Enzymatic Characteristics of I213T Mutant Presenilin-1/γ-Secretase in Cell Models and Knock-in Mouse Brains

FAMILIAL ALZHEIMER DISEASE-LINKED MUTATION IMPAIRS γ-SITE CLEAVAGE OF AMYLOID PRECURSOR PROTEIN C-TERMINAL FRAGMENT β

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Presenilin (PS)/γ-secretase-mediated intramembranous proteolysis of amyloid precursor protein produces amyloid β (Aβ) peptides in which Aβ species of different lengths are generated through multiple cleavages at the γ-, ζ-, and ε-sites. An increased Aβ42/Aβ40 ratio is a common characteristic of most cases of familial Alzheimer disease (FAD)-linked PS mutations. However, the molecular mechanisms underlying amyloid precursor protein proteolysis leading to increased Aβ42/Aβ40 ratios still remain unclear. Here, we report our findings on the enzymatic analysis of γ-secretase derived from I213T mutant PS1-expressing PS1/PS2-deficient (PS−/−) cells and from the brains of I213T mutant PS1 knock-in mice. Kinetics analyses revealed that the FAD mutation reduced de novo Aβ generation, suggesting that mutation impairs the total catalytic rate of γ-secretase. Analysis of each Aβ species revealed that the FAD mutation specifically reduced Aβ40 levels more drastically than Aβ42 levels, leading to an increased Aβ42/Aβ40 ratio. By contrast, the FAD mutation increased the generation of longer Aβ species such as Aβ43, Aβ45, and >Aβ46. These results were confirmed by analyses of γ-secretase derived from I213T knock-in mouse brains, in which the reduction of de novo Aβ generation was mutant allele dose-dependent. Our findings clearly indicate that the mechanism underlying the increased Aβ42/Aβ40 ratio observed in cases of FAD mutations is related to the differential inhibition of γ-site cleavage reactions, in which the reaction producing Aβ40 is subject to more inhibition than that producing Aβ42. Our results also provide novel insight into how enhancing the generation of longer Aβs may contribute to Alzheimer disease onset.

Amyloid β (Aβ) is a hydrophobic peptide that pathologically deposits in the brains of Alzheimer disease (AD) patients. The significant neurotoxicity of oligomeric and/or fibrillar Aβ aggregates indicates that accumulation of Aβ is a central pathogenic event in AD (1). Sequential cleavage of β-amyloid precursor protein (APP) by β- and γ-secretases releases Aβ into the luminal/extracellular space. β-secretase is a membrane-bound aspartic protease (identified as BACE) that cleaves the extracellular region of APP to produce membrane-spanning APP C-terminal fragment β (termed CTFβ or C99) and a N-terminal secreted form of APPβ. The intramembranous region of CTFβ is cleaved next by γ-secretase to produce Aβ and APP intracellular domain, and because of the loose site specificity of γ-secretase-mediated proteolysis, various C-terminal truncated Aβ species, including two major species, Aβ40 and Aβ42, are also generated (1). Aβ40 is the most predominant species of secreted Aβ. Aβ42 is hypothesized to be the trigger species for AD-related amyloid pathophysiology, because it has much faster aggregation potential than Aβ40. Indeed, histochemical and biochemical studies have revealed that Aβ42 primarily deposits within the brains of AD patients and several animal models (2–4). Most importantly, mutations of APP, presenilin-1 (PS1), and PS2 have been identified in familial Alzheimer disease (FAD) and have been found to specifically increase the Aβ42/Aβ40 ratio in cell medium or animal tissues and to accelerate the parenchymal accumulation of Aβ (5–9). In this way, γ-secretase-mediated Aβ metabolism may be critically involved in the onset of AD.

