Alzheimer Presenilin-1 Mutations Dramatically Reduce Trimming of Long Amyloid β-Peptides (Aβ) by γ-Secretase to Increase 42-to-40-Residue Aβ*

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Background: Mutations in presenilin-1 (PS1) cause early-onset familial Alzheimer disease (FAD).

Results: The PS1-γ-secretase complex trims the C terminus of long amyloid β-peptides (Aβ), and FAD mutations significantly reduce the efficiency of trimming.

Conclusion: This loss of carboxypeptidase function results in a gain of toxic Aβ42 compared to Aβ40.

Significance: Understanding the effects of FAD mutations on γ-secretase function is critical for developing effective treatments.

The presenilin-containing γ-secretase complex produces the amyloid β-peptide (Aβ) through intramembrane proteolysis, and >100 presenilin mutations are associated with familial early-onset Alzheimer disease (AD). The question of whether these mutations result in AD through a gain or a loss of function remains highly controversial. Mutations in presenilins increase ratios of 42- to 40-residue Aβ critical to pathogenesis, but other Aβs of 38–49 residues are also formed by γ-secretase. Evidence in cells suggests the protease first cleaves substrate within the transmembrane domain at the ε site to form 48- or 49-residue Aβ. Subsequent cleavage almost every three residues from the C terminus is thought to occur along two pathways toward shorter peptides to Aβ42/Aβ40. Here we show that the addition of synthetic long Aβ peptides (Aβ45–49) directly into purified preparations of γ-secretase leads to the formation of Aβ40 and Aβ42 whether the protease complex is detergent-solubilized or reconstituted into lipid vesicles, and the ratios of products Aβ42 to Aβ40 follow a pattern consistent with the dual-pathway hypothesis. Kinetic analysis of five different AD-causing mutations in presenilin-1 revealed that all result in drastic reduction of normal carboxypeptidase function. Altered trimming of long Aβ peptides to Aβ40 and Aβ42 by mutant proteases occurs at multiple levels, independent of the effects on initial endoproteolysis at the ε site, all conspire to increase the critical Aβ42/Aβ40 ratio implicated in AD pathogenesis. Taken together, these results suggest that specific reduction of carboxypeptidase function of γ-secretase leads to the gain of toxic Aβ42/Aβ40.

Deposition of the amyloid β-peptide (Aβ) in the form of neuritic plaques in the brain is a defining pathological characteristic of Alzheimer disease (AD) (1). A large body of evidence points to Aβ as the pathogenic initiator, most notably the identification of dominant missense mutations in the amyloid β-protein precursor (APP) and the presenilins that cause early-onset familial AD (FAD) (2) and the discovery of presenilin as the catalytic component of γ-secretase responsible for generation of the Aβ C terminus (3). More than 100 FAD presenilin mutations have been identified, and these mutations can cause a decrease in γ-secretase cleavage of substrates as well as an increase in the ratio of the more aggregation-prone 42-residue Aβ (Aβ42) over the major secreted 40-residue Aβ (Aβ40). These findings have led to a still unresolved controversy over whether presenilin mutations cause FAD through a loss or a gain of function (4–8).

In deciphering the effects of presenilin FAD mutations, it is critical to consider the various proteolytic functions of γ-secretase. First, upon assembly with the other three components of the protease complex, presenilin undergoes autoproteolysis (3, 9) within a large loop located between transmembrane domains 6 and 7 to form an N-terminal fragment (NTF) and C-terminal fragment (CTF) (10), resulting in activation of the enzyme for cleavage of substrates. FAD-mutant presenilins likewise undergo NTF/CTF formation, with one notable exception (Dexon9, which is active as a holoprotein (10)). Second, γ-secretase has endoproteolytic activity toward membrane stubs of APP and other substrates generated upon ectodomain release by sheddases (11). This cleavage occurs within the substrate transmembrane domain to release the intracellular domain. For APP, the endoproteolytic site (called the ε site) is located close to the transmembrane/cytosolic boundary (Fig. 1A) (12), with cleavage here releasing the APP intracellular domain (AICD). Reduction of ε cleavage is seen with many, although not all, FAD presenilin mutations (7, 13, 14).

In recent years evidence has mounted in support of a third type of proteolytic function of the γ-secretase complex, a car...

* This work was supported, in whole or in part, by National Institutes of Health Grant R03 NS081498 (to M. S. W.). This work was also supported by an HHMI Gilliam Fellowship (to M. A. F.).

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2 The abbreviations used are: Aβ, amyloid β-peptide; AD, Alzheimer disease; AICD, amyloid β-protein precursor intracellular domain; APP, amyloid β-protein precursor; CTF, C-terminal fragment; NTF, N-terminal fragment; FAD, familial Alzheimer’s disease; GSM, γ-secretase modulator; CHAPSO, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PS1, presenilin-1; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxy-methyl)propane-1,3-diol; ANOVA, analysis of variance; D1, PS1 D257A; Bicine, N,N′-bis(2-hydroxyethyl)glycine; DAPT, N-[(3,5-difluorophenacyl)-l-alanyl]-5-phenylglycine t-buty...
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boxy-peptidase activity that trims membrane-associated long Aβ peptides of 48 or 49 residues to shorter secreted forms of 38–43 amino acids (15–19). Biochemical and cellular studies suggest that the endoproteolytic cleavage of APP substrate occurs first and that the resultant long Aβ peptides are primarily trimmed in intervals of three amino acids along two product lines (Fig. 1A), with Aβ49 leading to Aβ46, Aβ43 and Aβ40 and Aβ48 leading to Aβ45, Aβ42, and Aβ38 (this last event removing a tetrapeptide). The expected tri- and tetrapeptide products have been detected by mass spectrometry (18). Despite the evidence, however, other studies suggest that Aβ and AICD production can be dissociated (20, 21) or that there is no precursor-product relationship between Aβ42 and Aβ38 (22, 23). The former contention has been disproven by the determination of equimolar Aβ and AICD production (24), and the latter has been unambiguously settled recently with the demonstration that synthetic Aβ42 is converted to Aβ38 by isolated γ-secretase (25).

