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γ -Secretase Catalyzes Sequential Cleavages of the A β PP Transmembrane Domain

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Abstract

The biogenesis of the amyloid- β peptide (A β) is a central issue in Alzheimer's disease (AD) research. A β is produced by β - and γ -secretases from the amyloid- β protein precursor (A β PP). These proteases are targets for the development of therapeutic compounds to downregulate A β production. γ -secretase has received more attention 1) because it generates the C-terminus of A β , which is important in the pathogenesis of AD because the longer A β species are more amyloidogenic, and 2) because it cleaves A β PP within its transmembrane domain. In the understanding the mechanism of γ -secretase cleavage, three major cleavage sites have been identified, namely, γ -cleavage site at A $\beta_{40/42}$, ζ -cleavage site at A β_{46} , and ϵ -cleavage site at A β_{49} . Moreover, the novel finding that some of the known γ -secretase inhibitors inhibit the formation of secreted A β_{40} and A β_{42} , but cause an intracellular accumulation of long A β_{46} , provided information extremely important for the development of strategies aimed at the design of γ -secretase inhibitors to prevent and treat AD. In addition, it has been established that the C-terminus of A β is generated by a series of sequential cleavages: first, ϵ -cleavage, followed by ζ -cleavage and finally by γ -cleavage, commencing from the membrane boundary to the middle of the A β PP membrane domain.

Keywords

Alzheimer's disease; amyloid; amyloid- β protein precursor; presenilin; secretase

INTRODUCTION

Alzheimer's disease (AD) is the most common type of dementia in the elderly population. AD is clinically characterized by progressive dementia, including loss of memory and the ability to learn and think. Pathologically, it is characterized by degeneration of neurons and accumulation of abnormal protein structures in the brain. This abnormal accumulation includes intracellular deposition of neurofibrillary tangles (NFT) that are composed of the microtubule-associated protein tau, and extracellular deposits of various types of amyloid- β (A β) in senile plaques. A β is a mix of peptides of 39 to 43 amino acids in length and is proteolytically produced from a large amyloid- β protein precursor (A β PP). Several lines of evidence support the hypothesis that progressive accumulation of A β is an early and critical event in the pathogenesis of AD [1]. Studies have revealed that the accumulation of A β initiates a series of downstream neurotoxic events, including synaptic failure [2] and hyperphosphorylation of tau, which results in neuronal dysfunction and death [3]. The strongest support for this "amyloid cascade" hypothesis comes from observations of molecular genetic studies. First, patients with Down

syndrome, who possess an extra copy of chromosome 21 on which the $A\beta$ PP gene is located, invariably develop AD [4]. Second, familial forms of AD (FAD) are linked to several missense mutations in the $A\beta$ PP gene that result in increased production of total $A\beta$ or of $A\beta_{42}$, which is the more amyloidogenic form [5–7]. Furthermore, FAD-linked mutations in the other two AD genes, presenilin 1 (PS1) [8] and presenilin 2 (PS2) [9,10], also cause abnormal production of $A\beta$, mostly resulting in an increase in the ratio of $A\beta_{42}$ to $A\beta_{40}$. Third, genetically engineered mice that carry the human FAD genes develop amyloid plaques as well as symptoms that mimic those of human disease [11]. The relationship of NFT and senile plaques with clinical disease has long been debated. There is an emerging appreciation that these changes themselves may not have a pathogenic or etiological role in AD, but are more likely by-products of a preceding, primary pathobiologic process that results in their accumulation [12,13]. However, evidence now suggests that the soluble forms of $A\beta$ oligomers, rather than the plaques, are indeed the principal “toxic intermediates” of synapse loss and neuronal injury [13,14]. Thus, elucidating how $A\beta$ oligomers are generated is essential for understanding AD and for developing strategies to prevent and treat this disease. For instance, such tactics might include development of small molecules that inhibit one or another secretase involved in $A\beta$ PP processing and $A\beta$ formation over long periods of time.

