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Review Article

γ-Secretase-Dependent Proteolysis of Transmembrane Domain of Amyloid Precursor Protein: Successive Tri- and Tetrapeptide Release in Amyloid β-Protein Production

Mako Takami[1,2] and Satoru Funamoto[1]

[1] *Department of Neuropathology, Graduate School of Life and Medical Sciences, Doshisha University, Kyotanabe, Kyoto 610-0934, Japan*
[2] *Pharma Eight Co. Ltd., Kyoto, Kyoto 602-0841, Japan*

Correspondence should be addressed to Satoru Funamoto, sfunamot@mail.doshisha.ac.jp

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γ-Secretase cleaves the carboxyl-terminal fragment (βCTF) of APP not only in the middle of the transmembrane domain (γ-cleavage), but also at sites close to the membrane/cytoplasm boundary (ε-cleavage), to produce the amyloid β protein (Aβ) and the APP intracellular domain (AICD), respectively. The AICD49–99 and AICD50–99 species were identified as counterparts of the long Aβ species Aβ48 and Aβ49, respectively. We found that Aβ40 and AICD50–99 were the predominant species in cells expressing wild-type APP and presenilin, whereas the production of Aβ42 and AICD49–99 was enhanced in cells expressing familial Alzheimer's disease mutants of APP and presenilin. These long Aβ species were identified in cell lysates and mouse brain extracts, which suggests that ε-cleavage is the first cleavage of βCTF to produce Aβ by γ-secretase. Here, we review the progress of research on the mechanism underlying the proteolysis of the APP transmembrane domain based on tri- and tetrapeptide release.

1. Introduction

The amyloid precursor protein (APP) is a type I membrane protein. After ectodomain shedding by β-secretase, the carboxyl-terminal fragment (βCTF) of APP becomes a direct substrate of γ-secretase and is processed into the amyloid β protein (Aβ) and the APP intracellular domain (AICD) [1–5]. γ-secretase is an enigmatic protease composed of presenilin 1/2, nicastrin, Aph-1, and Pen-2 that catalyzes proteolysis in the hydrophobic environment of the lipid bilayer [6–15]. Currently, over 50 molecules are reported as γ-secretase substrates, which reflects the physiological importance of this enzyme [16]. For instance, the Notch receptor on the plasma membrane is cleaved by γ-secretase upon ligand binding and the liberated Notch intracellular domain (NICD) translocates into the nucleus and activates the expression of transcription factors to suppress neuronal differentiation [17, 18]. This indicates that inhibition of γ-secretase for suppression of Aβ production causes harmful side effects. To avoid this risk in anti-Alzheimer's disease (AD) therapeutics, it is very important to elucidate the molecular mechanism underlying γ-secretase-dependent proteolysis. Recently, it was revealed that γ-secretase forms a hydrophilic pore and three water-accessible cavities [19–23]. Here, we review the progress of research on the mechanism underlying the proteolysis of the transmembrane domain of βCTF.

2. Discovery of ε-Cleavage during APP Processing

After the β-secretase-dependent cleavage of APP, the ectodomain of APP is released into the extracellular space and βCTF (as a stub in the lipid bilayer) is the direct substrate of γ-secretase [2, 3, 24]. βCTF is composed of 99 amino acids and is eventually processed into the 38–43-residue-long Aβ, suggesting that the counterparts of those Aβ species should

