γ-Secretase Associated with Lipid Rafts

MULTIPLE INTERACTIVE PATHWAYS IN THE STEPWISE PROCESSING OF β-CARBOXYL-TERMINAL FRAGMENT*

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Background: Intramembranous cleavages of β-carboxyl-terminal fragment (βCTF) by γ-secretase generate amyloid β-protein (Aβ).

Results: Three- to six-residue peptides are released successively along with Aβ generation by lipid raft-associated γ-secretase.

Conclusion: γ-Secretase cleaves βCTF through multiple interactive pathways for stepwise successive processing to generate Aβ.

Significance: This cleavage model provides insights into the precise molecular mechanism of Aβ generation.

γ-Secretase generates amyloid β-protein (Aβ), a pathogenic molecule in Alzheimer disease, through the intramembrane cleavage of the β-carboxyl-terminal fragment (βCTF) of β-amyloid precursor protein. We previously showed the framework of the γ-secretase cleavage, i.e. the stepwise successive processing of βCTF at every three (or four) amino acids. However, the membrane integrity of γ-secretase was not taken into consideration because of the use of the 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid-solubilized reconstituted γ-secretase system. Here, we sought to address how the membrane-integrated γ-secretase cleaves βCTF by using γ-secretase associated with lipid rafts. Quantitative analyses using liquid chromatography-tandem mass spectrometry of the βCTF transmembrane domain-derived peptides released along with Aβ generation revealed that the raft-associated γ-secretase cleaves βCTF in a stepwise sequential manner, but novel penta- and hexapeptides as well as tri- and tetrapeptides are released. The cropping of these peptides links the two major tripeptide-clearing pathways generating Aβ40 and Aβ42 at several points, implying that there are multiple interactive pathways for the stepwise cleavages of βCTF. It should be noted that Aβ38 and Aβ43 are generated through three routes, and γ-secretase modulator 1 enhances all the three routes generating Aβ38, which results in decreases in Aβ42 and Aβ43 and an increase in Aβ38. These observations indicate that multiple interactive pathways for stepwise successive processing by γ-secretase define the species and quantity of Aβ produced.

γ-Secretase is a membrane-embedded multimeric high molecular mass aspartic protease that determines the molecular species of amyloid β-protein (Aβ),3 a pathogenic molecule in Alzheimer disease (AD) (1). It cleaves β-carboxyl-terminal fragment (βCTF) of β-amyloid precursor protein (APP) in the middle of the membrane and releases Aβ and APP intracellular domain (AICD). Many familial AD (FAD)-associated mutations are found in presenilin (PS) 1/2, the catalytic subunit of γ-secretase (2). Those mutations appear to modulate the activities of γ-secretase, leading to qualitatively and/or quantitatively altered generation of Aβ species (3). Thus, how γ-secretase cleaves βCTF is a critical issue in the pathogenesis of AD. In fact, a number of clinical and preclinical AD therapeutic trials targeting Aβ and/or γ-secretase are ongoing based on the amyloid theory (4).

γ-Secretase cleaves the substrate within its transmembrane domain (TMD), i.e. the protein hydrolysis typically occurs in the hydrophobic environment of the lipid bilayer. Because of the unavailability of water molecules within the membrane, the cleavage that occurs within the membrane has remained an enigma ever since the identification of APP (5). However, advanced structural analyses of γ-secretase with cryo-electron

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3 The abbreviations used are: Aβ, amyloid β-protein; AD, Alzheimer disease; APP, β-amyloid precursor protein; βCTF, β-carboxyl-terminal fragment; AICD, APP intracellular domain; PS, presenilin; FAD, familial Alzheimer disease; TMD, transmembrane domain; DRM, detergent-resistant membrane; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; GSM, γ-secretase modulator; L-685,458, [1S-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; 5(3,5-difluorophenacetyl)-L-alanyl]-2-phenylethylcarboxyimido-15-3-methylbutylcarbamoyl)-2R-hydroxy-5-phénylpentyl]-carbamic acid tert-butyl ester; DAPT, N-(3,5-difluorophenacetyl)-L- alanly]-5-phenylglycine t-butyl ester; IP, immunoprecipitation.
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microscopy and the substituted cysteine method have provided a plausible explanation; γ-secretase itself provides the hydrophilic environment required for substrate cleavage by generating a water-accessible cavity surrounded by multiple transmembrane segments of its own components (1, 6), as demonstrated in another intramembrane-cleaving protease, site-2 protease (7). This view is supported by a recent study of the crystal structure of a PS/signal peptide peptidase homologue (8).