SECRETASES INVOLVED IN $A\beta$ PP PROCESSING AND $A\beta$ FORMATION

$A\beta$ is proteolytically derived from $A\beta$ PP, which is a ubiquitously expressed, type I transmembrane protein [15–20]. $A\beta$ PP can be processed via two alternative pathways, the amyloidogenic pathway and the non-amyloidogenic pathway (Fig. 1) [21]. In the nonamyloidogenic pathway, $A\beta$ PP is cleaved within the $A\beta$ sequence by α -secretase, resulting in the release of a soluble ectodomain, $sA\beta$ PP α , and a membrane-anchored C-terminal fragment, CTF α . Since α -cleavage occurs within the $A\beta$ sequence, it precludes the formation of full-length $A\beta$. Alternatively, in the amyloidogenic pathway, $A\beta$ PP is cleaved at the N-terminal of the $A\beta$ sequence by β -secretase to produce a soluble $sA\beta$ PP β and a membrane-anchored C-terminal fragment, CTF β . Both CTF α and CTF β are subsequently cleaved within their transmembrane domain by γ -secretase to produce the short peptide p3 from CTF α , and the full-length $A\beta$ from CTF β , in addition to the release of the $A\beta$ PP intracellular domain (AICD) from both CTF α and CTF β [22]. The $A\beta$ peptides generated in the amyloidogenic pathway vary in length from 38 to 43 amino acids, as detected in conditioned medium and brains of transgenic mice [23,24], with $A\beta_{40}$ as the predominant secreted $A\beta$ species. Although the longer forms of $A\beta$, mostly $A\beta_{42}$, are the minor species, it has been shown that they are more hydrophobic and particularly prone to aggregate and form amyloid fibrils [25,26]. In addition, longer forms of $A\beta$ are believed to play a pivotal role in the nucleation of amyloid plaques [27]. In contrast, p3 peptide produced from CTF α in the non-amyloidogenic pathway is not involved in amyloidogenesis [3]. AICD produced in both pathways is reported to bind to different target proteins and may be involved in various cellular events, including apoptosis, neuronal growth and regulation of gene expression [28]. Thus, these proteases involved in the processing of $A\beta$ PP are the therapeutic targets for developing compounds that down-regulate $A\beta$ production either by augmenting the non-amyloidogenic processing or by inhibiting the amyloidogenic processing of $A\beta$ PP.

α -secretase

Sequence analysis of the soluble secreted human brain $A\beta$ PP [29] and secreted $A\beta$ PP from various types of cells [30–32] indicates that α -cleavage mainly occurs after Lys-16 (numbered according to $A\beta$ sequence), but multiple minor cleavages around this site have been observed. While the enzyme accountable for the α -cleavage has not been finally identified, studies suggest that α -cleavage is likely mediated by members of the ADAM (a disintegrin and metalloproteinase) family of proteases (for review see [33]).

β -secretase

Since $A\beta$ is produced by β -secretase and γ -secretase, these two enzymes have attracted more attention. Almost simultaneously, several laboratories, using different approaches, identified β -secretase as a type-I transmembrane aspartyl protease characterized by the presence of two D^T/sG^T active site motifs, which is a conserved signature sequence of aspartic proteases. This enzyme was referred to as BACE (β -site $A\beta$ PP cleaving enzyme) or BACE1, Asp2, and memapsin 2 [34–38]. In contrast to α -secretase, β -secretase cleaves its substrate with loose, but primary sequence specificity [39–41]. Like other known aspartic proteases such as cathepsin D and E, napsin A, pepsin, and renin, BACE prefers a leucine residue at position P1 [40], and this may explain the fact that Swedish mutant $A\beta$ PP, in which the residues Lys-Met at the P2-P1 positions were substituted by Asn-Leu, serves as a better substrate for β -secretase cleavage. In addition, the facts that: 1) BACE1 is expressed at a higher level in the brain in comparison with other tissues [34,42]; 2) BACE1 knockout animals do not produce detectable levels of $A\beta$; and 3) BACE1 knockout mice are healthy and develop normally suggest that inhibition of β -secretase activity is a potentially promising therapeutic approach for AD (for review see [43,44]).

γ -secretase

The other important secretase involved in $A\beta$ formation is γ -secretase, which cleaves $A\beta$ PP at the C-terminal end of the $A\beta$ sequence to produce the C-termini of the $A\beta$ peptide (Fig. 1). γ -secretase attracts more attention not only because its cleavage determines the ratio of $A\beta_{42}/A\beta_{40}$, which is a key factor in the amyloidogenesis of AD implying an important role for γ -secretase as a therapeutic target, but also because it occurs within the transmembrane domain of $A\beta$ PP. This proteolysis within the hydrophobic environment of a membrane has attracted general biological interest. The current knowledge about the γ -secretase-catalyzed intramembrane processing of $A\beta$ PP is the focus of this review. In search for genetic factors of AD, in addition to $A\beta$ PP, two other related genes, PS1 and PS2, were identified to be associated with early-onset, auto-somal dominant FAD [8–10]. Shortly after their identification, it was found that the missense mutations in these two genes are associated with increased levels of the long and more aggregation-prone $A\beta_{42}$, suggesting that these mutations cause disease by altering $A\beta$ PP processing, especially the formation of the C-termini of $A\beta$ [45,46]. This notion was strongly supported by the finding that FAD-linked mutations in both PS1 and PS2 increase the production of $A\beta_{42}$ in transfected cells and transgenic mice [47–50]. Subsequently, the discovery that genetic knockout of both PS1 and PS2 resulted in total inactivation of γ -secretase activity suggests that presenilin may be functional γ -secretase [51–53]. The notion that presenilins bear the γ -secretase active site was strongly supported by the observations that mutation of either of the two conserved aspartate residues in PS1 (D257 and D385) or PS2 (D263 and D366) substantially reduced $A\beta$ production and caused a concomitant accumulation of CTF β [54,55]. The notion that presenilins bear the γ -secretase active site was further supported by the facts that γ -secretase activity was inhibited by aspartyl protease substrate-based peptidomimetic inhibitors [56,57] and, specifically, that aspartyl protease transition-state analog bound directly to PS1 and PS2 [58,59].

