Aβ42 Overproduction Associated with Structural Changes in the Catalytic Pore of γ-Secretase

COMMON EFFECTS OF PEN-2 N-TERM N ELONGATION AND FENOFRIBRATE

Noriko Iiso‡, Chihiro Sato‡, Hiroyuki Miyashita‡, Mitsuru Shinohara‡, Nobumasa Takasugi‡, Yuichi Morohashi‡1, Shoji Tsuji‡, Taisuke Tomita‡, and Takeshi Iwatsubo‡3

From the 1Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, and the 2Department of Neurology, Division of Neuroscience, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

γ-Secretase is an atypical aspartyl protease that cleaves amyloid β-precur sor protein to generate Aβ peptides that are causative for Alzheimer disease. γ-Secretase is a multimeric membrane protein complex composed of presenilin (PS), nicastrin, Aph-1, and Pen-2. Pen-2 directly binds to transmembrane domain 4 of PS and confers proteolytic activity on γ-secretase, although the mechanism of activation and its role in catalysis remain unknown. Here we show that an addition of amino acid domain 4 of PS and confers proteolytic activity on Aph-1, and Pen-2. Pen-2 directly binds to transmembrane protein complex composed of presenilin (PS), nicastrin, Aph-1, and Pen-2. Pen-2 generation was independent of the amino acid sequences, the expression system and the presenilin species. In vitro γ-secretase assay revealed that Pen-2 directly affects the Aβ42-generating activity of γ-secretase. The elongation of Pen-2 N terminus caused a reduction in the water accessibility of the luminal side of the catalytic pore of PS1 in a similar manner to that caused by an Aβ42-raising γ-secretase modulator, fenofibrate, as determined by substituted cysteine accessibility method. These data suggest a unique mechanism of Aβ42 overproduction associated with structural changes in the catalytic pore of presenilins caused commonly by the N-terminal elongation of Pen-2 and fenofibrate.

Amyloid β peptide (Aβ)4 deposited in the brains of patients with Alzheimer disease (AD), is derived from amyloid β-precursor protein (APP) through sequential proteolytic cleavages by β- and γ-secretases (1). γ-Secretase cleaves a scissile bond within the transmembrane domain (TMD) of APP and determines the C-terminal length of Aβ. Moreover, more than 50 type 1 single spanning transmembrane proteins, including Notch protein, are also endoproteolyzed by γ-secretase to secrete short peptides extracellularly, and simultaneously, release intracellular domain into cytosol. A set of intracellular domains mediates the cellular signaling, suggesting that the γ-secretase cleavage has dual roles in membrane protein metabolism: degradation and proteolysis-dependent signaling (2).

Genetic and biochemical studies suggest that γ-secretase is a high molecular weight membrane protein complex, composed of presenilin (PS), nicastrin (Nct), Aph-1, and Pen-2 (1, 3). Ablation of either of the genes abolished the γ-secretase activity in nematodes, flies, and mice. In contrast, the overexpression of the four proteins reconstituted the proteolytic activity, suggesting that these proteins are necessary and sufficient for the γ-secretase activity (4–6). Molecular cellular and chemical biological analyses revealed that PS forms a hydrophilic pore involving TMD6 and -7 where conserved aspartic residues, that are critical for the γ-secretase activity, reside (7, 8). Moreover, mutations in PS genes (PSEN1 and -2) account for the majority of early-onset familial AD (FAD), causing an overproduction of Aβ ending at position 42 (Aβ42), that most readily forms amyloid deposits (1). Thus, PS is a catalytic subunit of γ-secretase and regulates the property of endoproteolytic activity. Nct is a single pass transmembrane protein harboring a large extracellular region that captures the N-terminal tip of substrates (9). This binding is independent of the formation of an active γ-secretase complex, suggesting that Nct functions as a substrate binding site that is distinct from the active site (i.e. exosite) of γ-secretase. Molecular function of Aph-1, a putative multipass membrane protein that forms a subcomplex with Nct in early
secretory pathway (10), remains unknown. However, the loss of Aph-1 or Nct decreased the expression of PS and Pen-2. In contrast, the overexpression of Aph-1 together with Nct stabilizes the γ-secretase complex, suggesting the role of Aph-1 as a molecular scaffold for this atypical membrane-associated protease (5, 11).