Accumulating evidence strongly indicates that γ-secretase is a high molecular weight membrane protein complex in which PS serves as a catalytic subunit (10). PS proteins are hydrophobic multiple membrane-spanning proteins that become activated through endoproteolysis of its large cytosolic loop domain, producing N- and C-terminal fragments (11–14). The

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†1 The abbreviations used are: Aβ, amyloid β; PS, presenilin; APP, amyloid precursor protein; AD, Alzheimer disease; FAD, familial AD; WT, wild-type; CTFβ, C-terminal fragment β; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MEF, mouse embryonic fibroblast; PIPES, 1,4-piperazinediethanesulfonic acid; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
N- and C-terminal fragments incorporate into the stable high molecular weight complex, which consists of at least three minimum cofactors: nicastrin, APH-1, and PEN-2 (11–13). Two conserved aspartate (Asp259 and Asp385 in PS1) residues, which are located within transmembrane domains 6 and 7, form the predicted active center of the PS/γ-secretase complex (10). Various type I membrane protein substrates, including APP and Notch, undergo regulated intramembranous proteolysis within the hydrophobic lipid bilayer environment (14, 15).

On the basis of this model, we hypothesize that the mechanisms underlying the increased Aβ42/Aβ40 ratio observed in cases of FAD-linked PS mutations might directly reflect an alteration of the enzymatic characteristics of the PS/γ-secretase complex. More than 150 FAD-linked mutations have been identified in PS genes (1). These widely distribute to every mechanism, among individual studies make the study of PS mutations face an important challenge. Nevertheless, experimental differences, such as the variations in cell models, detection systems, and normalization methodology, among individual studies make the study of PS mutations through living cell-based methods a controversial issue (16, 17). Traditionally, γ-secretase was believed to mainly hydrolyze the covalent bonds at Val40–Ile41 and Ala42–Thr43 of CTFβ (termed γ-sites), generating Aβ40 and Aβ42, respectively (1). The γ-sites were thus regarded as the major cleavage sites targeted by γ-secretase. However, recent studies have revealed novel cleavage sites: γ-secretase also mediates the cleavage of ζ- and ε-sites, which are closer to the cytoplasmic membrane boundary (18–20). Cleavage at the ζ- and ε-sites produces various longer Aβ (>43) species, and evidence indicates that these species may be processed to shorter Aβ species in stages (21, 22). Taken together, these findings suggest that γ-secretase-mediated proteolysis consists of multiple complicated cleavage reactions that relate to each other along the transmembrane domain of APP-CTFβ. Nonetheless, it is still unclear how FAD mutations modify overall γ-secretase-mediated cleavage of APP, leading to increased Aβ42/Aβ40 ratios. The molecular mechanisms underlying this process also remain elusive. This crucial issue requires more careful assessment from the aspect of the enzymatic characteristics of the PS/γ-secretase complex.

These findings prompted us to evaluate the activity kinetics of the wild-type (WT) and FAD mutant PS1/γ-secretase enzyme by using a CHAPOS solubilization γ-secretase assay system (23, 24). This strategy has great advantages because it enables us not only to directly assess the effect of mutations on the enzyme in cell models but also in brain tissues, which should better reflect the physiological status of the enzyme in vivo. We previously generated PS1/PS2-deficient cell lines that stably express either the WT or FAD mutant forms of human PS1 (25). Consistent with previous reports (8, 9), we demonstrated that FAD mutations reduced the secretion of Aβ peptides (25). Thus, one possible mechanism underlying increased Aβ42/Aβ40 ratios is that FAD mutations might directly impair the enzymatic activity of γ-secretase, thereby modifying the individual cleavage reactions that produce Aβ40 and Aβ42. In this study, we specifically focused on the effect of the I213T FAD-linked PS1 mutation on γ-secretase and reported the enzymatic characteristics of γ-secretase derived both from cell models and from the brains of knock-in mice.

**Effect of FAD Mutation on Stepwise APP Proteolysis**

**Antibodies**—Anti-PS N-terminal mouse monoclonal antibody (AD3.4, 1:1,000) and anti-PS1 C-terminal rabbit polyclonal antibody (AD3C, 1:1,000) were described previously (25). Rabbit polyclonal antibodies against the C-terminal sequences of APH-1αL (ACS-01, 1:1,000) and PEN-2 (PCS-01, 1:1,000) were generated previously (25). Mouse monoclonal antibody against the N-terminal region of nicastrin was purchased from Chemicon. End-specific antibodies against Aβ N-terminal (82E1, 1:100); Aβ40 (1A10, 1:50); and Aβ42 C-terminal (1:50) were purchased from IBL.