Presenilins are multiple transmembrane proteins and have been suggested to span the membrane six, seven, eight, and even nine times [60–62]. After synthesis, the 43 ~ 47 KDa presenilin holoprotein is subsequently endoproteolytically cleaved within its large cytoplasmic loop between the sixth and seventh transmembrane domains to generate a 27 ~ 28 KDa N-terminal and a 16 ~ 18 KDa C-terminal fragment (NTF, CTF) [63–65]. The finding that NTF and CTF of presenilin remained physically associated in a high molecular complex [66–69], suggesting that other components that interact with PS1 may play a functional role in γ -secretase. In an effort to identify the putative components required for γ -secretase activity, nicastrin was first discovered by biochemical isolation [70]. Nicastrin is a 130 kDa type I

transmembrane protein with a large ~ highly glycosylated ectodomain. RNA interference (RNAi)-mediated knockdown experiments demonstrated that nicastrin was essential for γ -secretase-mediated cleavage of both A β PP and Notch proteins [70]. Subsequently, genetic screening for Notch signaling modulators in *C. elegans* led to the identification of two other novel genes, Aph-1 and Pen-2, which encode proteins of a 25 kDa with seven, and a 10 kDa with two, transmembrane domains, respectively [71,72]. As with nicastrin, knockdown of Aph-1 and Pen-2 by RNAi strongly reduced γ -secretase activity, indicating that these two are also essential components of γ -secretase [71–73]. Now, it is widely accepted that the active γ -secretase complex consists of at least four polypeptides – presenilin, nicastrin, Aph-1, and Pen-2 – with presenilin functioning as the catalytic subunit (Fig. 2, for review see [74]).

IDENTIFICATION OF THE ϵ -CLEAVAGE SITE

The α -secretase-catalyzed cleavage between the 16th (Lys¹⁶) and 17th (Leu¹⁷) amino acids of the A β sequence is referred to as the α -cleavage site; the β -secretase-catalyzed cleavage site at the first residue (Asp¹) of the A β sequence is referred to as the β -cleavage site. Since the majority of the C-termini of A β species generated by γ -secretase end at A β ₄₀ or A β ₄₂, the cleavage sites at these positions are commonly referred to as the γ -cleavage sites (Fig. 3). In addition, sequence analysis of the C-terminal fragments of A β PP produced in primary hippocampal neurons revealed that a minor species starts at position 12 in amino acids N-terminal to the β -cleavage site, and this fourth cleavage site was tentatively named the δ -cleavage site [32]. CTF α and CTF β produced by α - and β -cleavage undergo further γ -secretase mediated cleavage, resulting in the formation of a common AICD, the full-length A β from CTF β , and a p3 fragment from CTF α . Since most A β and p3 fragments end at A β ₄₀ or A β ₄₂, one may assume that AICD would start at either A β ₄₁ or A β ₄₃. That is why AICD time-after-time was referred to as CTF γ , probably based on the idea that it was produced by γ -cleavage. However, follow-up studies surprisingly revealed that most of the AICDs start from A β ₅₀, with some of them starting from A β ₄₉ [75–78]. Thus, these findings led to the identification of a new cleavage site, which is now known as the ϵ -cleavage site at A β ₄₉ [75–78]. Interestingly, this ϵ -cleavage site is found to be equivalent to the S3 site of Notch 1 [79]. In addition, a new intramembrane cleavage site, the S4 site in Notch 1, was reported in a recent study, and it is assumed to be homologous to γ -cleavage site in A β PP [80]. These findings further suggest that A β PP and Notch 1 undergo similar intramembrane cleavages, namely, γ (S4) and ϵ (S3) cleavages [80].

IDENTIFICATION OF THE ζ -CLEAVAGE SITE

Since the ϵ -cleavage site at A β ₄₉ is 7–9 residues distal to the γ -cleavage site at A β _{40/42}, the identification of the ϵ -cleavage site raised questions as to the relationship between the γ - and ϵ -cleavages and whether ϵ -cleavage is an essential step for generating the C-termini of A β [81]. There are several possibilities: one possibility is that CTF β is firstly cleaved at the γ -cleavage site at A β _{40/42} and that the resulting CTF γ (CTF41–99 and CTF43–99) undergoes further and rapid degradation by a particular aminopeptidase and/or endopeptidase or undergoes a second γ -secretase-dependent cleavage at the ϵ -site to produce CTF ϵ (CTF50–99, the AICD mentioned above); the second possibility is that the ϵ -cleavage at A β ₄₉ occurs first, followed by γ -cleavage at A β _{40/42} to produce CTF ϵ and A β _{40/42}; or thirdly, it is also possible that the ϵ -cleavage and γ -cleavage are two totally unrelated events occurring in parallel or even possibly involved in two separate proteolytic pathways [75–78]. Given the fact that the ϵ -cleavage is closer to the membrane boundary and is thus easily accessed by water molecules required for the hydrolysis of the peptide bond, the second possibility that ϵ -cleavage precedes γ -cleavage is more plausible. However, either one of these possibilities need to be experimentally tested. One of the obstacles to addressing these questions is that neither the long A β peptides, which end at the ϵ -cleavage site, nor the C-terminal fragment, which starts