Pen-2 is a membrane protein harboring two TMDs with a hairpin-like topology (12). Depletion of Pen-2 caused the loss of the γ-secretase activity accompanied by the accumulation of PS holoprotein–Nct-Aph-1 trimeric complex (5). The overexpression of Pen-2 together with PS, Nct, and Aph-1 provoked the endoproteolysis of PS to generate N- and C-terminal fragments (NTF and CTF, respectively), concomitantly with the acquisition of the γ-secretase activity. Mutational analyses revealed that Pen-2 directly binds to TMD4 of PS through its proximal two-thirds of the first TMD (13–15). Assembled γ-secretase complex is sorted out from the endoplasmic reticulum and exhibits proteolytic activity. Thus, Pen-2 is the functional activator of the γ-secretase complex in its biosynthetic pathway. It has been shown that the length and the amino acid sequences of the C terminus of Pen-2 affect the stability of PS fragments after maturation (13, 16–18). However, the role of Pen-2 in endoproteolytic activity remains unknown. Here we report on an unexpected observation that the length of the N terminus of Pen-2 affects the structure of the active site of γ-secretase in a way to increase Aβ42 generation, in a similar fashion to fenofibrate, an Aβ42-raising γ-secretase modulator.

**MATERIALS AND METHODS**

*Construction of Expression Plasmids, Cell Culture, Transfection, and Viral Infection*—Expression plasmids for *Drosophila* S2 cells encoding SC100, Psn, dNct-VS/His, dAph-1-FLAG, HA-dPen-2, HA-hPEN-2, and EGFP were described previously (5, 11, 19). All Pen-2 mutants, including untagged dPen-2 and hPEN-2, were generated by the long-PCR-based mutagenesis. For generation of the expression plasmid for SC100gal4, cDNA fragment encoding GAL4 amplified from pcDNA3–GAL4 (provided from Dr. M. Miura) was incorporated at nucleotides encoding Ser-59 and Ile-60 (amino acid number according to a rabbit polyclonal antibody 82E1. Simultaneously, synthetic Aβ peptides were loaded as forward and reverse primers, respectively; for dNct, 5′-tta ata cga ctc act ata gga aga ctt aac taa atc cca-3′ and 5′-tta ata cga ctc act ata gga aga cag tgg tgt ggg ttc-3′ were used as forward and reverse primers, respectively; for dNct, 5′-tta ata cga ctc act ata gga aga cag gag cgc aac act-3′ and 5′-tta ata cga ctc act ata gga aga cga aga tct cca cca-3′) or 60-bp synthetic cDNA corresponding to the 3′-UTR region of dPen-2 (5′-aca taa cta gag taa ttc gtt ggc aac taa tga tta aaa aga ctt cct taa atc cca-3′ for dPen-2) were used to amplify dsRNA templates by PCR using synthetic oligonucleotide containing T7 primer binding site (5′-tta ata cga ctc act ata gga aga ctc act ata gga aga tct tta gga atg aag-3′ were used as forward and reverse primers, respectively). For 3′-UTR-targeted RNAi against dAph-1, we used small interference RNA (5′-gcu uuu gua uaa cua uaa aaa-3′ and 5′-uuu aau guu aua cag cua-3′ were sense and antisense sequences, respectively), kindly provided by Dr. K. Ui-Tei (11). Generation of dsRNAs and transfection were performed as previously described (5, 11, 19). Establishment of reporter S2 cell line coexpressing EGFP, SC100gal4, and UASdestabilized luciferase reporter was generated by cotransfection of pAC5.1-EGFP, pIB-SC100gal4, and pGL3(r2.2)-UAS and subsequent selection by blasticidin. Full-length cDNAs encoding APP carrying Swedish mutation (APPNL), wild-type human PS1, and single-Cys mutants (I114C, A246C, or L250C) based on cysteine-less PS1 in pLPCX (Clontech) or pMX-puro (provided from Dr. T. Kitamura) were described (8). Human NCT (hNCT), APh-1 (hAPH-1b), and untagged and FLAG-tagged hPEN-2 in pMX vector were kindly provided from Dr. B. De Strooper) using ectopic packaging Plat-E cells (21) was performed as previously described (8, 14). Making recombinant baculoviruses, culturing Sf9 cells, baculoviral infection, and large scale preparation of membranes were described previously (6, 22).