In this study we developed a method for examining the carboxypeptidase cleavage of synthetic Aβ48 and Aβ49 by isolated or purified enzyme complexes, independent of their initial formation through proteolysis of APP substrate. In so doing, we confirm that these long Aβ peptides are indeed intermediates toward Aβ40 and Aβ42, provide support for the dual-pathway model, and determine the degree to which long Aβ peptides contribute to the critical Aβ42-to-Aβ40 ratio. This biochemical system also allowed evaluation of the role of the membrane in the carboxypeptidase activity. Most notably, kinetic analysis of Aβ40 and Aβ42 production from long Aβ peptides by FAD mutant presenilin-1-γ-secretase complexes revealed striking reductions in carboxypeptidase activity that have important implications for resolving the loss-of-function versus gain-of-function controversy and providing a unifying model for the pathogenic mechanism of presenilin mutations.

**EXPERIMENTAL PROCEDURES**

**γ-Secretase Preparations**—CHAPSO-solubilized membranes from S20 cells, which are CHO cells overexpressing all four γ-secretase components, were prepared as previously described (26). Briefly, 20 confluent 15-cm plates were scraped and lysed using a French pressure cell at 1000 p.s.i. The lysate was spun at low speed to remove nuclei and unbroken cells and then at 100,000 × g. The resulting membrane pellet was washed in sodium bicarbonate buffer and solubilized in 2 ml of 1% CHAPSO. For purified γ-secretase preparations, membranes from 160 confluent 15-cm plates of S20 cells were isolated, washed, and solubilized as described above, and γ-secretase complexes were purified by sequential affinity purifications via nickel-nitritoltriacetic acid-agarose beads (Sigma) and M2 immobilized anti-FLAG-agarose beads (Sigma). For the inactive mutant enzyme controls, both solubilized membranes and purified complexes were prepared from CHO D1 cells (overexpressing all four γ-secretase components but with D257A mutant PS1 containing an N-terminal Myc tag) in the same way as above, except the first affinity purification step involved anti-Myc antibody-agarose beads (Sigma). For the analysis of FAD PS1 mutant complexes, membranes were prepared from CHO cells overexpressing Pen2, Aph1, nicastrin, and either WT or mutant PS1 as previously reported (14). For these preparations, membranes were isolated from 20 confluent 15-cm plates and resuspended in 160 μl of 1% CHAPSO.

**γ-Secretase Assays**—For assays performed in CHAPSO (27), solubilized membranes were incubated with substrate in HEPES buffer at pH 7.0 with 0.1% phosphatidylcholine, 0.025% phosphatidylethanolamine, 0.00625% cholesterol, and a final CHAPSO concentration of 0.25%. For assays performed in vesicles (28), purified γ-secretase, 0.1% phosphatidylcholine, 0.025% phosphatidylethanolamine, and 0.00625% cholesterol were solubilized in 0.25% CHAPSO and incubated with styrene-based Biobeads (Bio-Rad) for 2 h at 4 °C to remove detergent. Substrate was added to the resulting detergent-solubilized preparations or proteoliposomes followed by incubation at 37 °C. To validate trimming of long Aβ substrates, reactions were carried out for 4 h, which was within the time frame needed for maximal product formation; to analyze the rate of trimming by WT or FAD mutant PS1-γ-secretase and to examine the effects of modulators (GSM-1 (22) and compound 29, obtained courtesy of T. Golde (University of Florida)) on the trimming process, reactions were carried out for 1.5 h, which was within the linear range of product formation. Synthetic, purified long Aβ substrates were purchased from Anapec, and APP C100-FLAG substrate was prepared as previously described (27, 30). Aβ40 and Aβ42 generated from Aβ49-45 were detected using Aβ40- and Aβ42-specific ELISAs (Invitrogen).

**Kinetic Analysis**—Samples of WT and FAD mutant membrane preparations were run on 4–12% Bis-Tris gels followed by Western blotting with mAb1563 (Millipore), which detects PS1 NTF. The signal was captured using ECL (GE Healthcare), densitometry was performed on the films using ImageQuant (GE Healthcare), and the amount of enzyme added to each reaction was normalized based on the measured level of PS1 NTF (or holoprotein in the case of the uncleavable Δexon9). Time course experiments were first performed with the FAD mutants to establish the linear range of product formation. Reactions were then carried out with varying concentrations of substrate within this linear time frame. Aβ40 and Aβ42 products were quantified by specific ELISAs (Invitrogen), and non-linear regression analysis with $K_m$ and $V_{max}$ determination was performed using GraphPad Prism 4.