with an N-terminal generated by γ -cleavage, has ever been detected. There are several considerable possibilities that may account for the inability to detect these intermediates. First, these intermediates might have been rapidly processed or degraded into short species, $A\beta_{40/42}$ and/or CTF ϵ , which are the final products of γ -secretase processing and were usually detected in most of the previous studies. Second, thus far, most of the attention has been concentrated on the secreted forms of $A\beta$, and less attention has been paid to intracellular or membrane-bound $A\beta$; however, it is very possible that the trace amount of the intermediate long $A\beta$ species may have been overlooked; and third, as discussed above, it may be that ϵ -cleavage and γ -cleavage are separate events, and the intermediates do not exist at all. Since $A\beta$ species are short and hydrophobic, it is difficult to distinguish them from each other using the conventional SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) under normal conditions. To solve this issue, researchers have developed a new SDS-PAGE system, the urea-SDS-PAGE system, which has been shown to be able to successfully separate $A\beta_{42}$ from $A\beta_{40}$ [82,83]. By using this system in combination with mass spectrometric analysis, two groups reported the identification of a long form of $A\beta$, $A\beta_{46}$, in cultured cells expressing $A\beta$ PP and in brain tissues of a transgenic mouse carrying the human $A\beta$ PP gene [84,85]. Since the production of this $A\beta_{46}$ is inhibited by transition state analog γ -secretase inhibitor and dominant negative presenilin, it is believed that this $A\beta_{46}$ is produced by γ -secretase activity [84]. Thus, this finding led to the discovery of a new γ -secretase-mediated cleavage site, which was designated as the ζ -cleavage site at $A\beta_{46}$ between the γ -cleavage site at $A\beta_{40/42}$ and ϵ -cleavage site at $A\beta_{49}$ [84].

$A\beta_{46}$ IS A PRECURSOR OF $A\beta_{40/42}$

The identification of the new ζ -cleavage site at $A\beta_{46}$ confirmed that the $A\beta$ species with a C-terminus end at $A\beta_{46}$, detected in human skeletal muscles in a previous study [86], is not a result of random degradation of $A\beta$ PP, but a product of γ -secretase activity. These findings strongly support the notion that, as with γ -cleavage and ϵ -cleavage, ζ -cleavage is a normal physiological event during secretase-mediated $A\beta$ PP processing. Now, the questions are, what is the relationship of this new ζ -cleavage with the other two known γ -secretase-mediated cleavages, namely γ -cleavage and ϵ -cleavage, and does this new ζ -cleavage at $A\beta_{46}$ play a role in the formation of secreted $A\beta_{40/42}$? In this regard, it is notable that the newly identified $A\beta_{46}$ was found to be tightly associated with γ -secretase complex [87]. Thus, it is fair and reasonable to speculate that this $A\beta_{46}$ may undergo further cleavage at $A\beta_{40/42}$, the γ -cleavage site, to produce secreted $A\beta_{40/42}$. This speculation is strongly supported by a very interesting finding that several well characterized γ -secretase inhibitors, such as DAPT, DAPM and compound E, which are known as non-transition state inhibitors, inhibited the formation of secreted $A\beta_{40/42}$ and caused a concomitant accumulation of intracellular membrane-bound $A\beta_{46}$ in a dose dependent manner, suggesting a possible precursor-product relationship between $A\beta_{46}$ and $A\beta_{40/42}$ [84]. However, the observation that ζ -cleavage can be differentially inhibited by γ -secretase inhibitors known as transition state analogs, such as L-685,458 and WPE-III-31C (31C), but less affected by non-transition state inhibitors, such as DAPT, DAPM and compound E, complicated this matter [84]. One may even question whether γ -cleavage and ζ -cleavage are catalyzed by different enzymes, which are both dependent on PS1 or a PS1-like protein, but have different sensitivities to transition state and non-transition state inhibitors. Thus, the roles of ϵ - and ζ -cleavages in the formation of secreted $A\beta$ and the relationship among these three cleavages, namely ϵ -, ζ -, and γ -cleavages, still remain elusive. To challenge these issues, one group, by employing a differential inhibition strategy, conducted a series of experiments to: 1) explore the relationship between $A\beta_{46}$ and $A\beta_{40/42}$; 2) determine the possible presence of $A\beta_{49}$ generated by ϵ -cleavage and its relationship with $A\beta_{46}$ and $A\beta_{40/42}$; 3) elucidate the relationship between the three major cleavages (ϵ -, ζ -, and γ -cleavages) within the transmembrane domain of $A\beta$ PP; and 4) establish the roles of ϵ - and ζ -cleavages in the formation of secreted $A\beta_{40/42}$ [87,88]. To determine whether $A\beta_{46}$ can be processed into