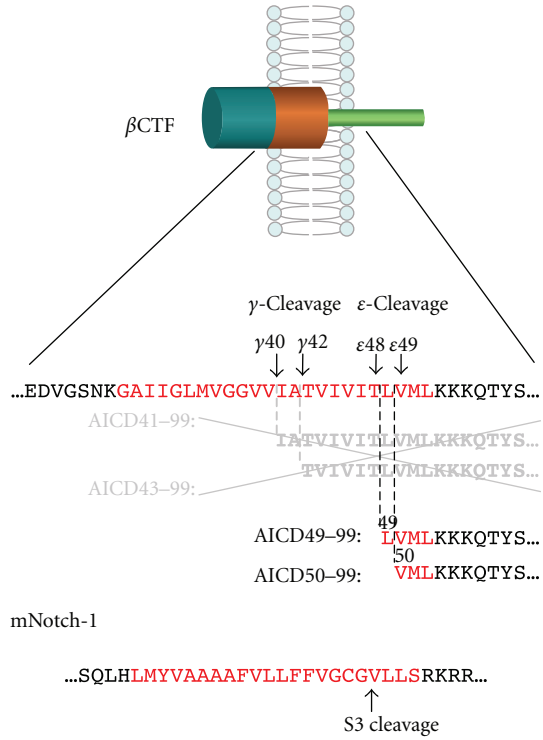

Figure 1: βCTF is cleaved at the membrane-cytoplasm boundary and not in the middle of the transmembrane domain (ε-cleavage), to release the AICD49–99 and AICD50–99 species. The production of AICD species was inhibited in the presence of a γ-secretase inhibitor. ε-Cleavage is analogous to the S3 cleavage of mNotch-1. Red indicates the transmembrane domain.

contain 56–61 residues [4, 25–29]. However, 50-51-residue-long AICDs were identified that correspond to residues 49–99 and 50–99 of βCTF (AICD49–99 and AICD50–99), instead of 56–61-residue-long species (Figure 1) [30–32]. These AICD species were suppressed by L-685,458, a transition state analogue γ-secretase inhibitor, and by expression of a dominant-negative mutant of presenilin (PS), suggesting that γ-secretase cleaves βCTF not only in the middle of the transmembrane domain (γ-cleavage), but also at sites close to the membrane/cytoplasm boundary (ε-cleavage), releasing AICD49–99 and AICD50–99. ε-Cleavage sites are analogues of the Notch S3 cleavage site, which is located at the membrane, near the cytoplasm (Figure 1). Cleavages similar to the APP ε-cleavage were identified in other proteins, such as amyloid precursor-like protein 1 (APLP-1), APLP-2, CD44, Delta 1, E-cadherin, ErbB4, and LRP1 [30, 33–37]. It is reasonable to consider that the water molecules required for proteolysis have access to the catalytic center of γ-secretase from the cytoplasm, rather than from the extracellular space, and that ε-cleavage precedes γ-cleavage during APP processing.

3. Relationship between γ- and ε-Cleavage

CHO cells expressing familial AD (FAD) mutants of PS or APP increase production ratio of Aβ42 (Aβ43) to Aβ40 compared to cells expressing wild-type PS or APP these longer Aβ species are more hydrophobic and more prone to form neurotoxic aggregates. CHO cells expressing wild-type PS preferentially release AICD50–99, whereas those expressing a subset of familial AD (FAD) mutants of PS or APP exhibit an increased proportion of AICD49–99 (Figure 2(a)) [42]. As those FAD mutations cause an increase in the Aβ42/Aβ40 ratio, a potential link between γ- and ε-cleavage was assumed. To test this, we expressed Aβ49 and Aβ48, which are potential counterparts of AICD50–99 and AICD49–99, respectively, in CHO cells. The cells expressing Aβ49 predominantly secreted Aβ40, whereas those expressing Aβ48 exhibited a significantly increased proportion of Aβ42/Aβ40 (Figure 2(b)) [43]. These data indicate that ε-cleavage sites determine the preference for γ- and ε-cleavage sites to produce Aβ40 and Aβ42. Long Aβ species, Aβ49 and Aβ48, have been identified in cell lysates and mouse brain extracts, which suggests that ε-cleavage is the first cleavage of βCTF to produce Aβ by γ-secretase [44]. On the other hand, ε-cleavage can be considered as endopeptidase activity of γ-secretase. FAD mutations did not consistently impair the endopeptidase activity on APP, Notch, ErbB4, and N-Cadherin, but altered γ-cleavage of APP, especially fourth cleavage to produce Aβ40 and Aβ38 from Aβ43 and Aβ42, respectively [45]. Such dissociation between ε-cleavage and γ-cleavage was also proposed by Quintero-Monzon et al. [46].