The underlying molecular mechanism of the cleavage within the membrane is another important issue in the context of developing a disease-specific therapeutic reagent. We have been concerned about the molecular mechanisms regarding developing a disease-specific therapeutic reagent. We have proposed the stepwise successive cleavage model for Aβ generation (11). The liquid chromatography-tandem mass spectrometry (LC-MS/MS) identification of βCTF-derived tri- (and tetra-) peptides generated along with Aβ has lent strong support to the model (13). In this model, the initial ε-cleavages are followed sequentially by cleavages after every three (or four) residues, releasing Aβ40 and Aβ42 (Aβ38) as the final products. There are two product lines as follows: Aβ49 > Aβ46 > Aβ43 > Aβ40 and Aβ48 > Aβ45 > Aβ42 > Aβ38. Similarly spaced residues with intramembrane cleavage have been identified in tumor necrosis factor-α-secretase within the membrane (9–12) and proposed the stepwise successive cleavage model (15). These observations indicate that the stepwise cleavage mechanism may be a characteristic of intramembrane proteolysis.

As the previous study to identify the released oligopeptides was carried out using CHAPSO-solubilized γ-secretase (13), the mechanism underlying intramembrane cleavage by γ-secretase remains to be clarified. Now it is known that some previous observations do not agree with the proposed model. For example, the different molecular species of Aβ and their corresponding AICDs are not produced in a one-to-one ratio (9). Cell-based expression of Aβ48 generates both Aβ42 and Aβ40 even if there is a preference for Aβ42 generation (10). These observations raise the possibility that there may be additional unidentified processing pathways for Aβ generation. Here, to address these issues, we sought to verify the stepwise cleavage model for Aβ generation using lipid raft membranes (16), where active γ-secretase is known to reside (17).

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The antibodies against Aβ used here were 82E1 (IBL), 6E10 (Covance), polyclonal antibodies specific for Aβ40, Aβ42, or Aβ43 (IBL), and a monoclonal antibody specific for Aβ38 (IBL). Antibodies against nicastrin and Pen-2 were from Sigma and Oncogene Science, respectively. The monoclonal antibodies against caveolin, flotillin, and calnexin were purchased from BD Transduction Laboratories. Anti-FLAG M2 monoclonal antibody was from Sigma.

**Membrane Preparation**—A microsomal fraction was prepared as described previously (11). Briefly, harvested CHO cells or cortices from 4-week-old Wistar rats were homogenized in buffer A (20 mM PIPES, pH 7.0, 140 mM KCl, 0.25 mM sucrose, 5 mM EGTA) containing various protease inhibitors. Following brief centrifugation at 800 × g for 10 min, the resulting postnuclear supernatants were centrifuged at 100,000 × g for 1 h. The pellets containing the total membrane fraction were suspended in buffer C (50 mM PIPES, pH 7.0, 0.25 mM sucrose, 1 mM EGTA).

Lipid rafts were obtained as the detergent-resistant membranes (DRMs) as described (17) with some modifications. The pellets containing the total membrane fraction were homogenized in 10% sucrose in MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl) containing 1% CHAPSO and various protease inhibitors. The homogenate was adjusted to 40% sucrose, placed at the bottom of an ultracentrifuge tube, and overlayed with 35% and then 5% sucrose in MES-buffered saline. The discontinuous gradient was centrifuged at 39,000 rpm for 20 h at 4 °C on an SW41 Ti rotor (Beckman). Lipid rafts accumulating at the 5–35% sucrose interface were carefully collected and resuspended in buffer C.

Reconstituted γ-Secretase Assay—βCTF (C99) tagged with FLAG at the carboxyl terminus (C99-FLAG) was prepared from Sf9 cells basically as described (18). Briefly, Sf9 cells were infected with recombinant baculovirus and cultured in the presence of 20 mM GM6001 to suppress α-secretase-like cleavage of C99-FLAG. C99-FLAG overexpressed in Sf9 cells was solubilized with the lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor mixture (Roche Applied Science)) and then immunopurified with anti-FLAG® M2-agarose beads (Sigma). The quantity and purity of the obtained C99-FLAG were assessed by gel electrophoresis and Coomassie Brilliant Blue staining.

The reaction mixture contained DRMs at a protein concentration of 80 μg/ml from CHO cells or 100 μg/ml from rat brains, defined amounts of C99-FLAG, and 0.25% CHAPSO in buffer C supplemented with protease inhibitor mixture (0.5 mM diisopropyl fluorophosphate, 1 μg/ml N-[tosyl-L-lysyl chloromethyl ketone, 10 μg/ml antipain, 10 μg/ml leupeptin, 1 mM thiorphan, 100 μM bestatin, 10 μM amastatin, 0.1 μM amastatin, and 5 mM EDTA). This mixture was incubated at 37 °C for the indicated times, and the reaction was stopped by placing the reaction mixture on ice.