$A\beta_{40/42}$, the investigators first cultured the cells in the presence of non-transition state inhibitor DAPM to accumulate intracellular membrane-bound $A\beta_{46}$ for a certain period, and then the DAPM was replaced with transition state analog L-685,458, which blocks new formation of $A\beta_{46}$ from CTF β , in a fresh medium. After two hours incubation, when the fresh medium was examined for the presence of possible secreted $A\beta_{40/42}$ during a time course of the subsequent culture, surprisingly, along with a decrease of $A\beta_{46}$, an increase in secreted $A\beta_{40/42}$ was observed. Under the same conditions, namely in the presence of transition state analog L-685,458, no $A\beta_{40/42}$ was detected in cells without pre-accumulated $A\beta_{46}$. These results indicate that, in the presence of L-685,458, the secreted $A\beta_{40/42}$ detected in the fresh medium during the subsequent culture should be produced solely from the pre-accumulated $A\beta_{46}$, but not from CTF β , which remains unchanged since the transition state analog L-685,458 blocked it from being further processed by γ -secretase. These results have been further confirmed using both living cells and cell-free systems and by metabolic labeling approaches [87,88]. Thus, these observations clearly established the precursor-product relationship between $A\beta_{46}$ and $A\beta_{40/42}$ [87,88].

SECRETED $A\beta$ IS PRODUCED BY γ -SECRETASE-MEDIATED SEQUENTIAL CLEAVAGES: FIRST THE ϵ -CLEAVAGE, FOLLOWED BY A RAPID ζ -CLEAVAGE, AND THEN BY A γ -CLEAVAGE

In these studies, one notable finding is that transition state analog L-685,458 showed no effect on the turnover of $A\beta_{46}$ in both living cell and cell-free systems, indicating that L-685,458 does not directly inhibit γ -cleavage at $A\beta_{40/42}$ [87]. On the other hand, L-685,458 showed a strong inhibitory effect on the formation of $A\beta_{46}$ by ζ -cleavage and the formation of CTF ϵ by ϵ -cleavage. These findings strongly suggest that this inhibitor inhibits the formation of secreted $A\beta_{42}$ by a mechanism other than direct inhibition of γ -cleavage at $A\beta_{40/42}$, but indirectly, by inhibiting the formation of $A\beta_{46}$ by ζ -cleavage. Therefore, the fact that the inhibition of upstream ζ -cleavage by L-685,458 completely prevents the downstream γ -cleavage from taking place strongly supports an important notion that γ -cleavage not only occurs secondarily, but also is dependent on ζ -cleavage occurring first [87]. The next question is, what is the relationship of ϵ -cleavage to ζ - and γ -cleavages? To answer this question, the key is to determine the presence of $A\beta_{49}$ produced by ϵ -cleavage. In an effort to determine the long-sought $A\beta_{49}$, the researchers performed immunoprecipitation of cell lysates using an $A\beta$ -specific antibody and successfully detected a modest level of $A\beta_{49}$ in cells cultured in the absence of any inhibitors [87]. The fact that $A\beta_{49}$, which contains the ζ -cleavage site at $A\beta_{46}$, is detectable in living cells in the absence of any inhibitors indicates that ϵ -cleavage occurs prior to ζ -cleavage; otherwise the ϵ -cleavage product $A\beta_{49}$ should not have had a chance of being formed. In addition, in the same study, it was observed that $A\beta_{49}$ can be processed into $A\beta_{40/42}$ in the absence of inhibitors and processed into $A\beta_{46}$ in the presence of non-transition state inhibitors, such as DAPT, DAPM, and compound E. These findings indicate that $A\beta_{49}$ is a direct precursor of $A\beta_{46}$. However, in the presence of transition state analogs, such as L-685,458 and 31C, neither $A\beta_{40/42}$ nor $A\beta_{46}$ was detected, indicating that these transition state analogs inhibit ζ -cleavage. Thus, as illustrated in Fig. 4, these observations strongly suggest that after β - or α -cleavage of $A\beta$ PP, the resulting CTF β and CTF α first undergo ϵ -cleavage, followed by a sequential but rapid ζ -cleavage and then a γ -cleavage, commencing at the site closest to the membrane boundary and proceeding towards the site within the middle of the transmembrane domain of $A\beta$ PP. More importantly, the fact that γ -cleavage not only occurs last but also is dependent on ϵ - and ζ -cleavages occurring prior to it, established that the ϵ -cleavage and ζ -cleavage are obligatory steps, and especially, that the ϵ -cleavage is the initial and rate-limiting step of the sequential ϵ , ζ , and γ -cleavages involved in the generation of the C-termini of secreted $A\beta$.