**Cloning, Cell Culture, Transfections, and Analysis**—All CHO cell lines were grown in DMEM with 10% fetal bovine serum. For the analysis of long Aβ trimming in cells, APP truncated at residues 49 and 48 (Aβ numbering) and harboring the Swedish double mutation were cloned and expressed using the Tet-On system (Clontech). 100,000 CHO cells/well were seeded into 24-well plates. Upon reaching ~50% confluence, cells were transiently co-transfected with a 1:5 ratio of the pTet-On Advanced plasmid (Clontech) and a pTRE-Tight plasmid (Clontech) carrying the truncated APP sequence. Six hours post-transfection, the medium was changed to DMEM complete containing 1 μg/ml doxycycline to induce the expression of the truncated APP. Inhibitor control transfections were also treated with 10 μM DAPT at this time. After 24 h cells and conditioned media were harvested. Aβ40 and Aβ42 in the media were measured by specific ELISAs (Meso Scale Discov-
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RESULTS

γ-Secretase Trims ε-Site Aβ Peptides to Aβ40 and Aβ42—We first examined the production of Aβ40 and Aβ42 from synthetic Aβ48 and Aβ49 in vitro, which allowed us to demonstrate the C-terminal trimming of ε-site Aβ peptides by γ-secretase using isolated enzyme and substrate. We could also directly examine the model in which Aβ48 processing leads primarily to Aβ42 and Aβ49 primarily to Aβ40 (18). We performed these assays in CHAPSO detergent (27, 30) as well as with purified enzyme complexes reconstituted into lipid vesicles (28) to ensure that the results are not artifacts arising from detergent solubilization and to determine if the membrane is critical to the trimming process. We and others have previously described the use of urea polyacrylamide gel electrophoresis followed by Western blotting with an N-terminally directed anti-Aβ antibody to detect the range of C-terminal variants of Aβ produced in vitro γ-secretase reactions (7, 14, 17, 31).

However, trimmed Aβ generated from the synthetic long Aβ substrates could not be detected by this method because the signal from the excess substrate streaked throughout the lane, completely obscuring any signals from the Aβ products (data not shown). The levels of Aβ40 and Aβ42 produced from each synthetic long Aβ were instead quantified using specific ELISAs. The data reveal that Aβ48 and Aβ49 are indeed trimmed by γ-secretase to Aβ40 and Aβ42 in both the CHAPSO-solubilized (Fig. 1B) and proteoliposome systems (Fig. 1C). Control reactions with Aβ substrate alone (in which no enzyme was added to the reaction mixture) give the base-line levels of cross-reactivity of the large amounts of long Aβ substrates in the assays with each ELISA detection antibody. Although there is some cross-reactivity, the results indicate that the signal obtained in the reactions with enzyme present is not simply background. Furthermore, the addition of the γ-secretase transition-state analog inhibitor L-685,458 to the assays results in inhibition of Aβ40 and Aβ42 production. Heat-inactivated or inactive PS1 D257A mutant PS1; boiled reactions contain heat-inactivated γ-secretase; γ-secretase reactions have no substrate added to the assay mixtures: Aβ reactions have no enzyme added to the reaction mixtures. For all of the non-control reactions, the level of Aβ40 produced was significantly different from that of Aβ42 (p < 0.01, Student’s t test). n = 3; error bars, S.D.

FIGURE 1. γ-Secretase trims synthetic Aβ49 and Aβ48 to Aβ40 and Aβ42 in vitro. A, APP substrate is thought to be cleaved sequentially by γ-secretase at the ε, ζ, and γ sites, indicated by arrowheads. These cleavage events result in Aβ peptides with the indicated C termini. Ihara and co-workers (16, 18) have proposed Aβ40- and Aβ42-generating pathways (top and bottom, respectively), in which ε cleavage that produces AICD50–99 primarily leads to Aβ40, whereas ε cleavage that produces AICD49–99 mainly produces Aβ42. B and C, Aβ40 and Aβ42 production from synthetic Aβ49 and Aβ48 and CHAPSO-solubilized membranes from CHO cells overexpressing the four γ-secretase complexes reconstituted into lipid vesicles (27, 30) as well as with purified enzyme complexes reconstituted into lipid vesicles (28) to ensure that the results are not artifacts arising from detergent solubilization and to determine if the membrane is critical to the trimming process. We and others have previously described the use of urea polyacrylamide gel electrophoresis followed by Western blotting with an N-terminally directed anti-Aβ antibody to detect the range of C-terminal variants of Aβ produced in in vitro γ-secretase reactions (7, 14, 17, 31). However, trimmed Aβ generated from the synthetic long Aβ substrates could not be detected by this method because the signal from the excess substrate streaked throughout the lane, completely obscuring any signals from the Aβ products (data not shown). The levels of Aβ40 and Aβ42 produced from each synthetic long Aβ were instead quantified using specific ELISAs. The data reveal that Aβ48 and Aβ49 are indeed trimmed by γ-secretase to Aβ40 and Aβ42 in both the CHAPSO-solubilized (Fig. 1B) and proteoliposome systems (Fig. 1C). Control reactions with Aβ substrate alone (in which no enzyme was added to the reaction mixture) give the base-line levels of cross-reactivity of the large amounts of long Aβ substrates in the assays with each ELISA detection antibody. Although there is some cross-reactivity, the results indicate that the signal obtained in the reactions with enzyme present is not simply background. Furthermore, the addition of the γ-secretase transition-state analog inhibitor L-685,458 to the assays results in inhibition of Aβ40 and Aβ42 production. Heat-inactivated or inactive PS1 D257A mutant PS1; boiled reactions contain heat-inactivated γ-secretase; γ-secretase reactions have no substrate added to the assay mixtures: Aβ reactions have no enzyme added to the reaction mixtures. For all of the non-control reactions, the level of Aβ40 produced was significantly different from that of Aβ42 (p < 0.01, Student’s t test). n = 3; error bars, S.D.

