay using native PS1 protein, PS1 or P88L mutant PS1 was expressed in DKO cells and solubilized in 10 mM HEPES buffer containing 1% CHAPSO. After addition of 2 ng of A β 48 peptide, the solubilized fraction was incubated with anti-PS1 antibody G1Nr5 at 4 degree overnight. PS1-A β 48 complex was then immunoprecipitated using Protein G sepharose 4 Fast Flow (GE Healthcare). Subsequently eluates (i.e., proteins bound to GST-fusion recombinant proteins or native PS1 proteins) were analyzed by immunoblotting. We loaded 0.75 and 20% of samples as "input" and "bound", respectively, in immunoblot of all pull down assay unless the amount of loaded proteins was otherwise indicated.

Abbreviations

AD: Alzheimer disease; CTF: Carboxyl-terminal fragment; DKO: *Psen1/Psen2* double knockout mouse immortalized fibroblasts; GSM: γ -secretase modulator; NTF: Amino-terminal fragment; mt: Mutant; PS: Presenilin; SCAM: Substituted cysteine accessibility method; TMD: Transmembrane domain.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YO and TT designed the research. YO, AT, SO and TT performed biochemical experiments. NS, TH, SY and TF synthesized the compounds. YO, TT and TI wrote the paper. All authors read and approved the final manuscript.

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Author details

¹Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. ²Department of Synthetic Natural Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan. ³Laboratory of Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, Nagoya University, Nagoya 464-8601, Japan. ⁴Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Bunkyo-ku, Tokyo 113-0033, Japan. ⁵Department of Neuropathology, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan.

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