THE SEQUENTIAL CLEAVAGE MODEL PROVIDES NEW INSIGHTS INTO THE MECHANISM OF γ -SECRETASE-MEDIATED A β PP PROCESSING

All proteases are also known as hydrolases because they catalyze the hydrolysis of a peptide bond; therefore, water molecules are required for the cleavage of the peptide bond. According to the catalytic mechanism of the aspartyl protease, in order to hydrolyze the peptide bond of the substrate, one of the two aspartate residues in the enzyme active site, disposed on opposite faces of the peptide bond to be cleaved, needs to first act as a general base to activate the water molecule. The activated water molecule then attacks and breaks the peptide bond, in cooperation with the second aspartate, which acts as general acid to protonate the departing amine product. Thus, one of the most mysterious questions in γ -secretase-catalyzed intramembrane processing of A β PP is how the water molecules, which are required for the peptide bond hydrolysis, enter the γ -secretase active site, which is localized within the hydrophobic environment of the lipid bilayer membrane. In this regard, the sequential cleavage model may provide an answer to this question. As shown in Fig. 5, the ϵ -cleavage site is close to the membrane boundary and is easily accessed by water molecules in the cytosol. The initial ϵ -cleavage may not only release the CTF ϵ (AICD), but may also create a path for the water molecule to have access to the next cleavage sites, namely the ζ -cleavage site and then the γ -cleavage site. Accordingly, without removal of the C-terminal fragment, water molecules may not be able to access the γ -cleavage site, resulting in the prevention of γ -cleavage from taking place. Thus, the blockage of water access may account, at least in part, for the fact that γ -cleavage depends on ϵ - and ζ -cleavages occurring first.

The second question is whether these cleavages, namely ϵ -, ζ -, and γ -cleavages, are catalyzed by the same γ -secretase or different enzymes. The finding that ϵ -, ζ -, and γ -cleavages can be differentially inhibited by different inhibitors, i.e. ϵ -/ ζ -cleavages are inhibited by transition state analogs and γ -cleavage is inhibited by non-transition state inhibitors, suggests several possibilities. First, these cleavages may be catalyzed by two enzymes, one of them sensitive to transition state analogs and the other sensitive to non-transition state inhibitors. Second, these cleavages may be catalyzed by one enzyme with two inhibitor binding sites, one for the transition state analogs, such as L-685,458, and the other for the non-transition state inhibitors, such as compound E, as suggested by a recent inhibitor-binding kinetic study [89]. In regard to the question of whether there is only one enzyme or multi-enzymes involved in the sequence of ϵ -, ζ -, and γ -cleavages, the precursor and product relationship between the long forms of A β species and the short forms of A β species, the sequential relationship of these cleavages, and specifically the finding that γ -cleavage is dependent on ϵ - and ζ -cleavages occurring first, strongly suggest that γ -, ζ -, and ϵ -cleavages are catalyzed by a single enzyme. If it is not the same enzyme, it is hard to imagine how, after ϵ - and ζ -cleavages, the intermediate A β_{46} is transferred from the enzyme that produced it to another enzyme for further processing into A $\beta_{40/42}$. In fact, the single enzyme model is further strongly supported by the finding reported by the same study that the intermediate A β_{46} is tightly associated with PS1, the catalytic subunit of the γ -secretase complex, indicating that the same PS1-dependent enzyme executes the whole series of the ϵ -, ζ -, and γ -cleavage [87]. The one enzyme model is also supported by the fact that both groups of inhibitors have been shown to bind to presenilins [58,90,91] and the finding that equimolar amounts of CTF ϵ and A β are produced from the cleavage of CTF β by γ -secretase [92].

The third question is, how does a single enzyme sequentially cleave a peptide at multiple sites over a sequence of ten amino acids? In this regard, several studies have reported the identification of intermediate A β_{43} ([84,85,88], see also Fig. 3). This A β_{43} was even detectable in the conditioned medium when the cells were cultured in the presence of transition state analog 31C, probably because 31C may cause a conformational change in the γ -secretase complex, leading to a looser association of A β_{43} with the γ -secretase complex and resulting in

the release of this intermediate [88]. The detection of the intermediates, $A\beta_{49}$, $A\beta_{46}$, and $A\beta_{43}$, revealed an interesting pattern of γ -secretase processing of CTF β , i.e., γ -secretase cleaves CTF β every three residues to produce the major secreted $A\beta$ species, $A\beta_{40}$ [85,87,88]. How does the one enzyme model account for the sequential cleavage of every three residues? Evidence suggests that the $A\beta$ PP transmembrane domain is in an α -helical conformation [56, 93]. Based on this α -helical model, as shown in Fig. 6A, in their native state, the peptide bonds between residues $A\beta_{46}$ and $A\beta_{47}$ and residues $A\beta_{43}$ and $A\beta_{44}$ are aligned to the center of the putative enzyme targeting side, this may account for the fact that the major intermediate $A\beta$ species are detected as $A\beta_{46}$ and $A\beta_{43}$ [88]. According to this model, the peptide bonds between residues $A\beta_{41}$ and $A\beta_{42}$ and between $A\beta_{48}$ and $A\beta_{49}$ are on the side that is precisely opposite to the side on which the peptide bonds between residues $A\beta_{46}$ and $A\beta_{47}$ and residues $A\beta_{43}$ and $A\beta_{44}$ are aligned. This may account for the fact that cleavages between these residues hardly occur; as a result, no $A\beta_{41}$ and only a very low level of $A\beta_{48}$ was reported by a recent study [85]. After each cleavage, the α -helical structure may be slightly loosened, unfolded and stretched. This slight unfolding may cause a counterclockwise turn of the α -helix and make the next cleavable peptide bond move toward the enzyme-attacking site; this may partially explain why the majority of the secreted $A\beta$ species is $A\beta_{40}$ rather than $A\beta_{42}$. In addition, the stretch of the slightly unfolded α -helix may also help the next cleavable peptide bond to move and reach the enzyme active site. It has been hypothesized that transition state analogs and non-transition state inhibitors bind to different sites, i.e., the transition state analogs bind to the catalytic site and the non-transition state inhibitors bind to a remote site [89]. Based on this hypothesis, it is plausible that the transition state analogs may inhibit the upstream cleavages, ϵ -cleavage and ζ -cleavage, by directly binding to the catalytic site. As a result, the downstream γ -cleavage is also prevented from taking place. On the other hand, the non-transition state inhibitors may bind to a remote site of the enzyme, inducing conformational changes in the enzyme and preventing the γ -cleavage site from having access to the catalytic site of the enzyme, resulting in the preferential inhibition of γ -cleavage with less effect on the ϵ - and ζ -cleavages.