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enzyme preparations. γ-Secretase modulators (GSMs) also had the effect on trimming that they typically have on Aβ production (32); levels of Aβ42 generated from Aβ48 and Aβ49 were decreased by two different GSMs (22, 29), whereas Aβ40 levels were not altered, providing further validation of C-terminal trimming by γ-secretase in vitro (Fig. 2, A and B). However, only the more potent GSM-1 compound showed a robust dose-response effect. Kinetic analysis of the levels of Aβ42 generated from APP C100FLAG and Aβ48 in the presence and absence of GSM-1 indicates that this modulator reduces the $V_{\text{max}}$ in both cases and does not alter the $K_m$ of these conversions (Fig. 2C). This result is consistent with a recent report demonstrating that modulators increase the $k_{\text{cat}}$ of Aβ42 conversion to Aβ38 (25).

FIGURE 2. γ-Secretase modulators lower Aβ42 produced from Aβ49 and Aβ48. Both GSM-1 and Rivkin-2 (compound 2 in Ref. 29) have previously been shown to selectively lower Aβ42 levels and concomitantly increase Aβ38 levels (22, 29). A, as expected, both compounds lower Aβ42 and increase Aβ38 in a concentration-dependent manner using APP C100-FLAG as substrate in CHAPS-solubilized γ-secretase assays, as detected using a Bicine/urea-polyacrylamide gel electrophoresis system. B, both compounds decreased the amount of Aβ42 generated from Aβ48 and Aβ49 without altering Aβ40 levels. * indicate values that were significantly different from the Aβ42 value with no compound added ($p < 0.05$). Aβ40 values were not significantly different at any concentration tested. C, cleavage reactions were performed in CHAPSO with the indicated concentrations of substrate. The levels of Aβ42 generated from C100-FLAG (left) or Aβ48 (right) were measured by ELISA. + GSM, reactions contained 25 μM GSM-1; − GSM, reactions with vehicle alone. $V_{\text{max}}$ values for C100 and Aβ48 substrates were significantly reduced in the presence of GSM-1 ($p < 0.05$, Student's t test). n = 3; error: S.D.
The ratios of Aβ42 to Aβ40 produced from each Aβ substrate in each system (taking into account the background signal that each substrate alone has in each ELISA) are shown in Table 1 and indicate that γ-secretase cleavage of Aβ49 primarily leads to Aβ40, with an Aβ42/Aβ40 ratio of ~1.7, and cleavage of Aβ48 primarily leads to Aβ42, with an Aβ42/Aβ40 ratio of ~9:1 in detergent and ~6:1 in vesicles (with no statistically significant differences in trimming between the CHAPSO and vesicle assays). These results are consistent with the model proposed by Ihara and co-workers (18) of two pathways in which Aβ49 is primarily converted to Aβ40 and Aβ48 primarily to Aβ42. We show that C-terminal trimming along these pathways is an inherent property of γ-secretase whether it is solubilized in detergent or incorporated in a membrane and also demonstrate that Aβ40 and Aβ42 can be produced by a small degree of crossover between the two pathways.

Although these results demonstrate the trimming of ε-site Aβ peptides in vitro, we nonetheless sought to validate our findings in a cellular assay. Previous studies have attempted to examine the trimming of Aβ49 and Aβ48 by γ-secretase in cells by direct expression of these long Aβs with a signal sequence (15). Although this system was used to demonstrate that Aβ49 expression primarily leads to Aβ40 secretion, the 1:1 ratio for Aβ42/40 observed from Aβ48 was inconclusive, as the levels of secreted Aβ were highly variable and apparently below the detection limits, likely due to the observed poor expression of Aβ48. Alternatively, APP truncated at position 49 (APPΔ49) can be expressed at high levels, inserted into the membrane, and sorted to the cell surface (33). Moreover, APPΔ49 was processed by β- or α-secretase and then γ-secretase to generate quantifiable amounts of secreted Aβ or p3, an N-terminally truncated Aβ generated through α/γ-secretase cleavage. Therefore, we generated constructs for the expression of APPΔ49 and the previously unreported APPΔ48 and introduced them into CHO cells overexpressing all four γ-secretase components (14). Expression of these truncated APPs was confirmed by Western blot of the cell lysates (Fig. 3A), and the levels of p3/Aβ40 and p3/Aβ42 secreted into the media were measured by specific ELISAs (Fig. 3B). We used 4G8, targeting the middle region of Aβ, as the detection antibody in the ELISA because it detects both p3 and Aβ, as secretion of Aβ alone from these cells was too low for quantification. The results confirm our in vitro findings, as APP49 expression primarily led to p3/Aβ40 secretion, and APP48 expression led to mainly p3/Aβ42 secretion. p3/Aβ production from each construct could be inhibited by the γ-secretase inhibitor DAPT. In principle, we could obtain Aβ42/40 ratios by subtracting the background observed from endogenous APP in untransfected cells; however, this may not be appropriate, as the overexpressed truncated APPs may compete with endogenous substrate for binding to secretases. The inability to accurately quantify ε-independent Aβ40 and Aβ42 production in this cellular system emphasizes the value of direct biochemical analysis with isolated or purified enzyme. In any event, whether production of p3/Aβ from endogenous substrate is completely inhibited or uninhibited by the overexpression, the conclusions about product preference (Aβ40 from APPΔ49 and Aβ42 from APPΔ48) still stand.