To assess γ-secretase activities in the total membrane fraction, the pellets containing the total membrane fraction were resuspended in buffer C to give a final protein concentration of 4.0 mg/ml. An equal volume of buffer C containing 2% CHAPSO and protease inhibitors (1 mM diisopropyl fluorophosphate, 2 μg/ml N-[tosyl-L-lysyl chloromethyl ketone, 20 μg/ml antipain, 20 μg/ml leupeptin, and 2 mM thiorphan) was added, and the fraction was kept on ice for 1 h. The fraction was diluted in 3 volumes of buffer C containing protease inhibitors (0.5 mM diisopropyl fluorophosphate, 1 μg/ml N-[tosyl-L-lysyl chloromethyl ketone, 10 μg/ml antipain, 10 μg/ml leupeptin, 1 mM thiorphan, 133 μM bestatin, 13.3 μM amastatin, 0.13 μM amastatin, and 6.7 mM EDTA) and defined amounts of C99-FLAG, and then the mixture was incubated at 37 °C for the indicated times. The experiments to compare γ-secretase activities between total membranes and lipid rafts were performed within a linear range of Aβ generation according to the concen-
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- The effect of the substrate and the protein concentration in the membrane fraction.

Aliquots from the reaction mixture were transferred into the SDS sample buffer and subjected to quantitative Western blotting. The remaining part of the reaction mixture was mixed with trichloroacetic acid and kept on ice for 15 min. The suspension was centrifuged at 100,000 × g, and the supernatant was filtered through a PVDF or nitrocellulose membrane (0.22-μm pore size, Merck). The filtrate was injected into an HP1100 (Hewlett-Packard) system equipped with an STR ODS-II column (4.6 × 150 mm, Shinwa Chemical Industries) to be concentrated. The column was washed with 0% B for 7 min, and the peptides were eluted as a mixture by using a steep 0–90% B gradient for 0.1 min (A, 0.05% formic acid; B, 0.05% formic acid in 100% acetonitrile). The eluate was subjected to LC-MS/MS analyses.

Western Blotting and Quantification — The proteins were separated on either a 16.5% conventional Tris/Tricine gel or an 11% Tris/Tricine long gel (20 cm length) containing 8M urea, separated on either a 16.5% conventional Tris/Tricine gel or an 11% Tris/Tricine long gel (20 cm length) containing 8M urea, and the peptides were eluted as a mixture by using a steep 0–90% B gradient for 0.1 min (A, 0.05% formic acid; B, 0.05% formic acid in 100% acetonitrile). The eluate was subjected to LC-MS/MS analyses.

LC-MS/MS Quantification of Tripeptides and Other Oligopeptides — The quantification of the expected peptides by LC-MS/MS was performed as described previously (13). A Quattro Premier™ XE tandem quadrupole mass spectrometer accompanied by ultra-performance liquid chromatography (Waters) equipped with a column (ACQUITY UPLC® HSS T3, 1.8 μm, 2.0 × 150 mm) was used to identify and quantify the tripeptides and other oligopeptides. To quantify each analyte, the precursor ion-product ion pairs were monitored using the device’s multiple-reaction monitoring mode as follows: m/z = 304.1 and 185.0 for IAT; 330.1 and 185.0 for VIV; 346.1 and 215.1 for ITL; 332.1 and 173.0 for TVI; and 332.1 and 185.0 for VIT. The precursor and product m/z values used for other peptides were as follows: MVG, 305.9 and 202.9; VGG, 231.9 and 156.9; GGV, 232.1 and 117.8; GVV, 274.1 and 128.9; VVI, 330.1 and 199.1; VIA, 302.1 and 185.0; ATV, 290.1 and 173.0; IVI, 344.1 and 213.1; TLV, 332.1 and 187.1; LVM, 362.1 and 213.1; GGVV, 331.1 and 213.9; GVVI, 387.1 and 231.1; VIA, 401.3 and 203.0; VIAT, 403.5 and 184.9; IATV, 403.0 and 219.0; ATV, 403.3 and 254.1; TVIV, 431.1 and 231.0; VIVI, 443.1 and 231.0; IVIT, 444.9 and 233.0; VITL, 444.9 and 232.9; GVIV, 458.0 and 256.0; VIATV, 502.7 and 199.1; VIAT, 502.0 and 185.0; IATVI, 516.0 and 173.0; ATTV, 502.0 and 254.0; TVIV, 544.0 and 200.9; VIVI, 544.0 and 185.0; IVITL, 558.2 and 213.1; IATIV, 615.2 and 268.0; VIVITL, 657.3 and 184.9. FLF (m/z = 426.3 and 261.2) was used as an internal standard. Standard curves for peptides were obtained within a linear range from 1.5 to 100 fmol.

Mass Spectrometric Analyses of Shorter Aβ Species and AICDs — After 3 h of incubation of the reconstituted Aβ generation system, the produced Aβ was immunoprecipitated with 6E10 and protein G-coupled to Sepharose beads. The produced AICD was immunoprecipitated subsequently with anti-FLAG® M2-agarose beads. Aβ and AICD bound to the recovered beads were eluted with 30% acetonitrile in 1% trifluoroacetic acid. Molecular masses of the peptides were determined with a matrix-assisted laser desorption ionization-TOF-mass spectrometer, 4800 Plus MALDI TOF/TOF™ analyzer (AB SCIEX) using α-cyano-4-hydroxycinnamic acid as a matrix (18).