However, the one catalytic site model fails to explain the fact that $A\beta_{46}$ is still processed to $A\beta_{40/42}$ by γ -cleavage in the presence of the transition state analog, which is assumed to bind to the catalytic site [89]. Therefore, the other possibility is likely that these sequential cleavages may be catalyzed by an enzyme that has two catalytic sites, one catalyzing the ϵ -cleavage at $A\beta_{49}$ and the ζ -cleavage at $A\beta_{46}$, both of which are specifically inhibited by transition state analogs, and the other one catalyzing the cleavages at $A\beta_{43}$, $A\beta_{40/42}(\gamma)$, which are inhibited by non-transition state inhibitors [87]. Regardless of whether there is one or two catalytic sites, according to the one enzyme model, at high concentrations the non-transition state inhibitors, which preferentially inhibit γ -cleavage, may also inhibit ϵ - and ζ -cleavages by an allosteric mechanism, i.e., these compounds may induce conformational changes into the γ -secretase complex, resulting in partial inhibition of ϵ - and ζ -cleavages. The other possible explanation for the fact that non-transition state inhibitors also cause accumulation of CTF β is that in the presence of non-transition state inhibitors, which inhibit the turnover of $A\beta_{46}$ into $A\beta_{40/42}$, the accumulated intermediate $A\beta_{46}$ occupies the binding site of the γ -secretase complex and prevents the further binding of CTF β to the γ -secretase complex, resulting in accumulation of unprocessed CTF β .

The fourth question is, how does the one enzyme model account for the production of both secreted $A\beta_{40}$ and $A\beta_{42}$? Whether $A\beta_{40}$ and $A\beta_{42}$ are produced by the same enzyme or by different enzymes has been debated for a long time, and the answer still remains elusive. Based on the observations that the calpain inhibitor had different effects on the production of $A\beta_{40}$ and $A\beta_{42}$, it has been hypothesized that the different C-termini of $A\beta$ are generated by different protease activities [94,95]. A recent study also hypothesized that $A\beta_{40}$ is generated by an $A\beta_{40}$ -secretase that acts from one side of the α -helical wheel and cleaves $A\beta$ PP every three

residues (i.e., $A\beta_{49}$ to $A\beta_{46}$ then to $A\beta_{43}$ and finally to $A\beta_{40}$) and that $A\beta_{42}$ is generated by an $A\beta_{42}$ -secretase acting from the other side of the α -helical wheel and cleaving $A\beta$ PP every three residues (i.e., $A\beta_{48}$ to $A\beta_{45}$ and then to $A\beta_{42}$) [85]. However, as it has been pointed out, there is insufficient evidence to support this hypothesis of the coordinated cleavages for $A\beta_{42}$, $A\beta_{45}$, and $A\beta_{48}$ [85]. Moreover, other studies have shown that both $A\beta_{40}$ and $A\beta_{42}$ are generated from the same intermediate $A\beta_{46}$ [87,88]. In these studies, it was hypothesized that $A\beta_{46}$ generated by ζ -cleavage is mostly cleaved at $A\beta_{43}$, which is further processed at $A\beta_{40}$, resulting in the formation of major secreted $A\beta$ species $A\beta_{40}$. However, $A\beta_{46}$ may also be cleaved with low efficiency at $A\beta_{42}$ (Fig. 4). The $A\beta_{42}$ produced from $A\beta_{46}$ can be either released into the medium or undergo further processing to produce shorter $A\beta$ species such as $A\beta_{38(39)}$ [88]. It should be pointed out that, any genetic or environmental changes could cause a conformational change between the enzyme and substrate, resulting in a shift of the cleavage from $A\beta_{43}$ to $A\beta_{42}$. For example, as shown in Fig. 6B, if this relative conformational change between enzyme and substrate can be mimicked by rotating the α -helical wheel clockwise to a certain degree, the peptide bond between residues $A\beta_{43}$ and $A\beta_{42}$ would move toward the center of the enzyme-attacking site and become more susceptible to the γ -secretase cleavage. This may explain why certain mutations in $A\beta$ PP and presenilin could cause increased production of $A\beta_{42}$ with a concomitant decrease in $A\beta_{40}$. However, as shown in Fig. 6B, when the peptide bond between $A\beta_{42}$ and $A\beta_{43}$ is aligned to the center of the enzyme-attacking site, the peptide bond between $A\beta_{48}$ and $A\beta_{49}$ is still not in the favorable position. Therefore, the cleavage at $A\beta_{48}$ may occur under some circumstance, but may not be necessarily required for the production of $A\beta_{42}$.