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![FIGURE 3. γ-Secretase trims p3/Aβ49 and p3/Aβ48 to p3/Aβ40 and p3/Aβ42 in cells. A, the expression of APPΔ49 and APPΔ48 in CHO cells was confirmed by Western blot of cell lysates using anti-APP antibody 22C11. The truncated APPs run slightly faster than full-length, endogenous APP. B, Aβ42 and Aβ40 secretion by cells expressing APPΔ49, APPΔ48, or full-length APP. Untransfected controls (no txn) show the endogenous background signal from the cells; for + inhibitor transfections, cells were treated with 10 μM DAPT. *, p < 0.05; **, p < 0.01 (Student’s t test). n = 3; error bars, S.E.}

\textbf{TABLE 1}
\textbf{Aβ42/Aβ40 ratios from trimming of ε- and γ-cleaved Aβs}

| Conversion   | Aβ45 | Aβ46 | Aβ48 | Aβ49 |
|-------------|------|------|------|------|
| CHAPSO      | 41 ± 2.4 | 0.14 ± 0.03 | 8.8 ± 1.1 | 0.14 ± 0.06 |
| Vesicles    | 40 ± 10 | 0.18 ± 0.06 | 6.1 ± 1.4 | 0.14 ± 0.08 |

The background of each substrate in each ELISA (i.e. Aβ alone controls) was first subtracted. Error: S.D.
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FIGURE 4. γ-Secretase trims synthetic Aβ45, Aβ46, and Aβ47 to Aβ40 and Aβ42 in vitro. A, enzyme assays using CHAPSO-solubilized membranes from CHO cells overexpressing the four γ-secretase components. B, enzyme assays using purified γ-secretase complexes reconstituted into lipid vesicles. Control reactions are the same as in Fig. 1. The level of Aβ40 generated in all non-control reactions was significantly different from that of Aβ42 (p < 0.01 for Aβ45 and Aβ46 substrates; p < 0.05 for Aβ47 substrate, Student’s t test). n = 3; error bars, S.D.

-1:7, whereas Aβ45 led to Aβ42 almost exclusively, with a large Aβ42/Aβ40 ratio of ~40:1. A higher ratio of Aβ42/Aβ40 generated from Aβ45 than from Aβ48 is also consistent with the Ihara model, which posits only one cleavage event between Aβ45 and Aβ42. Importantly, we again found that the Aβ42/Aβ40 ratios from each substrate were the same in both the solubilized and proteoliposome systems (Table 1). These results consistently indicate that dual-pathway C-terminal trimming is an intrinsic property of γ-secretase and that the membrane is not essential for the enzyme to trim with apparent precision along these two pathways. We also attempted to confirm these results in cells by expressing APPe46 and APPe45 in the same CHO cell line that was used for APPe49 and APPe48 expression; however, we could not detect an increase in the level of secreted p3/Aβ above endogenous background when these constructs were expressed, likely due to the inability of these truncated APPs to remain inserted in the membrane. With synthetic Aβ47 as a substrate for isolated γ-secretase, we found that the levels of Aβ40 and Aβ42 produced were substantially reduced compared with that seen with the natural long Aβ substrates. This is consistent with the fact that the expected major cleavage products of Aβ47 based on the Ihara model are Aβ44 and Aβ41, which, like Aβ47, are not naturally observed γ-secretase products.