**Antibodies and Immunological Methods**—The rabbit polyclonal antibodies against *Drosophila* Psn NTF (GDN1) and Psn CTF (GDL1) were raised as described before (5, 19), dPNT1 was a rabbit polyclonal antibody raised against synthetic peptides encoding the N terminus (MDISKAPPRKLELCKRYFFAG-FAFL) of *Drosophila* Pen-2. Anti-PS1NT against the N terminus of human PS1 was kindly provided by Dr. G. Thinkaran. Anti-Aβ (82E1) mouse monoclonal antibody (IBL), anti-FLAG M2 mouse monoclonal antibody (Sigma), anti-HEMA (3F10) rat monoclonal antibody (Roche Applied Science), and anti-V5 mouse monoclonal antibody (Invitrogen) were purchased. The samples were analyzed by immunoblotting and two-site enzyme-linked immunosorbent assays as described (5, 23, 24). Proteolytic activity of reconstituted γ-secretase was measured by in vitro γ-secretase assay using recombinant substrates as previously described (6, 22, 25). N-[N-(3,5-Difluorophenacyl)-1-alanyl]-(S)-phenylglycine t-buty1 ester (DAPT) was provided by Drs. T. Kan and T. Fukuyama (26). Fenofibrate was purchased from Sigma. Analyses of the water accessibility of the substituted cysteines using stable DKO cell lines were performed as previously described (8).

**Analysis of Immunoprecipitated Aβ Peptides**—Conditioned media supernatants from S2 cells and mouse embryo fibroblast cells were incubated with 20 μl of anti-Aβ antibody BAN50 (provided by Takeda Pharmaceutical Co.) in a rotator at 4 °C for 5–18 h. Protein G-agarose (Invitrogen) was added, and rotational incubation was continued for additional 2 h. For immunoblot analysis, precipitated Aβ peptides were separated by 10% Tris/Bicine/urea gel (27, 28) and probed by anti-Aβ antibody 82E1. Simultaneously, synthetic Aβ peptides were loaded and used as molecular standards.

**RESULTS**

*N-terminally Tagged Drosophila Pen-2 Specifically Increased Aβ42 Generation*—It has been well established that the percentage of secreted Aβ42 that comprises the total secreted Aβ
N-terminally Tagged Pen-2 Overproduces Aβ42

![Diagram](Image)

**TABLE 1**
Relative fold change in %Aβ42 of S2 cells expressing the tagged dPen-2 together with Psn, dNct-V5/His, and dAph-1-FLAG

| Exogenous dPen-2 genotype | Fold change in %Aβ42 |
|---------------------------|---------------------|
| mock                      | 1                   |
| dPen-2                    | 1.04 ± 0.08         |
| HA-dPen-2                 | 2.46 ± 0.17**       |
| myc-dPen-2                | 1.42 ± 0.12*        |
| FLAG-dPen-2               | 1.59 ± 0.11*        |
| Hx-dPen-2                 | 1.63 ± 0.06**       |
| HA2-dPen-2                | 2.60 ± 0.24**       |

*p < 0.05 by Student’s t test.
**p < 0.005 by Student’s t test.

(%Aβ42) is ~10% in almost all types of mammalian cultured naïve cells (29). We previously reported that *Drosophila* S2 cells harbor endogenous γ-secretase activity with a property quite similar to that of mammalian cells, %Aβ42 being at ~15–20% (5, 19). Furthermore, the overexpression of *Drosophila* PS (Psn), nicastrin (dNct), Aph-1 (dAph-1), and Pen-2 (dPen-2) increased the levels of Psn fragments as well as of total γ-secretase activity in *Drosophila* S2 cells (Fig. 1A) (5). We measured secreted Aβ40 and Aβ42 from cells expressing tagged *Drosophila* γ-secretase components (i.e. Psn, dNct-V5/His, dAph-1-FLAG, or HA-dPen-2) together with SC100, the latter corresponding to the C-terminal fragment of human APP with a signal peptide. Unexpectedly, S2 cells expressing all the four components secreted significantly increased levels of Aβ42 compared with that from cells expressing Psn, dNct-V5/His, and dAph-1-FLAG (“mock” in Fig. 1B). %Aβ42 was significantly increased to almost 2.5-fold (11.7% in mock, and 28.5% in HA-dPen-2 transfected cells), although the coding sequences of all the γ-secretase components were of wild-type (shown as fold changes in %Aβ42 compared with mock in Table 1). In contrast, the C-terminal V5/His-tagged dPen-2 had no effect on %Aβ42 (1.2-fold compared with mock, p = 0.339 (n = 3)). To ascertain whether the addition of the tag sequence to the N terminus of dPen-2 caused this effect, we overexpressed dPen-2 without a tag together with Psn, dNct-V5/His, and dAph-1-FLAG and examined the secretion of Aβ. In contrast to HA-dPen-2, %Aβ42 from cells expressing untagged dPen-2 was almost comparable to that in mock-transfected cells (Fig. 1B and Table 1). To specifically examine the effects of tagged Pen-2 or other γ-secretase components by eliminating endogenous components, we took a UTR-targeted RNAi/rescue approach on each component (11), which enables us to analyze the function of exogenous proteins under a null-phenotype of the gene of interest in *Drosophila* S2 cells. The levels of Psn fragments and of the γ-secretase activity were decreased by the UTR-targeted RNAs against the chosen UTR sequences (5’-UTR for Psn and dNct, 3’-UTR for dAph-1 and dPen-2, respectively) did suppress the gene expression. The overexpression of exogenous components (i.e. Psn, dNct-V5/His, dAph-1-FLAG, and HA-dPen-2) in respective knockdown cells restored both the expression of Psn fragment and Aβ generation (Fig. 2, A and B). Intriguingly, however, only Pen-2–rescued cells showed a statistically significant overproduction of Aβ42 (Fig. 2B and Table 2). These data suggest that the reconstitution of the *Drosophila* γ-secretase complex harboring dPen-2 tagged with HA at the N terminus caused an increase in the Aβ42 generation in *Drosophila* S2 cells.