Other Methods and Reagents — The γ-secretase inhibitors [15-benzyl-4R-(1S-carbamoyl-2-phenylethylcarbamoyl-1S-3-methylbutylcarbamoyl)-2R-hydroxy-5-phenylpentyl] carbamic acid tert-butyl ester (L-685,458) (19) and N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester (DAPT) (20) were purchased from Calbiochem. γ-Secretase modulator 1 (GSM-1) was synthesized (21). Tripeptides and other oligopeptides were custom-made by the Peptide Institute. Their quantities were determined by amino acid analysis (Peptide Institute). Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce).

RESULTS

Generation of Aβ and AICD by Lipid Rafts — Our previous study showed that lipid rafts contain all four components (PS1/2, nicastrin, Aph-1, and Pen-2) required for the active γ-secretase complex and exhibit higher γ-secretase activity in the membrane-based cell-free Aβ generation system using an endogenous substrate (17). This indicates that lipid rafts are one of the sites involved in Aβ generation within the cell. The following studies provided several lines of evidence for this assumption (22, 23). In addition, the lipid composition of the rafts is favorable for γ-secretase activities (24). Thus, we decided to use lipid rafts as a model system for the intramembrane cleavage of βCTF by γ-secretase.

Lipid rafts were prepared from CHO cells by sucrose density gradient centrifugation in the presence of 1% CHAPSO. The raft fraction was determined by its flotation, the presence of flotillin (a lipid raft marker), and the absence of calnexin (a non-DRM marker). The majority of mature nicastrin, Pen-2, and the carboxyl-terminal fragment of PS were fractionated into the lipid raft fraction (Fig. 1A) (17).

An in vitro reconstituted γ-secretase assay was performed using lipid rafts together with 0.25 μM C99-FLAG that was purified from Sf9 cells. γ-Secretase activity was assessed by the amounts of Aβ generated. Although only small amounts of proteins were recovered, the raft fraction yielded specific γ-secretase activity 10-fold higher than that of the total membrane-associated γ-secretase (Fig. 1B). Aβ was produced in a time-dependent manner by incubation at 37 °C (Fig. 2, A and B). The production of Aβ40 and Aβ42 proceeded in a similar profile (Fig. 2, A and C). L-685,458 completely suppressed the Aβ production, indicating that it was mediated by γ-secretase. The molecular species of Aβ produced were examined by a long SDS-urea gel (Fig. 2D). The most robustly produced was Aβ40, followed by Aβ42. In addition to Aβ40 and Aβ42, significant amounts of Aβ43 and small amounts of Aβ45 were detected. A weak signal migrating at the Aβ46 position...
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A

![Graph showing preparation of lipid rafts and their Aβ generation activity.](image)

B

![Graph showing generation of Aβ by raft-associated γ-secretase.](image)

The analysis of the produced AICD, a counterpart of Aβ, by immunoprecipitation (IP)/TOF-mass spectrometry clearly showed that raft-associated γ-secretase generated the two counterparts, AICD(50–99) and AICD(49–99) (Fig. 2F), as expected. Other longer or shorter AICDs were undetectable or below the detection limit. Thus, the molecular species of Aβ and AICD produced by γ-secretase associated with lipid rafts in vitro were overall indistinguishable from those produced by CHAPSO-solubilized γ-secretase (13, 18).

Release of the “Predicted” Five Triptides and One Tetrapeptide by Raft-associated γ-Secretase—To learn how βCTF is cleaved by γ-secretase within membranes, we first examined using LC-MS/MS whether the same five triptides and one tetrapeptide released in the CHAPSO-solubilized system in a stepwise manner (13) were produced by raft-associated γ-secretase. As shown in Fig. 3 B, five triptides, IAT, VIV, ITL, TVI, and VIT, were released concomitantly with Aβ generation. Their levels increased linearly in a time-dependent manner. The levels of VVIA, a tetrapeptide, also increased in a time-dependent manner, although its amount was much smaller than those of the five triptides (~1/5 that of TVI and ~1/9 that of ITL). The generation of all six peptides was completely suppressed by the addition of L-685,458 at 1 μM (data not shown). Consistent with our model, the quantitative relationships IAT < VIV < ITL and VVIA ≪ TVI < VIT, as seen previously in the CHAPSO-solubilized system (13), were invariably maintained. These results indicate that raft-associated γ-secretase cleaves the intramembrane region of βCTF in a stepwise successive manner at every three or four residue to generate Aβ40 and Aβ42 (and then Aβ38).

According to the stepwise cleavage model, the differences between the amounts of the successively released peptides determine the amounts of the Aβ species produced (13); it is assumed that Aβ40 = IAT (when further cleavage does not occur) and Aβ42 = TVI − VVIA (Fig. 3A). Thus, the amounts of Aβ40 and Aβ42 calculated from the released peptide quantified by LC-MS/MS were compared with those Aβ levels quantified by Western blotting using end-specific Aβ antibodies to further test the cleavage model. These two Aβ measures assessed by two different methodologies were roughly consistent (Fig. 3C).