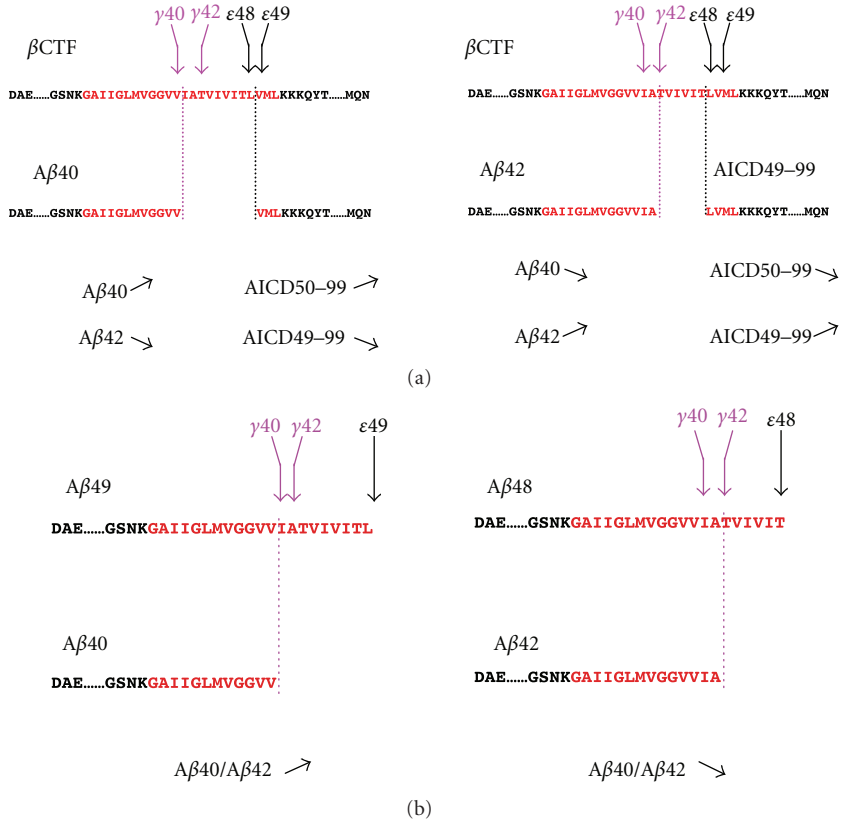

Figure 2: Relationship between γ- and ε-cleavage. (a) Cells expressing wild-type PS or APP predominantly produce Aβ40 and AICD50–99, while cells expressing a FAD mutant of PS or APP exhibited increased proportion of Aβ42 and AICD49–99. (b) Expression of Aβ49 results in an increase in Aβ40/Aβ42 ratio, whereas expression of Aβ48 leads to opposite results. ↗ increase, ↘ decrease.

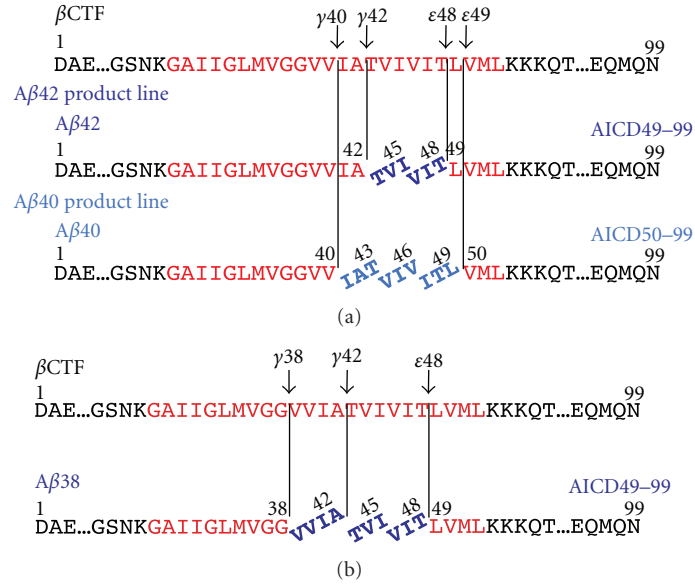

Figure 3: Tri- and tetrapeptide release from βCTF. (a) Upon ε-cleavage at ε48, γ-secretase releases the VIT and TVI tripeptides successively to produce Aβ42. (b) In the Aβ40 product line, after ε-cleavage at ε49, βCTF is converted into Aβ40 by releasing ITL, VIV, and IAT. Aβ42 is a direct substrate during Aβ38 production, which acts by releasing the VVIA tetrapeptide.