C-terminal Trimming by FAD-mutant γ-Secretase—Having established a system to examine γ-secretase trimming of synthetic long Aβ from the ε to γ sites, we next examined the effects of FAD mutations in PS1 on this trimming process. To accomplish this, we determined the levels of Aβ40 and Aβ42 that five FAD-mutant PS1-γ-secretase complexes produced from Aβ49 and Aβ48 and their rates of formation compared with wild-type (WT) complexes. In this system, the levels of Aβ40 and Aβ42 generated are solely based on the efficiency of trimming, independent of effects on cleavage at the ε site (which could either alter the rate of ε site cleavage or the specificity of cleavage at positions 48 and 49). The PS1 mutations are G384A, Δexon9, L166P, A246E, and L286V, and all except L286V have been previously shown to reduce the endoproteolytic cleavage at the ε site that leads to AICD production (14). However, all five mutations have been shown to generate an increased proportion of long Aβ peptides (Aβ42+) compared with WT enzyme (14). Membranes from CHO cells stably overexpressing the four γ-secretase components with either WT or mutant PS1 were solubilized in CHAPSO, and equal amounts of each enzyme were used in the reactions based on PS1 NTF levels (or PS1 holoprotein in the case of the uncleavable but proteolytically active Δexon9) in each enzyme preparation. We first performed time course experiments to determine the linear range for Aβ40 and Aβ42 production from Aβ48 and Aβ49 followed by kinetic analysis of C-terminal trimming by each mutant. The rates of Aβ40 and Aβ42 production for each mutant were measured across different concentrations of substrate, and curves were generated for each possible conversion (Aβ49 → Aβ40, Aβ49 → Aβ42, Aβ48 → Aβ40, and Aβ48 → Aβ42) using non-linear regression analysis (Fig. 5). The $K_m$ and $V_{max}$ values were then calculated from these curves (Table 2). Because each reaction contained equal amounts of enzyme, $k_{cat}$ is proportional to $V_{max}$, and $V_{max}/K_m$ is, therefore, a measure of...
catalytic efficiency (Table 2). When the efficiencies of these conversions by WT PS1 are set to 100% and compared with the efficiencies of the mutants (Table 3), all of the FAD mutant complexes displayed clear and substantial reduction of the normal carboxypeptidase trimming function of \( \beta \)-secretase, with efficiencies ranging from 2 to 9% of WT for A\(_{49} \) trimming and from 17 to 40% of WT for A\(_{48} \) trimming. The \( K_m \) and \( V_{\text{max}} \) values in Table 2 indicate that these reductions in trimming efficiencies are primarily the result of dramatic decreases in \( V_{\text{max}} \), suggesting that the mutations affect the turnover of the long A\( \beta \)s more than their affinity for the enzyme. Moreover, the trimming of these synthetic e-cleaved A\( \beta \)s was altered in ways that contribute to an increase in the A\( \beta_{42}/A\beta_{40} \) ratio. First, as shown in Table 3, the trimming of A\( \beta_{49} \) to A\( \beta_{40} \) was significantly more reduced than the trimming of A\( \beta_{48} \) to A\( \beta_{42} \) by all of the mutant complexes when compared with WT. The catalytic efficiency values in Table 2 show that although the WT enzyme trimmed A\( \beta_{49} \) to A\( \beta_{40} \) ~2.5 times more efficiently than A\( \beta_{48} \) to A\( \beta_{42} \), the \( \Delta \)exon9 and L286V complexes trimmed A\( \beta_{48} \) to A\( \beta_{42} \) more efficiently than A\( \beta_{49} \) to A\( \beta_{40} \), and the L166P, G384A, and A246E complexes trimmed A\( \beta_{48} \) to A\( \beta_{42} \) with nearly equal efficiency as A\( \beta_{49} \) to A\( \beta_{40} \).

In addition to these differences seen between the processing of A\( \beta_{49} \) and A\( \beta_{48} \), the L166P and \( \Delta \)exon9 mutations resulted in a significantly greater reduction of the major A\( \beta_{49} \) to A\( \beta_{40} \) conversion compared with the crossover A\( \beta_{49} \) to A\( \beta_{42} \) conversion, and the \( \Delta \)exon9 and L286V mutations led to a greater reduction of the crossover A\( \beta_{48} \) to A\( \beta_{40} \) conversion compared with the major A\( \beta_{48} \) to A\( \beta_{42} \) conversion (Table 3).

We also attempted to examine the effects of FAD mutations on the trimming of \( \zeta \)-site A\( \beta \)s (A\( \beta_{46} \) and A\( \beta_{45} \)) in an effort to determine if a specific step in the trimming process is reduced.
more than others. However, due to the high background that the Aβ45 and Aβ46 substrates have in the Aβ42 ELISA (Fig. 4), we were not able to detect any Aβ46 signal above background when we attempted to monitor the rates of trimming of these substrates by the FAD mutants (data not shown). In addition, due to the low efficiency of the conversion of Aβ45 to Aβ40

**TABLE 2**

\( V_{\text{max}} \) and \( K_m \) of Aβ trimming events from WT and PS1 FAD-mutant γ-secretase

| Conversion | \( V_{\text{max}} \) | \( K_m \) | Catalytic efficiency |
|------------|-----------------|--------|-------------------|
| Aβ49 → Aβ40 | WT 9.18 ± 0.70 | 0.84 ± 0.17 | 11.13 ± 2.03 |
|            | L166P 0.35 ± 0.11 | 0.73 ± 0.33 | 0.50 ± 0.12 |
|            | Δexon9 0.53 ± 0.13 | 2.56 ± 1.46 | 0.25 ± 0.12 |
|            | G384A 0.74 ± 0.07 | 1.07 ± 0.49 | 0.75 ± 0.22 |
|            | L286V 0.60 ± 0.30 | 2.10 ± 1.92 | 0.37 ± 0.20 |
|            | A246E 0.79 ± 0.04 | 0.89 ± 0.34 | 0.94 ± 0.24 |