Generation of Other Triptides, Tetrapeptides, and Pentapeptides Indicates Multiple Pathways for Stepwise Processing—As lipid rafts exhibited extremely high γ-secretase activity for Aβ generation, we searched, using LC-MS/MS, more extensively and systematically for βCTF TMD-derived oligopeptides in addition to the six peptides already found.

Sixteen oligopeptides out of 29 peptides examined were released in a time-dependent manner besides the six peptides (Fig. 4A). Surprisingly, the levels of VIVI, VVIAT, and VIVIT increased to almost the same extent as VVIA. Of the peptides...
A, Western blotting with 82E1 (total Aβ) and Aβ40- and Aβ42-specific antibodies. Aβ peptide standards were applied onto each rightmost lane. B and C, quantification of the signal intensities for total Aβ (closed circles in B), Aβ40 (closed squares in C), and Aβ42 (open triangles in C) on the blots, which were expressed as picomoles of Aβ generated per ml of the reaction mixture. Data are represented as means ± S.E. from four independent experiments. D, Western blotting with 82E1 using a long SDS-urea gel. Authentic Aβ species from Aβ37 to Aβ49 were loaded onto the rightmost three lanes (M1: Aβ37, Aβ38, Aβ40, Aβ42, Aβ43, and Aβ46 to Aβ49; M2: Aβ39, Aβ41, and Aβ44). In this gel system, Aβ46 and longer Aβ species (Aβ47 to Aβ49) were not separated. Aβ40 was robustly produced by lipid rafts. Aβ38, Aβ42, Aβ43, Aβ45, and Aβ46 and longer species were also produced in a time-dependent manner. Asterisks in D indicate C99-FLAG or its carboxyl-terminally truncated fragments. E, IP/TOF-mass spectrometry for shorter Aβ species. An aliquot of the reaction mixture was subjected to Western blotting with 82E1 (upper panel). Aβ in the reaction mixture was immunoprecipitated with 6E10 and subjected to TOF-mass spectrometry (lower panel). Among the candidate Aβs, only Aβ1–16 and Aβ1–15 were identified after incubation. However, L-685,458 at 2 µM suppressed Aβ generation almost completely (upper panel) but not generation of the shorter Aβs, indicating that the generation of Aβ1–16 and Aβ1–15 was not mediated by γ-secretase (26). Aβ(1–17) and longer species were undetectable in this system. F, IP/TOF-mass spectrometry for AICDs. An aliquot of the reaction mixture was subjected to Western blotting with FLAG antibody (upper panel). AICD produced was subjected to IP/TOF mass spectrometry (lower panel). The signals corresponding to AICD(50–99) and AICD(49–99) were identified after incubation. They were suppressed by L-685,458 at 2 µM, indicating γ-secretase-mediated generation.

FIGURE 2. Aβ and AICD species generated by the reconstituted lipid raft Aβ generation system. Lipid rafts prepared from CHO cells were subjected to the reconstituted Aβ generation assay. Following incubation for the indicated times, the reaction mixtures were subjected to either Western blotting or IP/TOF mass spectrometry.
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A schematic illustration of the γ-secretase-mediated stepwise cleavages of βCTF, for which the results from the CHAPS-solubilized system (13) are shown. Following e-cleavages that generate Aβ49 and Aβ48 and their counterparts AICD(50–99) and AICD(49–99), respectively, γ-secretase successively cleaves Aβ49 and Aβ48 in the direction from the e- to the γ-cleavage sites by releasing tripeptides and finally produces Aβ40 and Aβ42, respectively. A small fraction of Aβ42 is further converted to Aβ38 by the release of a tetrapeptide. According to the model, the quantitative relationships among the peptides, IAT < VIV < ITL and VVIA < TVI < VIT, should be maintained. The differences between the successively generated tripeptides determine the amounts of the different Aβ species produced. B, quantification by LC-MS/MS of the predicted five tripeptides and one tetrapeptide released in the reaction mixture of the lipid raft Aβ generation system. The release of three tripeptides (IAT, VIV, and ITL) from the Aβ40 product line and two tripeptides (TVI and VIT) and a tetrapeptide (VVIA) from the Aβ42 product line proceeded in a time-dependent manner (p < 0.01, Spearman’s rank correlation). Data are represented as means ± S.E. from four independent experiments. Note that the quantitative relationships ITL > VIV > IAT and VIT > TVI >> VVIA were maintained at every time point. C, quantitative comparison of Aβ levels between Western blotting and LC-MS/MS. The reaction mixture was divided into two halves. One-half was not incubated; the other was incubated for 60 min. One-half of the reaction mixture with or without incubation was subjected to Western blot analyses using Aβ species-specific antibodies (closed columns). The other half of the reaction mixture was subjected to LC-MS/MS analyses, and the levels of Aβ species were assessed from the peptide amounts based on the stepwise processing model (open columns). Data are represented as means ± S.E. from five independent experiments. Note that there are no significant differences between the Western blotting and LC-MS/MS results (p = 0.5924, 0.2582, 0.5482, and 0.4089 for Aβ40, Aβ42, Aβ43, and Aβ38, respectively, Student’s t test).