**Plasmid DNA Constructs and Cell Cultures**—PS+/+ and PS1/PS2-deficient (PS/−/−) mouse embryonic fibroblast (MEF) cells (kindly provided by Dr. B. De Strooper, K. U. Leuven and Flanders Interuniversitary Institute for Biotechnology, Belgium) (26, 27) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 μg/ml gentamicin at 37 °C in a 5% CO2 incubator. PS/−/− MEF-based cell lines stably expressing either WT or I213T FAD mutant human PS1 were described previously (25). To establish cell lines that stably express dominant negative mutant D257A human PS1, we generated cDNA by using a conventional two-step PCR protocol and a primer pair for D257A (5′-ATT TCA GTA TAT GCT TTA GTG GCT GTT-3′ and 5′-AAC AGC CAC TAA AGC ATA TAC TG AAT-3′). The cDNA was then subcloned into the EcoRI/XbaI site of a pCIS-BIs vector; the DNA sequence was confirmed with an ABI DNA sequencer. After transfection into PS/−/− MEF cells, several clonal lines were selected in 7.5 μg/ml blasticidin S. All stable cell lines were maintained in growth medium containing 5.0 μg/ml blasticidin S.

I213T Knock-in Mice—We genotyped 11.0–12.5-month-old homozygous (I213TI213T, n = 4), hemizygous (+/I213T, n = 9), and control (+/+, n = 5) I213T mutant PS1 knock-in mice for WT and mutant alleles by PCR, as described previously (28, 29). Fresh whole brains were immediately dissected and used for further biochemical preparations and enzyme assays. All procedures involving animals and their care were approved by the Animal Care Use Committee of the RIKEN Brain Science Institute.

**Preparation of Solubilized γ-Secretase and Recombinant APP-C99-FLAG Substrate**—Semi-confluent cultured cells (60 × 100-mm dish) were washed with phosphate-buffered saline and disrupted in homogenization buffer (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 10% (v/v) glycerol, 5 μg/ml antipain, 5 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mM PMSF) using a Teflon homogenizer (1,200 rpm, 20 strokes). After removal of nuclei and cell debris through two centrifugations (1,500 × g, 4 °C, 10 min), the post-nuclear supernatant was ultracentrifuged (100,000 × g, 4 °C, 1 h). The precipitate was washed with homogenization buffer, ultracentrifuged (100,000 × g, 4 °C, 1 h), and finally collected as crude microsomal membranes. These membranes were then resuspended.
with resuspension buffer (50 mM PIPES-NaOH, pH 7.0, 250 mM sucrose, and 1 mM EGTA), and the amount of protein was quantitated with a BCA kit (Pierce). Protein was adjusted to a final concentration of 10 mg/ml. To prepare enzyme fractions, we added equal amounts of 2× NK buffer (50 mM PIPES-NaOH, pH 7.0, 250 mM sucrose, 1 mM EGTA, 2% (w/v) CHAPSO, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM pH blocker, 20 μg/ml TLCK, 10 mM 1.10-phenanthroline, and 2 mM thiorphan) to the membrane suspension and incubated the mixture for 1 h on ice. After ultracentrifugation (100,000 × g, 4 °C, 1 h), the supernatant containing solubilized γ-secretase was collected and stored at −80 °C.