| Aβ49 → Aβ42 | WT 3.83 ± 0.93 | 1.76 ± 0.53 | 2.21 ± 0.16 |
|            | L166P 0.18 ± 0.05 | 1.02 ± 0.44 | 0.18 ± 0.03 |
|            | Δexon9 0.17 ± 0.06 | 1.48 ± 0.43 | 0.17 ± 0.07 |
|            | G384A 0.20 ± 0.01 | 1.45 ± 0.64 | 0.15 ± 0.08 |
|            | L286V 0.22 ± 0.02 | 2.01 ± 1.15 | 0.13 ± 0.06 |
|            | A246E 0.56 ± 0.25 | 3.06 ± 0.76 | 0.18 ± 0.04 |

| Aβ48 → Aβ42 | WT 7.40 ± 0.08 | 1.85 ± 0.50 | 4.17 ± 0.95 |
|            | L166P 1.49 ± 0.47 | 2.41 ± 1.58 | 0.70 ± 0.19 |
|            | Δexon9 1.43 ± 0.29 | 1.50 ± 0.65 | 0.98 ± 0.27 |
|            | G384A 2.59 ± 0.24 | 3.18 ± 0.80 | 0.83 ± 0.13 |
|            | L286V 3.18 ± 0.02 | 2.05 ± 0.57 | 1.62 ± 0.44 |
|            | A246E 2.13 ± 0.32 | 2.13 ± 0.15 | 1.00 ± 0.08 |

| Aβ48 → Aβ40 | WT 1.07 ± 0.34 | 1.53 ± 0.49 | 0.70 ± 0.05 |
|            | L166P 0.13 ± 0.02 | 1.18 ± 0.23 | 0.12 ± 0.04 |
|            | Δexon9 0.17 ± 0.08 | 1.48 ± 0.42 | 0.11 ± 0.02 |
|            | G384A 0.17 ± 0.06 | 1.20 ± 0.43 | 0.16 ± 0.01 |
|            | L286V 0.22 ± 0.05 | 1.46 ± 0.01 | 0.15 ± 0.04 |
|            | A246E 0.27 ± 0.04 | 2.24 ± 0.20 | 0.12 ± 0.01 |

**FIGURE 6.** FAD-mutant PS1-γ-secretase complexes increase Aβ42/40 independent of effects on e-site endoproteolysis. Aβ42/40 ratios generated in vitro γ-secretase assays with either WT or the indicated FAD-mutant PS1 from a 70:30 mixture of Aβ49/48 (similar to what is observed normally from APP). ***, p < 0.01 (one-way ANOVA and Dunnett’s post test), n = 3; error bars, S.D.

**TABLE 3**

Catalytic efficiencies of trimming of Aβ49 and Aβ48 to Aβ40 and Aβ42 as a percent of WT PS1

Percentages are calculated from the catalytic efficiency values in Table 2. For all of the mutants, the values for each conversion were significantly lower than wild type (\( p < 0.01 \), one-way ANOVA, and Dunnett’s post test). In addition, all mutations resulted in a greater reduction of the trimming of Aβ49 to Aβ40 than Aβ48 to Aβ42 (\( p < 0.05 \), Student’s \( t \) test). For L166P and Δexon9, the conversion of Aβ49 to Aβ40 was more reduced than Aβ49 to Aβ42, and for L286V and Δexon9, the conversion of Aβ48 to Aβ42 was less reduced than Aβ48 to Aβ40 (\( p < 0.05 \), Student’s \( t \) test). Error: S.D.

| Conversion | WT L166P Δexon9 G384A L286V A246E |
|------------|---------|--------|--------|--------|--------|
| Aβ49 → Aβ40 | 100 5 ± 1 2 ± 1 7 ± 1 4 ± 2 9 ± 1 |
| Aβ49 → Aβ42 | 100 8 ± 1 7 ± 3 7 ± 3 6 ± 2 8 ± 2 |
| Aβ48 → Aβ40 | 100 17 ± 1 23 ± 2 21 ± 9 40 ± 2 27 ± 5 |
| Aβ48 → Aβ42 | 100 17 ± 4 16 ± 3 23 ± 2 21 ± 4 18 ± 2 |

**TABLE 4**

Rate of trimming of Aβ46 and Aβ49 to Aβ40 as a percent of WT PS1

The rates of trimming of 1 μM Aβ46 and Aβ49 (ps of Aβ46/h) were measured by ELISA. For both conversions, the values for the mutants were significantly lower than wild type (\( p < 0.01 \), one-way ANOVA and Dunnett’s post test); \( n = 3 \); error: S.D.

| Conversion | WT L166P Δexon9 G384A L286V A246E |
|------------|---------|--------|--------|--------|--------|
| Aβ46 → Aβ40 | 100 1.2 ± 0.2 1.4 ± 0.2 0.7 ± 0.2 2.3 ± 0.2 5.1 ± 1.6 |
| Aβ49 → Aβ40 | 100 3.8 ± 0.7 2.9 ± 0.3 0.8 ± 0.6 3.7 ± 0.5 11.5 ± 3.8 |
strate associated with each FAD mutant alone, independent of ε proteolysis, can indeed increase the Aβ42/40 ratio.

**DISCUSSION**

Our findings have important implications for the normal biochemical function of the γ-secretase complex as well as for the mechanism of pathogenesis of FAD presenilin mutations. First, we demonstrate that the carboxypeptidase activity is an intrinsic function of the enzyme independent of the membrane. Synthetic Aβ peptides of 45–49 amino acids in length were converted to Aβ40 and Aβ42 in a γ-secretase-dependent manner whether the enzyme was isolated from membranes and detergent-solubilized or purified and reconstituted into lipid vesicles. Little or no difference in the ratio of Aβ42/Aβ40 was seen between detergent-solubilized and membrane-incorporated protease complexes. Our results are consistent with the model that Aβ48 and Aβ49, formed upon initial ε endoproteolysis by γ-secretase, are intermediates toward Aβ40 and Aβ42.