examined, only those three increased prominently. This indicates that γ-secretase releases significant amounts of pentapeptides in addition to tri- and tetrapeptides. The levels of GVV, VVI, VIA, IVI, TLV, ATVI, and IVIT increased significantly in a time-dependent manner (Fig. 4A). ATV, LVM, TVIV, VITL, GVVI, and VIVITL increased slightly but significantly. Their release was almost completely suppressed by 1 μM L-685,458 (data not shown), indicating that their generation is mediated by γ-secretase. Thus, Aβ generation by raft-associated γ-secretase accompanied the release of many three- to six-residue peptides in addition to the above six peptides (Fig. 4B). There should be novel routes by which Aβ48 and Aβ47 are processed to Aβ43, releasing a pentapeptide VIVIT and a tetrapeptide VVI, respectively, and by which Aβ43 is processed to Aβ38, releasing a pentapeptide VVIAT (Fig. 4B). The presence of small amounts of the hexapeptide VIVITL suggests that the cleavage at every three residues is rarely skipped.

Overall, there are three routes to generate Aβ43, i.e. those via Aβ46, Aβ47, and Aβ48 (see Figs. 4B and 9). The comparison of the amounts of the released peptides (VIV, VVI, and VIVIT) indicates that the routes via Aβ46, Aβ47, and Aβ48 contribute to Aβ43 generation in the ratio 50:6:~5. There are also three routes by which to generate Aβ38. Most Aβ38 is generated either from Aβ42 (~50%) or Aβ43 (~36%) by the release of a tetrapeptide (VVIA) or a pentapeptide (VVIAT), respectively. A minor fraction of Aβ38 could be generated from Aβ41 (~14%) by the release of a tripeptide (VVI).

To validate the above assumption for multiple cleavage pathways by γ-secretase, the levels of Aβ38 and Aβ43 quantified by Western blotting were compared with those calculated from peptide amounts quantified by LC-MS/MS. There are two routes processing Aβ43 (see Figs. 4B and 9). Thus, it is assumed that Aβ43 = (VIV + VVI + VIVIT) – (IAT + VVIAT) and Aβ38 = VVIAT + VVIA + VVI. The results show that the measures obtained from the two methodologies are roughly consistent (Fig. 3C).

Notably, the release of VIVIT cross-links the Aβ40 and Aβ42 product lines at the Aβ43 level, making the two lines interactive (see Fig. 4B). The release of VVIAT also links the Aβ40 product line to Aβ38 generation. The minor release of a tetrapeptide makes it possible to link the Aβ product lines. Ultimately, the clipping of these oligopeptides permits production of multiple alternative pathways for stepwise cleavages, linking the Aβ product lines in an interactive manner.

Uniform Suppression of Peptide Release by γ-Secretase Inhibitors—L-685,458, a transition state analog inhibitor, uniformly suppressed all the Aβ species and peptides generated by the raft-based γ-secretase in a dose-dependent manner, as expected (data not shown). DAPT, a nontransition state analog inhibitor, similarly suppressed Aβ generation (Fig. 5). Unexpectedly, there were no accumulations of Aβ43 and Aβ46 as observed with the cell system (Fig. 5A) (11). The release of the major and minor peptides was also uniformly suppressed, but
VIA and VIVITL did not exhibit a significant decrease (Fig. 5, B and C). Sulfonamide, which induced a build-up of A
H9252
43 and A
H9252
46 within the cells (28), also failed to duplicate in this system (data not shown). It is likely that an intact cell system is required for the accumulation of longer A
H9252
species by DAPT, because significant DAPT-induced accumulations of longer A
H9252
species were not observed even by use of isolated membranes.

**GSM-1 Affects Multiple A
H9252
38-generating Pathways**—GSM, a compound that suppresses A
H9252
42 generation without affecting the total A
H9252
amount generated, would be beneficial for therapeutic development, because it could avoid the adverse effects elicited through other A
H9252
-secretase substrates, especially Notch (1). According to the stepwise processing model, decreased A
H9252
42 and increased A
H9252
38 levels caused by nonsteroidal anti-inflammatory drugs could be explained by an enhancement of the final (fourth) processing step of the A
H9252
42 product line, i.e. the increased release of VVIA (13). However, the effect may not be so straightforward, because three routes generating A
H9252
38 were found here. Thus, we sought to clarify which route among the three was affected with GSM-1.