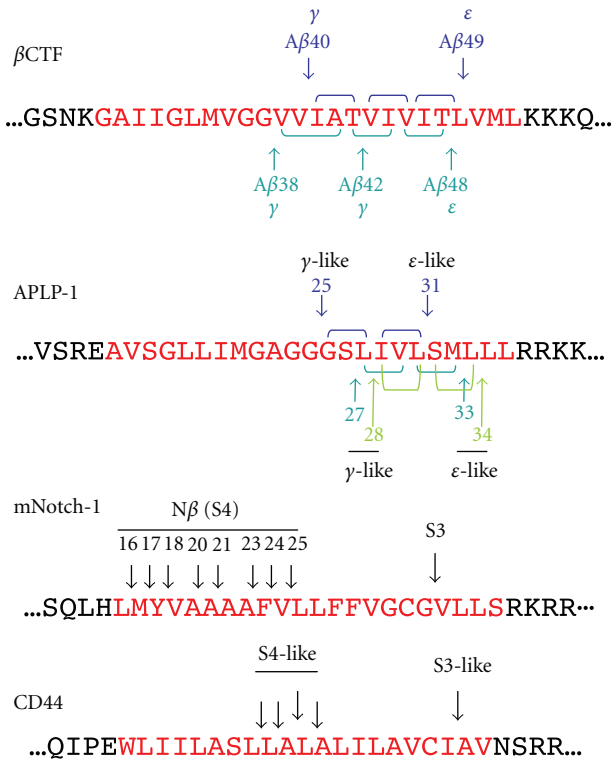

Figure 4: Multiple cleavage sites on the transmembrane domain of γ-secretase substrates. APP [38], APLP-1 [30, 39], mNotch-1 [40], and CD44 [41].

4. Tripeptide Hypothesis

Treatment with N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT), a γ-secretase inhibitor, suppressed extracellular Aβ in cells expressing APP [47]. The levels of the intracellular Aβ40 and Aβ42 species also decreased after DAPT treatment; however, intracellular Aβ43 and Aβ46 increased in a dose-dependent manner [44, 48, 49]. Tryptophan substitutions of γ-cleavage site (41–43) of APP attenuated Aβ secretion, but accumulated Aβ45 species in cell lysate. Tryptophan substitutions of ε-cleavage site (48–52) of APP decreased Aβ production and allowed longer AICD46–99 production. Tryptophan substitutions of ξ-cleavage site (45–47) also suppressed Aβ production. These substitution studies also implied successive cleavage of APP for Aβ production after ε-cleavage [50].

γ-Secretase containing mature nicastrin accumulates in lipid rafts, which indicates that active γ-secretase mainly localizes to the lipid raft of cells [51]. Lipid rafts are an ideal material to investigate Aβ production in the membrane environment. Aβ46 was the dominant species in a lipid raft isolated from DAPT-treated cells. Interestingly, incubating this lipid raft in the absence of DAPT resulted in production of Aβ40 and Aβ43, but not of Aβ42 [52]. These data suggest that Aβ46 is mainly converted into Aβ40 by releasing VIV and IAT tripeptides (successive tripeptide release, tripeptide hypothesis; Aβ40 product line) (Figure 3(a)). On the other hand, CHO cells expressing an FAD mutant of presenilin 2 exhibited a decrease in intracellular Aβ42 and a concomitant increase in intracellular Aβ45 levels in the presence of DAPT, suggesting that Aβ45 is a precursor of Aβ42 by releasing TVI (Aβ42 product line) (Figure 3(a)) [53]. It is reasonable to consider that two major product lines lead to Aβ40 and Aβ42 production (Figure 3(a)).