Second, our results are completely consistent with the dual-pathway model originally hypothesized by Ihara and co-workers (16, 18) in which Aβ49 → Aβ46 → Aβ43 → Aβ40 and Aβ48 → Aβ45 → Aβ42 → Aβ38. Aβ49 substrate resulted in Aβ40 along with a small level of Aβ42, and Aβ48 gave Aβ42 as well as some Aβ40. Nevertheless, the production of Aβ42 from Aβ49 and Aβ40 from Aβ48 reveals that crossover between the two pathways does occur to some degree, and therefore these crossover pathways contribute to the overall Aβ42-to-Aβ40 ratio. In addition, Aβ46 trimming results in Aβ40 in high preference to Aβ42, and Aβ45 is cleaved to Aβ42 to the virtual exclusion of Aβ40, again consistent with the dual-pathway model. Interestingly, use of Aβ47 as substrate resulted in only small degrees of conversion to either Aβ40 or Aβ42. This peptide, along with its expected trimmed products Aβ44 and Aβ41, is virtually absent in analyses of Aβ from *in vitro* γ-secretase assays, cell culture, or brain tissue. The corresponding tripeptide intermediates have also not been detected (18). Further study of Aβ47 is needed to determine if Aβ44 and Aβ41 are indeed produced as expected.

Most importantly, we uncovered a surprising and striking effect of PS1 FAD mutations on the carboxypeptidase function of the γ-secretase complex. Our biochemical system provided a means to study trimming by these mutant complexes independently of ε proteolysis. All mutant complexes that we examined, located in different regions of PS1 and associated with average ages of onset from 24 to 53 years displayed dramatic reductions in rates of conversion of Aβ49 and Aβ48 to Aβ40 and Aβ42. Most unexpectedly, the rates of Aβ49 conversion to Aβ40 with the PS1 mutants were extremely low, with catalytic efficiencies of 2–9% that of wild-type enzyme. These relative conversion rates of Aβ49 to Aβ40 by the PS1 mutants were much lower than those seen from Aβ48 to Aβ42, which gave catalytic efficiencies ranging from 17 to 40% that of wild-type enzyme. As Aβ49 is the major ε cleavage-product leading to Aβ40 (34), this difference in FAD-mutant PS1-γ-secretase in handling Aβ49 and Aβ48 leads to increased Aβ42/Aβ40, primarily through reduction in Aβ40.

Differences were also revealed in the rates of Aβ49 conversion to Aβ40 and Aβ42, as the crossover conversion of Aβ49 to Aβ42 was not decreased as much as its primary conversion to Aβ40 for two of the PS1 mutations (L166P and Δexon9). Moreover, the crossover conversion of Aβ48 to Aβ40 was decreased more than the major conversion to Aβ42 for two PS1 mutations (L286V and Δexon9). These effects, although minor in comparison to the overall difference in handling between Aβ49 and Aβ48, likewise contribute to net increases in Aβ42/Aβ40. Interestingly, some (34) but not all (35) PS1 mutations have been reported to shift ε cleavage to increase AICD51–99 versus AICD50–99, the other products generated along with Aβ48 and Aβ49, respectively (Fig. 1). Shifting ε cleavage toward Aβ48 in this way would also increase Aβ42/Aβ40. Finally, a new report showed that PS1 FAD mutations can also slow the conversion of Aβ42 to Aβ38 (25). Thus, multiple effects of PS1 FAD mutations all conspire to raise Aβ42/Aβ40.

Despite these multiple effects, the most striking and consistent change is the decreased carboxypeptidase trimming of ε cleavage products Aβ48 and Aβ49 to Aβ40 and Aβ42, most particularly the dramatic reduction in the Aβ49 (or Aβ46) to Aβ40 conversion. This loss, although not complete, is severe and clearly leads to increases in Aβ42/Aβ40 by virtue of reducing Aβ40 formation, providing a simple reconciliation of the loss-of-function versus gain-of-function controversy. These mutations do cause a loss of function: a specific loss of carboxypeptidase function, particularly the ability to trim Aβ49 or Aβ46 to Aβ40. Our findings are consistent with recent reports suggesting that FAD-mutant presenilins can cause a reduction in the conversion of Aβ43 and Aβ42 to Aβ40 and Aβ38, respectively (7, 25). This loss of carboxypeptidase function results in a gain of function; that is, the elevation of the critical Aβ42/Aβ40, thereby increasing the propensity of Aβ to aggregation and neurotoxicity (36). It should be noted that this specific loss of function also elevates longer Aβ peptides (7, 14) and that the gain of neurotoxic function may be through these forms of Aβ. Investigation of this possibility is, therefore, warranted and currently under way.

Acknowledgments—We thank colleague O. Holmes for CHO D1 cells expressing γ-secretase with the inactive PS1 D257A mutation and T. Golde (University of Florida) for γ-secretase modulators GSM-1 and Riv2.

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