GSM-1 caused a dose-dependent decrease in the A
H9252
42 levels and a reciprocal increase in the A
H9252
38 levels without affecting the total A
H9252
levels (Fig. 6A). The A
H9252
40 levels were almost constant up to 1
M
 of GSM-1 and then declined a little. Peptide analyses by LC-MS/MS showed that although the levels of all five major tripeptides were not altered, the levels of VVIA increased remarkably (2.4-fold increase at 2.5
M
 of GSM-1) in a dose-dependent manner (Fig. 6B). Additionally, the penta-peptide VVIAT and the tripeptide VVI increased in a dose-dependent manner remarkably (2.4-fold) and moderately (1.8-fold), respectively (Fig. 6C). The increases in the amounts of the released peptides VVIA, VVIAT, and VVI are in the ratio 7:4:1. These results indicate that GSM-1 enhances the cleavage step not only from A
H9252
42 to A
H9252
38 but also from A
H9252
43 to A
H9252
38 and to a lesser extent from A
H9252
41 to A
H9252
38.
A treatment with GSM-1, which may reflect a slight decrease in did not alter (13), but the levels of GVV tended to increase upon possible that the post-mortem time affects the activity of found in the CHO raft are also at work in the brain raft. As it is well (29), we next examined whether the cleavage pathways was produced in a time-dependent manner. Both A \beta 41 levels were not evaluated because they were under the detection limits on the SDS-urea gel, and a specific antibody was not available.

Regarding the putative A \beta 40 product line, the levels of IAT did not alter (13), but the levels of GVV tended to increase upon treatment with GSM-1 (Fig. 6A). The A \beta 41 levels were not evaluated because they were under the detection limits on the SDS-urea gel, and a specific antibody was not available.

A \beta was suppressed almost completely by L-685,458. The LC-MS/MS analyses of oligopeptides released during incubation showed that 15 oligopeptides out of 22 peptides generated by the CHO raft were released by the brain raft in a time-dependent manner (Fig. 7B). These included two pentapeptides and one hexapeptide observed in the CHO raft. The relative quantitative relationships among the peptides were very similar in the CHO and brain rafts, except for IVI and VVIAT (Figs. 4A and 7C), the amounts of which were smaller in the brain raft. The most abundant were the original five tripeptides, followed by VVIA (Fig. 7B). Quantification showed that the amounts increased mostly in the order IAT = VIV < ITL and VVIA << TVI < VIT. These results indicate that the two major stepwise cleavage pathways, which generate A \beta 40 and A \beta 42 (A \beta 38), respectively, are conserved in brain rafts as well. The peptides that were generated to lesser extents by the CHO raft were not found in the brain raft, probably because of the detection limit. This is because the CHO raft exhibited much higher \gamma-secretase activity than the brain raft (Figs. 3, 4, and 7). Thus, the cleavage mechanisms appear to be largely shared by the CHO and brain rafts, and the unusual oligopeptides such as penta- and hexapeptides are detectable in both systems.

Treatment with L-685,458 suppressed the release of the five major tripeptides and of other minor oligopeptides (Fig. 8A). The suppression was uniform and dose-dependent. DAPT also
suppressed the generation of all the peptides uniformly and in a dose-dependent manner (Fig. 8B). Again, we were unable to observe transient accumulations of longer $\alpha\beta_{9252}$ species in brain rafts.

We next examined to what extent GSM-1 affects intramembrane cleavages of $\alpha\beta_{9253}$CTF by brain raft-associated $\alpha\beta$-secretase (Fig. 8C). The LC-MS/MS analyses of the released peptides showed a dose-dependent increase in VVIA and VVIAT, as observed in the CHO raft, whereas VVI did not increase. The levels of all other peptides stayed the same. The increase in VVIAT was remarkable, being almost equivalent to that in VVIA. These results indicate that GSM-1 causes an increase in $\alpha\beta_{9253}$ levels largely through an enhancement of both the $\alpha\beta_{9254}$-to-$\alpha\beta_{9253}$ and $\alpha\beta_{9255}$-to-$\alpha\beta_{9253}$ cleavage steps in brain rafts.

**DISCUSSION**

In this study, we have shown using a lipid raft system that the intramembranous cleavage of $\beta$CTF by membrane-associated $\gamma$-secretase proceeds in a stepwise successive manner similar to that observed with $\gamma$-secretase in a CHAPSO-solubilized form.
**Multiple Pathways of Stepwise βCTF Processing by γ-Secretase**

![Graphs and Figures]

(13). However, the stepwise cleavage occurs not only at every three residues but also at four or five residues, and even cleavage at six residues was observed (Fig. 9). Thus, the cropping of these oligopeptides links the two major Aβ product lines identified previously (13) at several points, leading to multiple interactive pathways for the stepwise cleavages.

It is possible that the integration of γ-secretase in the raft membrane allows it to maintain its conformation adequately for exhibiting higher activity. If so, this could cause qualitative differences in the cleavages between the raft system and the CHAPSO-solubilized system. It is also possible that the extremely high activities of raft-associated γ-secretase (see Fig. 1B; the activities amounted to 78% of the total cell membrane activities) reveal minor cleavage pathways that were below the detection limit in the CHAPSO-solubilized system. For example, although Aβ37 was not detected in the CHAPSO-solubilized system (13), the detection of GVV and its response to γ-secretase inhibitor treatment indicate that a very small fraction of Aβ40 is processed to Aβ37 in the Aβ40 product line. This agrees with the previous observation that Aβ37 was produced by the SH-SYSY in vitro-reconstituted Aβ generation system (30). Thus, when all the available data are taken into account, it is reasonable to consider the progressive stepwise cleavage to be a basic mechanism of the intramembranous cleavage of βCTF by γ-secretase, whereas the quantitative variability derived from the employed system may result in some differences of the molecular species produced.