5. Identification of Tri- and Tetrapeptides Released from βCTF

The most effective approach to confirm tripeptide release from βCTF is the identification of those tripeptides directly in the reaction mixture of Aβ production. CHAPSO soluble γ-secretase was isolated and incubated with the βCTF substrate. LC-MS/MS analysis identified five major tripeptides, and γ-secretase inhibitors abolished the production of these molecules. ITL, VIV, and IAT were predicted tripeptides in the Aβ40 product line (Figure 3(a)). The amounts of Aβ40 and Aβ43 in the reaction mixture, as assessed using Western blotting, corresponded roughly to the predicted Aβ40 and Aβ43 levels, respectively [38]. VIT and TVI were also detected in the Aβ42 product line, as predicted (Figure 3(a)). Interestingly, the VVIA tetrapeptide was detected in the reaction mixture only in the absence of γ-secretase inhibitors (Figure 3(b)). We postulated that VVIA was released from Aβ42 to produce Aβ38. No significant difference was detected between the level of Aβ42 by Western blot quantification and that by LC-MS/MS quantitative estimation. These results indicate that γ-secretase releases

tri- and tetrapeptides successively upon ε-cleavage of βCTF, to produce Aβ species. These tri- and tetrapeptides released from βCTF were detected even in the lipid raft fraction (Takami, unpublished observation).

6. Is Tripeptide Release a General Property of Substrate Cleavage by γ-Secretase?

Successive tripeptide release was observed in βCTF processing by γ-secretase. We also found that γ-secretase released tri- and tetrapeptides successively from αCTF substrate (Takami, unpublished observation). Recently, tripeptide spacing of endoproteolysis on presenilin has been reported [54]. These suggest that successive tri- and tetrapeptide release is a general property of γ-secretase-mediated intramembrane proteolysis.

Yanagida et al. reported that APLP-1 was also cleaved into three Aβ-like peptides [39]. As three ε-like cleavages are known, it is likely that APLP-1 is processed in three product lines by successive tripeptide release [30] (Figure 4). The transmembrane domain of mNotch-1 is cleaved by γ-secretase after ectodomain shedding to liberate NICD (S3 cleavage). NICD containing V1744 was found as the prominent species produced by S3 cleavage [55]. To date, it seems reasonable to suppose that there is a single cleavage site in S3. γ-Secretase also cleaves mNotch-1 at the lumen-membrane boundary (S4 cleavage) to release Notch β peptides (Nβ) (Figure 4) [40, 56, 57]. Fenofibrate treatment increased the proportion of Nβ25, but not that of Nβ21, which implies that Nβ25 and Nβ21 correspond to Aβ42 and Aβ40, respectively [57]. However, it is unlikely that several Nβ product lines exist in Notch processing because of the single S3 site. The production of Nβ species may not fit the tripeptide-processing model (Figure 4). CD44 is cleaved not only at the membrane-cytoplasm boundary, but also at the middle of the transmembrane domain, which results in the release of Aβ-like peptides [33, 41]. Similar to Notch, the processing of the CD44 transmembrane domain may not fit the tripeptide-processing model (Figure 4).

7. Conclusion and Perspectives

The tripeptide hypothesis was confirmed in the processing of the APP transmembrane domain, which accounts for the production of Aβ species. Although the physiological significance of the multiple cleavage of the transmembrane domain is unknown, it is important to illustrate the cleavage mechanisms of other γ-secretase substrates, because the limitation of this stepwise mechanism would help to elucidate the substrate-specific inhibition of Aβ production. As shown in Figure 4, APLP-1 may be cleaved by tripeptide release; however, Notch and CD44 do not fit this processing model [40, 41]. γ-Secretase is widely believed to be a promiscuous protease; however, the cleavage mechanisms of APP and Notch, at least, seem to be different (Figure 4), which indicates that γ-secretase distinguishes substrates during proteolysis. Perhaps absence of helix breaker glycine residues in mid-portion of transmembrane domain allows multiple S4 cleavages even after single S3 cleavage in Notch. From this point of view, uncovering the mechanisms underlying γ-secretase-dependent cleavage offers a basis for new therapeutic approaches that are aimed at substrate-specific Aβ inhibition.

Abbreviations

Aβ:	Amyloid β protein
AICD:	APP intracellular domain
APP:	Amyloid precursor protein
βCTF:	Carboxyl terminal fragment of APP
DAPT:	N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butylester
FAD:	Familial Alzheimer's disease
LC-MS/MS:	Liquid chromatography-tandem mass spectrometry
Nβ:	Notch β peptide
NICD:	Notch intracellular domain
PS:	Presenilin.

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