Baculovirally expressed recombinant C99-FLAG substrate was prepared as described previously (24). Briefly, lysates from C99-FLAG-overexpressing Sf9 cells were extracted in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, was prepared as described previously (24). Briefly, lysates from C99-FLAG substrate at 37 °C. As previous studies mentioned, CHAPSO-free assay buffer (50 mM PIPES-NaOH, pH 7.0, 250 mM sucrose, and 1 mM EDTA, 1.10-phenanthroline, 1 mM thiorphan, and 0.133% (w/v) phosphatidylcholine) and incubated with 0.1–2.5 μM recombinant C99-FLAG substrate at 37 °C. As previous studies mentioned, 0.1% phosphatidylcholine enhances the basal levels of γ-secretase generation without affecting the Aβ42/Aβ40 ratio (24, 30). The final concentration of C99-FLAG stock solution containing Nonidet P-40 in the reactions was roughly estimated to be <0.07%, a concentration that does not noticeably affect de novo Aβ production. To quantify de novo generated Aβs, samples were collected at different time points and excess lipids in the assay mixture were removed by adding CHCl3:MeOH (2:1) and CHCl3:MeOH:MiliIQ (1:2:0.8). After drying, sample pellets were re-dissolved in 1× Sample buffer and then subjected to SDS-PAGE/immunoblot analysis with Aβ N- or C-terminal end-specific antibodies. To detect various longer Aβ species, Aβ1–37 through Aβ1–46 were separated by SDS-PAGE and 8 M urea-containing modified Tris-Tricine gels, and 82E1 antibody was used as a probe (21). All quantitated data were calculated by comparing chemiluminescence signal intensities of Aβ species to synthetic Aβ1–40 or Aβ1–42 standard calibration curves (AnaSpec or Peptide Inc.). Inhibition analyses were performed with two γ-secretase-specific inhibitors, DAPT and WPE-III-31C, purchased from Calbiochem (final dimethyl sulfoxide concentration was <1.0% (v/v)).

**Calculation of Kinetics Constants**—Michaelis-Menten kinetics constants (V\text{max} and K\text{m}) were calculated by fitting experimental data to Lineweaver-Burk plots according to the equation 1/[V] = K\text{m}/V\text{max}[S] + 1/V\text{max}, where [V] is the reaction velocity of Aβ generation, and [S] is the concentration of the APP-C99-FLAG substrate. To calculate the values of V\text{max} and K\text{m}, experimentally measured values of [V] determined from experiments using a range of high C99-FLAG concentrations were used for linear fitting because [V] measured from experiments using low C99-FLAG concentrations contains a large experimental error, which interferes with appropriate linear fitting.

**Statistical Analysis**—Statistical significance of data were tested with a Student’s t test or a Tukey-Kramer test. Data were analyzed with InStat version 3.0a (GraphPad).

**RESULTS**

**Enzymatic Characteristics of Wild-type PS1/γ-Secretase in PS\textsuperscript{−/−} MEF Cell Models**—To characterize γ-secretase activity using a CHAPSO-solubilized assay system, first we solubilized purified crude microsomal membranes from PS\textsuperscript{−/−} MEF, PS\textsuperscript{−/−}/MEF, and WT line 38 (WT38) cells, which stably express endogenous levels of WT human PS1 on a PS\textsuperscript{−/−} MEF background, and incubated these membranes with 500 nM C99-FLAG to detect any Aβ even after 4 h of incubation, thus completely excluding possible PS-independent nonspecific de novo Aβ generation activity due to contaminating proteases. As expected, expression of WT human PS1 significantly restored de novo Aβ generation activity to levels similar to those detected in PS\textsuperscript{−/−} MEF lysates (Fig. 1, A and B). We observed a slightly lower Aβ42/Aβ40 ratio in reaction mixtures containing WT38 lysates compared with those containing PS\textsuperscript{−/−} MEF lysates. This difference might reflect the absence of PS2 or differences between mouse and human PS1 species.