To examine if this effect is specific to the HA tag sequence (MYPYDVPDYA), or its length, we constructed dPen-2 tagged at the N terminus with “double” HA (MYPYDVPDYAYPYDVPDYA), Myc (MEQKLISEEDL), FLAG (MDYKDDDDK), or His/Xpress (MPRSHHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGSELE) (HA2-dPen-2, Myc-dPen-2, FLAG-dPen-2, and Hx-dPen-2, respectively) and expressed them together with Psn, dNct-V5/His, and dAph-1-FLAG. Immunoblot analysis revealed that all the tagged dPen-2 increased the generation of Psn fragments (Fig. 1A). Moreover, all N-terminally tagged dPen-2 increased %Aβ42 irrespective of the tag sequences, among which HA-dPen-2 and HA2-dPen-2 showed the highest augmentation effect (Fig. 1B and Table 1). The degree of increase in endoproteolysis of Psn was not correlated to that of the Aβ42 overproduction (Fig. 1A). These data strongly suggest that an N-terminal tag of dPen-2, especially the HA sequence, caused an increase in the production of Aβ42.

Kim and Sisodia reported, using deletion mutants, that the proximal (residues 3–9 in human PEN-2 (hPEN-2)), but not the
distal (residues 10–16), portion of the hydrophilic N-terminal region of hPEN-2 is dispensable for the acquisition of the \( \gamma \)-secretase activity, although the levels of secreted A\( \beta \)42 were not documented (13). To determine whether the HA tag effect on A\( \beta \)42 overproduction is dependent on the length and/or integrity of the N terminus of dPen-2, we generated a series of N-terminal length-mutants of dPen-2: dPen-2/2–10 lacking residues 2–10 of dPen-2, HA-dPen-2/2–10, a HA-tagged version of dPen-2/2–10, and dPen-2/10HA11 with a HA tag sequence inserted between residues 10 and 11 (Table 3).

Because the HA sequence consists of nine amino acid residues, HA-dPen-2/2–10 and dPen-2/10HA11 have N-terminal amino acid lengths equal to those of untagged dPen-2 and HA-dPen-2, respectively. These mutants increased Psn fragments as well as total A\( \beta \) secretion in the presence of Psn, dNct-V5/His, and dAph-1-FLAG (data not shown), suggesting that the proximal region of the N terminus of dPen-2 is dispensable for the \( \gamma \)-secretase activity, in agreement with the result in hPEN-2 (13). However, %A\( \beta \)42 in cells expressing dPen-2/2–10 or HA-dPen-2/2–10 was similar to that in cells expressing untagged dPen-2 (Table 3). Thus, the presence of an HA tag sequence at the N terminus of dPen-2 is not sufficient to increase A\( \beta \)42 production. In contrast, dPen-2/10HA11 significantly increased %A\( \beta \)42 in a similar manner to those with a series of the N-terminally tagged dPen-2 (Table 3). Finally, we examined the effects of untagged or HA-tagged dPen-2/2–17/rep, which harbor several substitutions of amino acid residues (MDISKAPNPRKLELCRK to MELCRGPQPKRVDISKR) within the entire length of the N-terminal region of dPen-2. This mutant carries a totally different amino acid sequence at the N terminus with similar characteristics in terms of bulkiness and charges other than the proline residues. HA-dPen-2/2–17/rep significantly increased the generation of A\( \beta \)42, whereas dPen-2/2–17/rep did not affect %A\( \beta \)42 at a statisti-
N-terminally Tagged Pen-2 Overproduces Aβ42

tially significant level (Table 3). Collectively, these data strongly suggest that the Aβ42 overproduction closely correlates with the extension of the N-terminal length of dPen-2, regardless of the amino acid sequences of the N-terminal luminal region of dPen-2, as well as of the types of the tag sequence added.