Quantitative analyses of the released oligopeptides indicate that the major processing pathways are the two successive tripeptide-releasing pathways starting at ε-cleaved Aβ49 and Aβ48 and ending at Aβ40 and Aβ42, respectively (Fig. 9). Aβ generated by these pathways amounts to ~75% of total Aβ generation. The released amounts of four tetra- and pentapeptides (VVIA, VIVI, VVIAT, and VIVIT) are less than those of the above tripeptides. The amounts of VVIA correspond to 19% of Aβ42 (TVI + TVIV) assumed to be generated, indicating the conversion of ~20% of Aβ42 generated to Aβ38. The amounts of VVIAT and VIVIT released are ~10% those of the tripeptides IAT and VIT released from the same Aβ through the major pathway. VIVI is released prominently, but we do not know its origin, because the corresponding AICDs are barely detectable (9, 18).

Interestingly, three pathways, each releasing a tri-, tetra-, or penta peptide, converge at the levels of Aβ38 and Aβ43 (Fig. 9). This indicates that the peptide bonds between Aβ38 and Aβ39 (Gly-38–Val-39) and between Aβ43 and Aβ44 (Thr-43–Val-44) are favorably attacked by γ-secretase. Because Gly is a small amino acid with a strong helix-destabilizing effect, the double-Gly configuration of Gly-37–Gly-38 may induce helix instability and facilitate cleavage at this position to release Aβ38. In the case of Aβ43, Thr-43 is a relatively hydrophilic residue embedded in a stretch of hydrophobic residues, and it may promote cleavage at Thr-43–Val-44.

The presence of multiple interacting pathways shown here could provide a better explanation for several previous observations apparently inconsistent with the stepwise processing model. The observation that the produced amounts of Aβ species and their corresponding AICDs (AICD(50–99) and Aβ40; AICD(49–99) and Aβ42) are correlated but not in a one-to-one ratio (9) could be explained by the presence of multiple minor cleavage pathways linking the major pathways in an interactive manner. The observation that the exogenously expressed Aβ48 produces both Aβ40 and Aβ42, whereas Aβ49 produces predominantly Aβ40 (10), fits the present cleavage model well. The

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S. Wada-Kakuda, M. Morishima-Kawashima, and Y. Ihara, unpublished observations.
significant release of the pentapeptide from Aβ43 found here may explain why a decrease in Aβ42 and an increase in Aβ38 are uncoupled in unusual cases (21, 31).

The release of the pentapeptide VVIAT by γ-secretase was reported quite recently by Okochi et al. (32) with a cell-based system and an in vitro reconstituted system using synthetic Aβ as a substrate. This study further identified two pentapeptides and estimated the relative contribution of those pentapeptides in Aβ-producing pathways, using βCTF and analyzing the released oligopeptides systematically by LC-MS/MS. This indicates that cropping of a pentapeptide is not an unusual pathway specific for Aβ38 generation. Furthermore, the release of VIVIT links the Aβ40 and Aβ42 product lines and may have an important role to define the Aβ species generated (see below).

One may think that FAD-associated mutations simply enhance the Aβ42 product line, resulting in increased Aβ42; however, that may be unlikely, because multiple pathways can be involved in the production and metabolism of Aβ42. There are many potential sites where mutations may produce effects. Those effects could modify the cleavage pattern in various ways that would lead to the common phenotype. Only the mutations associated with increased Aβ42 (in most cases) may have been selected as FAD mutations. In this context, it would be interesting to determine whether the release of VIVIT, which brings
about the conversion of Aβ48 to Aβ43, is influenced by FAD mutations, because FAD-associated mutations of PS cause an increase in Aβ43 (33) in addition to increases in Aβ42 and AICD(49–99), a counterpart of Aβ43 (9). This remains to be investigated in the future and is beyond the scope of this study.

GSM-1 enhances all three routes generating Aβ43 (33) in addition to increases in Aβ42 but not to Aβ42, in the low density membrane domains. J. Biol. Chem. 283, 733–738

In conclusion, this study has revealed the overall profile for Aβ generation pathways: the successive tripeptide-cropping pathway is the framework by which membrane-integrated γ-secretase cleaves βCTF. The recent NMR investigations of the TMD of βCTF lend substantial support to the model (35, 36). However, the pathways are not as simple as we previously thought. In particular, the concomitant release of tetra- and pentapeptides leads to cross-talk between the stepwise processing pathways, giving rise to diverse Aβ species generated through variable pathways. These observations extend our understanding of the intramembranous cleavage of βCTF by γ-secretase and may contribute toward the eventual development of an efficient therapeutic strategy against AD.

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