To examine the reaction kinetics of in vitro γ-secretase, next we monitored the time course of Aβ generation. The levels of each Aβ species increased in a linear fashion for up to 5 h, indicating that the initial velocity of the enzyme-substrate reaction was stable during this period (data not shown). The rate of Aβ generation fit to a Michaelis-Menten-like reaction curve (Fig. 1C). V\text{max} values for PS\textsuperscript{−/−} MEF and WT38 lysates were 790.60 ± 37.01 pm/min and 686.80 ± 198.73 pm/min, respectively, and K\text{m} values were 2.08 ± 0.13 μM and 2.26 ± 1.40 μM, respectively (Fig. 1C). These results approximately corresponded to those of previous studies (23, 24, 31) and suggest that the enzymatic characteristics of γ-secretase in both types of cell lysates are not much different. The V\text{max} and K\text{m} values of Aβ40 for PS\textsuperscript{−/−} MEF lysates were 150.99 ± 33.77 pm/min and 0.90 ± 0.30 μM, respectively, and the V\text{max} and K\text{m} values of Aβ40 for WT38 were 124.27 ± 12.68 pm/min and 0.70 ± 0.07 nm, respectively. However, we could not estimate the V\text{max} and
Km of Aβ42 because de novo Aβ42 levels continuously increased with increasing concentrations of C99-FLAG, resulting in a linear fit to the Lineweaver-Burk plot, which did not provide us with appropriate intercept values of the 1/V axis and 1/[S] axis. This difference in kinetics suggests the possibility that Aβ40 and Aβ42 are generated from distinct reactions. As recent studies have mentioned, the Aβ42/Aβ40 ratio gradually increases with increasing C99-FLAG concentrations (24, 32).

Accumulating evidence strongly indicates that PS itself works as the catalytic subunit of the γ-secretase complex. We confirmed this premise by demonstrating that expression of dominant negative human D257A mutant PS1 failed to restore de novo Aβ synthesis in PS−/− MEF cells and that 1% Triton X-100 solubilization, which disrupts the interaction of PS complex components, completely abolished Aβ generation (data not shown). The γ-secretase-specific inhibitors DAPT (data not shown) and WPE-III-31C (Fig. 1D) significantly suppressed the generation of each Aβ species in a dose-dependent manner at IC50 values of ~100 nM DAPT and ~10 nM WPE-III-31C. To evaluate expression levels of PS1 and de novo generation level of Aβ, we analyzed six independent stable cell lines, including the WT38 line (supplemental Fig. 1) (25). After incubation of the membrane fractions with 500 nM C99-FLAG, a linear correlation between PS1 levels and Aβ levels was observed (Fig. 1E), even though the Aβ42/Aβ40 ratio remained unchanged among these cell lines (supplemental Fig. 1C). Taken together, these results indicate that this assay system reflects exactly genuine PS-dependent γ-secretase activity. Thus, in all of the following experiments, we regarded PS1 protein levels as a relative unit of active enzyme.
I213T FAD-linked Mutations Partially Impair γ-Secretase-mediated γ-Site Cleavage—In our previous study, we reported that expression of FAD mutant PS1 in PS−/− MEF cells failed to restore the ability of these cells to secrete Aβ peptides, leading us to propose that FAD mutations might attenuate γ-secretase reactions directly modifying the enzyme characteristics of γ-secretase (25). To test this hypothesis, we assessed the rate that PS1/γ-secretase generated Aβs in cells expressing either WT PS1 or I213T FAD mutant PS1. WT and I213T FAD mutant cell lysates were coincubated with C99-FLAG at 0–2.5 μM concentrations, and the rate of Aβ generation was measured. Cell lysates expressing similar levels of each PS1 variant were chosen in order to minimize experimental error (Fig. 2A), and the relative amounts of Aβ per molecule of WT and I213T FAD mutant PS1/γ-secretase were evaluated after normalization with PS1 protein levels. As shown in Fig. 2B, the I213T mutation moderately attenuated the rate of Aβ generation compared with that of WT. Suppression was specific and significant at high concentrations (2.0–2.5 μM) of C99-FLAG substrate. Moreover, V_{max} of Aβ generation also tended to decrease by 30% (Fig. 2B and C). This suggests that the I213T mutation directly and specifically impaired γ-secretase enzymatic activity responsible for overall Aβ generation.