N-terminal Tags of Drosophila Pen-2-modulated γ- but Not ε-Cleavage—Several lines of evidence indicate that the γ-secretase complex executes “dual” cleavages, namely, γ- and ε-cleavages at middle and proximal positions to cytoplasm, respectively, within the transmembrane domain (2, 30). Almost all FAD mutations in PSEN genes affect the γ-cleavage to increase %Aβ42, whereas the effect of FAD mutations on ε-cleavage remain controversial. However, the γ-secretase-mediated cleavage of Notch at the site corresponding to the ε-cleavage is required for several biological functions (1–3). Thus, the modulation of the γ-cleavage site (i.e. specific decrease in Aβ42 generation) by small molecule compounds without affecting the ε-cleavage is one of the plausible therapeutic strategies for AD (1). To monitor the effect of the tagged dPen-2 on the ε-cleavage, a reporter S2 cell line stably expressing SC100 fused to gal4 within the cytoplasmic domain (SC100gal4) and EGFP, together with UAS-controlled, destabilized luciferase plasmid, was established. Upon the ε-cleavage of SC100gal4 by the γ-secretase, the intracellular domain of SC100gal4 enters the nucleus to activate the transcription of the luciferase. Luciferase activity and fluorescence light units of EGFP were dependent on the cell number (data not shown). Treatment with DAPT, a potent γ-secretase inhibitor, decreased the luciferase activity, whereas the fluorescence light units were unaffected (Fig. 3A). Thus, using this reporter cell line, we can assess the degree of the ε-cleavage of SC100gal4 by the relative luciferase activity standardized with the fluorescence light units. When we overexpressed the untagged or HA-tagged dPen-2 together with Psn, dNct-V5/His, and dAph-1-FLAG in the SC100gal4 reporter cell line, the relative luciferase activity of the cells expressing HA-dPen-2 was almost similar to that with untagged dPen-2 (Fig. 3B), suggesting that the tagged dPen-2 affected only the γ-cleavage site.

Several lines of evidence show an inverse correlation between Aβ38- and Aβ42-generating the γ-secretase activities in mammalian cells. For example, a subset of non-steroidal anti-inflammatory drugs known as γ-secretase modulators (GSMs) increase Aβ38 production (28, 31), whereas fenofibrate, which acts as an Aβ42-raising GSM, decreases an Aβ38 generation (supplemental Fig. S1) (28, 32). To ascertain whether the γ-cleavage site was modulated by the N-terminally tagged dPen-2 in Drosophila cells in a similar manner to that by the Aβ42-raising GSM, we examined the effect of the tagged dPen-2 on Aβ secretion from S2 cells expressing SC100 by an immunoprecipitation followed by an immunoblot analysis using Tris/Bicine/urea SDS-PAGE (Fig. 4) (27, 28). Unexpectedly, S2 cells showed robust Aβ38 secretion at an almost comparable level to that of Aβ40 at a steady state. However, the overexpression of HA-dPen-2 decreased the level of Aβ38 concomitantly with an increase in Aβ42. Collectively, these data suggest that an N-terminally tagged dPen-2 reciprocally modulates the Aβ38- and Aβ42-generating activities but has little effect on the ε-cleavage, in Drosophila cells, that was similar to the effect of Aβ42-raising GSMs in mammalian cells.