We further examined the generation rate of both Aβ40 and Aβ42 species. As expected, the I213T mutation drastically attenuated Aβ40 levels compared with WT levels (Fig. 2, B and D) by about 80% at the maximum concentration of C99-FLAG. Although the I213T mutation attenuated Aβ42 levels at C99-FLAG concentrations of 1.5–2.5 μM (Fig. 2, B and D), levels observed at 0.1–1.0 μM C99-FLAG were comparable with those measured from cells expressing the WT enzyme. The reduction of Aβ40 levels was more drastic than that of Aβ42, indicating that the I213T mutation certainly increases the Aβ42/Aβ40 ratio (Fig. 2E). Taken together, these results indicate that the I213T mutation impairs γ-site cleavage reactions affecting both Aβ40 and Aβ42 generation and that differential efficiency in reducing Aβ40 and Aβ42 ultimately results in a higher Aβ42/Aβ40 ratio.

I213T Mutant PS1 Enzyme Facilitates the Generation of Longer Aβ Species—Recent studies have identified various longer Aβ species (>AB43) generated from γ-secretase-mediated α- and ε-site cleavage (21, 33). The results shown in Fig. 2 demon-
strate that the I213T mutation clearly impairs the production of A/β species, affecting the rate at which these A/βs are generated. Nevertheless, the overall levels of A/β produced were not greatly affected by the I213T mutation. This suggests that the mutant enzyme may facilitate the generation of longer A/β species. To address this possibility, we measured the levels of each A/β species in the reaction mixtures by SDS-PAGE of 8M urea-containing modified Tris-Tricine gels, which enabled us to separate various long A/β species by the lengths of their C-terminal amino acid residues (21). We successfully observed PS-dependent de novo generation of not only A/β40 and A/β42 but also of A/β43, A/β45, and >A/β46 species in samples containing WT (line 67) enzyme are shown. conc., concentration. Dots and error bars represent mean values ± S.D. from three individual experiments. C, comparative kinetics profiles of A/β43, A/β45, and >A/β46 generation per molecule of γ-secretase are shown. Dots and error bars represent mean values ± S.D. from three individual experiments. Relative amounts of A/β were evaluated after normalization with PS1 protein levels. The value of WT enzyme with the maximum point was set to equal 1. The statistical significance of the data was tested with a Student’s t test. *, p < 0.05; **, p < 0.01.
we can strictly evaluate the characteristics of native γ-secretase activity without having to consider possible artifacts stemming from overexpression and/or integration of the transgene. To stimulate Aβ generation, we incubated CHAPSO-solubilized fractions containing γ-secretase with 500 nM C99-FLAG for 4 h. We detected de novo Aβ generation in all samples, regardless of genotype; 10 μM WPE-III-31C inhibitor almost completely blocked γ-secretase activity (Fig. 4B). As we observed with the cell models, in I213T PS1 knock-in mice, the I213T mutation significantly reduced Aβ40 levels without obviously affecting Aβ42 levels, leading to higher Aβ42/Aβ40 ratios (Fig. 4, C and D). The mutant enzyme also increased Aβ43 and Aβ45 levels (Fig. 4, E and F). Interestingly, the effects of the I213T mutation on γ-secretase activity were much more pronounced in brain samples from homozygous (I213T/I213T) mice than in samples from hemizygous (+/I213T) mice, suggesting that the effect of the I213T mutation is dose-dependent. These findings strongly support the results obtained from the cell models and suggest that FAD mutations directly lead to abnormal alterations in γ-secretase activity, even in the brain under physiological conditions.