N-terminally Tagged Human PEN-2 Directly Affected the Structure and the Activity of the γ-Secretase Complex—We have reported that hPEN-2 can compensate for the loss of function of dPen-2 in RNAi-treated Drosophila S2 cells (5). To test the effect of the tag sequence at the N terminus of hPEN-2, we measured the levels of Aβ secreted from cells expressing hPEN-2 or HA-hPEN-2 together with Psn, dNct-V5/His, and dAph-1-FLAG. %Aβ42 of cells expressing HA-hPEN-2 was dramatically increased, whereas the overexpression of untagged hPEN-2 only slightly increased it (Table 4). To test this effect in a mammalian cell expression system, recombinant retrovirus encoding untagged or N-terminally FLAG-tagged hPEN-2 was co-infected with retrovirus encoding human PS1, human NCT (hNCT), and human APH-1b (hAPH-1b) into fibroblast cells.
**TABLE 4**

Relative fold change in %Aβ42 of S2 cells expressing hPEN-2 together with SC100, Psn, dNct-V5/His, and dAph-1-FLAG

| Exogenous hPEN-2 genotype | Fold change in %Aβ42 |
|---------------------------|---------------------|
| mock                      | 1                   |
| hPEN-2                    | 1.33 ± 0.07         |
| hA-hPEN-2                 | 3.04 ± 0.28**       |

*p < 0.05 by Student’s t test.

**TABLE 5**

Relative fold change in %Aβ42 of DKO cells expressing hPEN-2 together with APP carrying Swedish mutant, PS1, hNCT, and hAPH-1b

| Exogenous hPEN-2 genotype | Fold change in %Aβ42 |
|---------------------------|---------------------|
| hPEN-2                    | 1                   |
| FLAG-hPEN-2               | 1.91 ± 0.16**       |

*p < 0.005 by Student’s t test.

obtained from Psen1/Psen2 double knock-out mice (DKO cells) stably expressing APP Swedish mutant (20). Generation of Aβ42 from cells expressing FLAG-hPEN-2 was dramatically augmented at 1.9-fold compared with that from untagged hPEN-2-expressing cells (Table 5). In contrast, consistent with the result of dPen-2/Δ2–10, the deletion of 2–10 of hPEN-2 (hPEN-2/Δ2–10) did not affect the Aβ42 generation (supplemental Table S1). These data strongly suggest that the effect of the N-terminal tag of hPEN-2 on Aβ42 production is observed beyond species and independent of the expression system.

To gain insights into the molecular mechanism whereby the tagged hPEN-2 increases %Aβ42, we directly assessed the enzymatic activity of reconstituted human γ-secretase complex using a baculovirus/Sf9 cell system. We have reported that the reconstituted γ-secretase complex containing Hx-hPEN-2 showed high %Aβ42 in de novo generated Aβ (6). However, the reconstituted enzymatic enzyme containing untagged hPEN-2 exhibited lower Aβ42 production (Fig. 5A and Table 6), whereas the expression levels of components as well as the endoproteolytic processing of PS1 were unchanged (data not shown). Then, we examined a Michaelis-Menten plot of the reconstituted γ-secretase activities for Aβ40 and Aβ42 generation (γ40- and γ42-secretase activities, respectively, Fig. 5, B and C). γ40-Secretase activity showed similar profiles in the reconstituted γ-secretase complex containing untagged or tagged hPEN-2. However, the γ-secretase complex reconstituted with the tagged hPEN-2 exhibited a significantly higher γ42-secretase activity at almost 2-fold in Vₘₐₓ value (11.5 pM/h for hPEN-2, 24.0 pM/h for Hx-hPEN-2). Notably, Kₘ values of these reconstituted enzymes were almost similar (1.56 μM for hPEN-2 and 1.67 μM for Hx-hPEN-2), suggesting that the binding affinity of substrate to enzyme was unaffected. Thus, it seems quite likely that the tagged hPEN-2 directly and specifically increased the γ42-secretase activity.

Recently, using a substituted cysteine accessibility method, we reported that PS forms a catalytic pore embedded within the lipid bilayer (8). Using this method, we can gain insights into the structure of PS1 by the water accessibility of substituted cysteines in cysteine-less PS1. In particular, single-Cys PS1

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**FIGURE 5.** Enzymatic analysis of human γ-secretase complex reconstituted in the Sf9/baculovirus expression system. A, de novo Aβ generation from recombinant substrate by reconstituted human γ-secretase complex containing the untagged or tagged hPEN-2. DAPT treatment (10 μM) abolished these activities. B and C, Michaelis-Menten plot for the generation of Aβ40 (black open circle) and Aβ42 (open circle) by reconstituted γ-secretase containing the untagged hPEN-2 (open circles) or tagged hPEN-2 (filled circles) (n = 3, mean ± S.E.).

**TABLE 6**

Relative fold change in %Aβ42 of enzymatic activity of reconstituted γ-secretase complex containing hPEN-2 together with PS1, hNCT, and hAPH-1aL

| Exogenous hPEN-2 genotype | Fold change in %Aβ42 |
|---------------------------|---------------------|
| hPEN-2                    | 1                   |
| Hx-hPEN-2                 | 1.65 ± 0.10**       |

*p < 0.005 by Student’s t test.
mutants harboring A246C or L250C were labeled by methanethiosulfonate ethylammonium (MTSEA)-biotin, a membrane-impermeable thiol-directed reagent, from the extracellular side. These labels were sensitive to a transition-state analogue inhibitor, L-685,458. These residues were hypothesized to function as subsites that directly bind to a substrate and are involved in the proteolysis. Moreover, it was suggested that GSMs allosterically inhibit the binding of L-685,458 to the catalytic pore and directly affect the structure of PS1 (25, 33–35).

To examine whether the structural change in the catalytic pore was correlated to the overproduction of Aβ42, the water accessibility of A246C and L250C from extracellular side was analyzed in DKO cells expressing untagged or N-terminally FLAG-tagged hPEN-2 together with single-Cys mutant PS1, hNCT, and hAPH-1b (Fig. 6) (32). Water accessibility of I114C, predicted to be located far from the catalytic pore, was unaffected. Surprisingly, the biotinylation of A246C and L250C was reduced upon expression of FLAG-hPEN-2, suggesting that the N-terminal tag of hPEN-2 decreased the water accessibility of the residues at the luminal side of the catalytic pore. Notably, this effect was more prominent in L250C, whereas the biotinylation of A246C was slightly, but constantly, reduced. Then, we examined the effect of fenofibrate, which modulates the γ-secretase in a way to enhance Aβ42 production and decrease Aβ38 without any effect on Aβ40 generation (supplemental Fig. S1 and Table S1), on the labeling of these residues (Fig. 6). Both the biotinylation of A246C and L250C, but not I114C, was decreased. Similar to the effect by the N-terminally tagged hPEN-2, the labeling of L250C was more strongly reduced. Taken together, our results suggest that the N-terminally tagged PEN-2 increases the γ-secretase activity through a direct effect on the catalytic pore in a similar manner to those by the Aβ42-raising GSM.

DISCUSSION

In this study, we found that the N-terminal extension of Pen-2 with various tag sequences directly affected the enzymatic property of the γ-secretase complex in a way that increases Aβ42 production both in insect and mammalian cell systems. Notably, the N-terminal tagged Pen-2 caused a decrease in the water accessibility of the luminal side of the catalytic pore in a similar manner to that caused by an Aβ42-raising GSM, fenofibrate. Thus, our results suggest that the structure of the catalytic pore determines the γ-cleavage position of substrates and that Pen-2, especially its N-terminal portion, is involved in the γ-secretase cleavage through structural regulation of the catalytic pore.

The N-terminal tag of Pen-2 increased Aβ42 generation regardless of the expression system and the tag sequence, whereas the HA tag in dPen-2 was most potent. The precise molecular function of Pen-2 still remains unknown. However, our in vitro reconstitution data clearly suggest a direct effect of Pen-2 on the enzymatic activity, especially modulation of the cleavage position. Pen-2 exhibits a hairpin-like topology with both the N and C termini being oriented to the luminal side (36, 37). Kim and Sisodia (13) reported that the proximal part of the N terminus (residues 10–16) of hPEN-2 is required for its function, although preservation of the exact amino acid sequence of this region is unnecessary. In fact, the replacement of most of the amino acid residues at the N terminus as well as the distal part (i.e. dPen-2/Δ2–17/rep and HA-dPen-2/Δ2–10) retained the activity of dPen-2, suggesting that the electrostatic composition of this proximal region determines its function related to the assembly and the activation of the γ-secretase complex. Because recent structural analyses of the γ-secretase complex still remain at low resolution, the location and stoichiometry of each component are unknown (22, 38). However, recently, we and others showed that hPEN-2 binds to PS1 TMD4 (14, 15).