**DISCUSSION**

Although an increased Aβ42/Aβ40 ratio is a common consequence of FAD-linked PS1 mutations, the molecular mechanism underlying this increase still remains controversial (16, 17). Possibly, the increased Aβ42/Aβ40 ratio may result from direct alterations in the cleavage reactions catalyzed by mutant PS/γ-secretase such that Aβ42 generation is facilitated, Aβ40 generation is attenuated, or both Aβ40 and Aβ42 generation change with a different manner. To clarify this crucial issue, we examined the enzymatic characteristics of WT and I213T FAD mutant PS1/γ-secretase in this...
study. First, we observed that de novo Aβ generation depended on PS protein levels, indicating that our assay system reflects genuine PS-dependent γ-secretase activity. Kinetics analyses revealed that the I213T mutation reduced de novo Aβ generation compared with that measured under WT conditions and specifically inhibited the generation of Aβ40 more drastically than of Aβ42. Importantly, these findings were clearly confirmed in experiments of γ-secretase derived from I213T mutant knock-in mouse brains, in which reduction of de novo Aβ generation was mutant allele dose-dependent. We reported previously that expression of FAD mutant PS1 in PS−/− MEF cells failed to restore the ability of these cells to secrete Aβ (25). Here, our findings suggest that the reduced secretion levels of Aβ directly correspond to a reduction in the rate of mutant PS1/γ-secretase-mediated cleavage of γ-sites. Taken together, these observations have led us to conclude that the mechanism underlying the increased Aβ42/Aβ40 ratio observed in cases of FAD mutations most likely has to do with the differential reduction of Aβ40 and Aβ42, in which Aβ40 generation is reduced more than Aβ42 generation. Accordingly, the I213T mutation would be expected to lead to a partial loss of γ-site cleavage, which would affect both Aβ40 and Aβ42 generation.

Besides γ-site cleavage at Val40 and Ile42 of the C terminus, γ-secretase also cleaves APP-CTFβ at two novel sites located closer to the cytoplasmic membrane boundary; these sites are termed ζ-site (Val46 of the C terminus) and ε-site (Thr48 and Leu49 of the C terminus) (18–20). Funamoto et al. (34) demonstrated that cell models expressing Aβ49 or Aβ48 prefer to secrete Aβ40 or Aβ42, respectively. In addition, recent studies identified various longer Aβ species (Aβ43 to Aβ49) and demonstrated that DAPT-treated cells contained elevated Aβ43 and Aβ46 levels but reduced Aβ40 levels (21, 22). On the basis of this evidence, it is hypothesized that Aβ40 and Aβ42 are generated as end-products of the γ-secretase-mediated stepwise cleavage of Aβ49 and Aβ48, which occurs at every third residue along the α-helix structure of Aβ49 and Aβ48 after the ε-cleavage site (21, 22). Interestingly, we observed that in cells expressing I213T mutant γ-secretase, de novo levels of Aβ43 and >Aβ46 species significantly increased in stages in a C99-FLAG concentration-dependent fashion. If all Aβ species are spontaneously generated from Aβ49/Aβ48 through a one-step reaction after ε-site cleavage, then one would expect the rate of Aβ40, Aβ43, and >Aβ46 generation to demonstrate similar reaction curves because these reactions should have similar $K_m$ values. However, in cells expressing WT PS1 γ-secretase, we clearly observed that only Aβ40 levels reached a plateau at C99-FLAG concentrations over 1.0 μM, whereas Aβ43 and >Aβ46 levels increased linearly in parallel with increasing C99-FLAG concentrations. This finding indicates that each Aβ species is generated from stepwise cleavage (Aβ49 → Aβ46 → Aβ43 → Aβ40) rather than spontaneous cleavage. This would suggest that Aβ43 → Aβ40 might be the rate-limiting step of this reaction pathway. Thus, a reasonable explanation for our results is that the I213T mutation specifically inhibits the γ-site cleavage reaction Aβ43 → Aβ40 and disrupts the kinetic balance of pre-γ-site cleavage reactions (Aβ49 → Aβ46 → Aβ43), leading to higher concentrations of longer Aβ intermediates. Our finding that the Aβ43-generating reaction curve of the I213T mutant enzyme clearly reached a plateau, as did the Aβ40-generating reaction, supports this idea well.