Thus, the N terminus of Pen-2 might be located close to the luminal side of the catalytic pore, the latter being formed by TMD6 and -7 of PS1. Consistent with this model, the N-terminal tag of hPEN-2 decreased the water accessibility of the luminal side of the catalytic pore. Therefore, it is tempting to speculate that the N terminus of Pen-2 might behave like a “lid” of the pore, or directly affect the structure of the pore in such a way to regulate the cleavage positions of substrates. However, the length of the tag sequences did not correlate with the degree in the Aβ42 overproduction, because 9 and 40 amino acid lengths of FLAG and His/Xpress tags, respectively, yielded comparable increases in %Aβ42. In addition, the HA tag showed the most potent effect, although the number of amino acid residues of HA, myc, and FLAG tags are almost similar. The HA2 tag caused an almost comparable increase with the HA tag in dPen-2/Δ2–17, although the number of amino acid residues of HA, myc, and FLAG tags are almost similar.
rrower and less accessible to labeling by MTSEA-biotin. It has been shown that antibodies to the N-terminal region of dPen-2 or hPen-2 failed to pull down the active \(\gamma\)-secretase complex in CHAPS- or CHAPSO-solubilized lysates, whereas free Pen-2 polypeptides were efficiently immunoprecipitated from Triton X-100-solubilized lysate, in which the \(\gamma\)-secretase complex is dissociated (Refs. 37 and 39 and data not shown). Moreover, little biotinylation of hPen-2 by MTSEA-biotin in the active \(\gamma\)-secretase complex was observed, despite hPen-2 carrying one luminal cysteine residue closely located to TMD1.6 In contrast, the overexpressed hPen-2 that was not bound to PS1 was labeled by MTSEA-biotin.6 Thus, it is highly likely that the N-terminal region of Pen-2 is buried and hidden within the active \(\gamma\)-secretase complex. Taken together, the N terminus of Pen-2 might function as a molecular chaperone that contributes to the structural arrangement of TMDs as well as of the catalytic pore. The tags, that contain hydrophilic and charged residues, might change an electrostatic configuration of the N terminus of Pen-2 and modify the structure of the catalytic pore in a way to increase the \(\beta\)42 generation.

Recently much attention is focused on the molecular mechanism of the \(\beta\)42-lowering GSMs as the plausible therapeutics without Notch-related adverse effects for AD (1). Several GSMs, including a subset of non-steroidal anti-inflammatory drugs, directly regulate the proteolytic cleavages at \(\beta\)38 and \(\beta\)42 positions, without affecting \(\epsilon\)-cleavage activity in a cell-based assay (25, 31, 32). Although the precise molecular mechanism whereby GSMs modulate the \(\gamma\)-secretase activity remains unknown, enzymatic analyses indicated that the \(\beta\)42-lowering GSMs modulate the binding site for transition-state analogue inhibitors in a noncompetitive, allosteric manner (25, 35). Moreover, FLIM assay showed that the \(\beta\)42-lowering GSMs increase the proximity of PS1 NTF and CTF (33), whereas FAD-linked PS mutations that augment \(\beta\)42 generation decrease the distance (34). Collectively, these results suggest that the structural changes in the catalytic site formed by PS1 NTF and CTF affect the enzymatic property regarding cleavage positions. Here we found that an \(\beta\)42-raising GSM and the N-terminally tagged Pen-2 caused a similar effect on the \(\beta\)38/42-generating activity and the water accessibility of the PS1 TMD6, thus constituting the luminal side of the catalytic pore. We previously reported that the biotinylation of L250C was inhibited by L-685,458 and DAPT, potent, non-selective \(\gamma\)-secretase pore. We previously reported that the biotinylation of the luminal part of the catalytic pore prompts us to speculate that the compound targets the position where the N-terminal region of Pen-2 is located. Further attempts to clarify the mode of binding as well as the molecular targets of GSM would resolve this issue, in the same way that we and others have identified the functional domains in the \(\gamma\)-secretase components using small compounds harboring photolabile moieties as a molecular probe (26, 41, 42).

In summary, we have identified a novel mode of the \(\gamma\)-secretase to increase \(\beta\)42, in which the extension of the N-terminal region of Pen-2 affects the regulation of the \(\gamma\)-secretase cleavage position, in a similar manner to the effect of the \(\beta\)42-raising GSM. Nevertheless, whatever the precise underlying mechanism may be, our findings are pharmacologically relevant and could have major therapeutic implications, because it is strongly suggested that the N terminus of Pen-2 and/or the structural changes occurring in the catalytic pore are one of the major determinants for the \(\gamma\)-secretase cleavage positions. Further investigations using a chemical biological approach should provide us with clues to the development of the \(\beta\)42-lowering GSMs for AD therapeutics.

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5 C. Sato, T. Iwatsubo, and T. Tomita, unpublished result.
6 N. Isoo, C. Sato, T. Iwatsubo, and T. Tomita, unpublished result.
N-terminally Tagged Pen-2 Overproduces Aβ42