The Aβ42-producing pathway remains controversial. In previous work, Qi-Takahara et al. (21) also pointed out that DAPT treatment did not lead to the accumulation of Aβ45 and Aβ48 species, even though it did have a clear inhibitory effect on secreted levels of Aβ42. In the case of PS2/I141I FAD mutant cells, a small accumulation of Aβ45 was observed after DAPT treatment (22). Based on our observations, cleavage reactions of Aβ42 and Aβ45 at C99-FLAG concentrations of >1.5 μM should fit well to stepwise cleavage reactions similar to Aβ48 → Aβ45 → Aβ42. Nevertheless, under lower concentrations of C99-FLAG, we noticed that the I213T mutation constantly facilitated the generation of Aβ45 without affecting Aβ42 levels, suggesting that an additional cleavage pathway that generates Aβ42 may exist. Zhao et al. (33) reported recently that Aβ46 may be converted not only to Aβ43 but also to Aβ42, although not through a major pathway. If this were the case, then one reasonable explanation for our observations is that in cells expressing I213T mutant γ-secretase, the Aβ45 → Aβ42 reaction, which is inhibited, may be partially compensated by the Aβ46 → Aβ42 reaction. In this case, a portion of the Aβ42 pool may be a by-product of a mis cleavage reaction of Aβ46 → Aβ43. Further detailed enzymological assessments will be required to clarify this important issue.

Increasing the Aβ42/Aβ40 ratio would be sufficient to cause parenchymal Aβ accumulation and formation of toxic Aβ oligomers. In our previous work, we observed that certain Aβ42/ Aβ40 ratios accelerate Aβ aggregation and cell toxicity (35). Recently, Kim et al. (28) also reported that luminal/extracellular overproduction of Aβ40 clearly attenuates amyloid pathology in Tg2576 mouse brain (36). These observations indicate that Aβ40 has protective effects against the aggregation of Aβ42. As a previous study demonstrated, formic acid-extracted fractions derived from I213T mutant knock-in mouse brains actually contain enhanced levels of Aβ42. Here, we demonstrated that, even though the I213T mutation impaired γ-site cleavage, Aβ42/Aβ40 ratios in the brains of both hemizygous and homozygous knock-in mice remained elevated compared with that in control mice. Thus, in mutant mouse brains, imbalanced Aβ metabolism would ultimately shift the equilibrium of Aβ aggregation potential to a situation that facilitates the accumulation of Aβ42. If this were indeed the case, one would expect the levels of non-aggregated soluble Aβ in the brains of FAD patients to be partially reduced. Recent studies indicate that Aβ may have physiological functions, such as regulation of synaptic plasticity, neuronal survival, or intracellular lipid metabolism (37–40). Thus, the partial lack of functional Aβ species within brain terminals and/or synaptic terminals may also contribute to part of the neurodegenerative process in FAD patients.

Little evidence has been accumulated on longer Aβ species in AD patients and in other Aβ-related abnormal events, and thus it is still unclear whether longer Aβ species contribute to AD pathophysiology. The majority of longer Aβ species are less efficiently secreted and are detected specifically in low density lipid fractions, as they are partially anchored to the membrane (41). Thus, if these membrane-anchored Aβs start to concen-
trate and form abnormal Aβ clusters on low density lipid domains, it is conceivable that these clusters can act as scaffolds to further the formation of toxic Aβ oligomers and/or fibrillar aggregates, which can disrupt membrane fluidity and/or receptor-mediated signaling functions. In this case, the I213T mutation could specifically enhance the steady state levels of longer Aβ species and accelerate abnormal membrane-related Aβ metabolism. Our preliminary results indicate that, like the I213T mutation, some FAD mutations also facilitate the generation of longer Aβ species. Different compositions of different Aβ species may explain the morphological variations in amyloid plaques reported by some immunohistochemical studies of FAD patient brains (42). To understand the overall Aβ-related cascade leading to the onset of AD, continuous effort will be necessary in the near future to elucidate the detailed molecular mechanisms underlying abnormal, FAD-related Aβ metabolism and γ-secretase function.

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EFFECT OF FAD MUTATION ON STEPWISE APP PROTEOLYSIS