THE PATHOLOGICAL AND PHARMACOLOGICAL SIGNIFICANCE OF THE IDENTIFICATION OF THE NEW ζ -CLEAVAGE SITE AT $A\beta_{46}$

First, according to the amyloid hypothesis that the long $A\beta$ is more amyloidogenic and more pathogenic, these long $A\beta$ species, $A\beta_{43}$, $A\beta_{46}$ and $A\beta_{49}$ may play an important role in disease development. Therefore, the discovery of these long $A\beta$ species may provide new targets for use in diagnosis and treatment of the disease. Second, the fact that $A\beta_{46}$ can be detected in the absence of inhibitor and accumulate to a high level in cells cultured in the presence of non-transition state inhibitors strongly indicates that the newly identified ζ -cleavage at $A\beta_{46}$ is another major γ -secretase-mediated cleavage site, besides the previously identified γ -cleavage at $A\beta_{40/42}$ and ε -cleavage at $A\beta_{49}$. It is also notable that the newly identified ζ -cleavage site at $A\beta_{46}$ is incidentally the AD-linked $A\beta$ PP717 mutation (also known as the London mutation) site [96,97]. Given the fact that the other AD-linked Swedish mutation site is at the β -cleavage site this finding revealed an interesting fact that the two AD-linked mutations, the Swedish mutation and the $A\beta$ PP717 mutation, actually both occur at cleavage sites that are vital for the generation of the $A\beta$ peptide. Thus, the identification that the ζ -cleavage site at $A\beta_{46}$ happens to be the $A\beta$ PP717 mutation site may open a new avenue for studying the mechanism by which this AD-linked mutation causes increased production of the more amyloidogenic $A\beta_{42}$.

The identification of ζ -cleavage is also pharmacologically significant. Prior to the identification of the new ζ -cleavage site and its product (the $A\beta_{46}$), all γ -secretase inhibitors, including both the transition state analogs and non-transition state inhibitors, were characterized by their inhibitory effects on the turnover of the initial substrate CTF β and the formation of the final product, the secreted $A\beta_{40/42}$. This is why both transition state and non-transition state inhibitors were thought to similarly inhibit γ -cleavage. The identification of $A\beta_{46}$ produced by ζ -cleavage makes it possible for the first time to determine the specificity of the γ -secretase inhibitors. Studies examining the effects of the known inhibitors on the formation and turnover of the newly identified $A\beta_{46}$ led to a novel finding that some of the compounds, such as DAPT, DAPM, and compound E, which were previously known to inhibit the formation of secreted $A\beta_{40}$ and $A\beta_{42}$, caused an intracellular accumulation of an even longer $A\beta$ species,

$A\beta_{46}$ [84,87]. According to the amyloid theory, the long $A\beta$ is more pathogenic. Therefore, these inhibitors, which prevent the formation of secreted $A\beta_{40/42}$, but cause accumulation of the long $A\beta_{46}$, are rather pharmacological poisons than potential therapeutic compounds. Thus, these findings provide information extremely important for the strategies to prevent and treat AD by the design and use of γ -secretase inhibitors. Thus, the identification of the novel ζ -cleavage at $A\beta_{46}$ and the finding the $A\beta$ peptide is produced by a series of sequential cleavages not only contribute to a better understanding of the molecular mechanism by which $A\beta$ is generated, but could also contribute to the development of treatments and methods of AD prevention.

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Fig. 1.

Secretase-mediated A β PP processing pathways. The amyloid- β protein precursor (A β PP) is a type I transmembrane protein with a large extracellular domain, a single transmembrane, and a short intracellular domain. In the predominant non-amyloid pathway, A β PP is first cleaved by α -secretase and results in the release of the large N-terminal soluble ectodomain (sA β PP α) and the generation of the 83 amino acid-long membrane anchored C-terminal fragment (CTF α). In the amyloidogenic pathway, A β PP is first cleaved by β -secretase, resulting in the release of sA β PP β and the generation of the membrane-anchored CTF β of 99 amino acids. Both CTF α and CTF β undergo further cleavage by γ -secretase within the membrane domain leading to the release of the intracellular domain (AICD) and the generation of a p3 fragment from CTF α and A β from CTF β . The gray area represents the membrane.

Fig. 2. γ -secretase complex. The γ -secretase complex is composed of four integral membrane proteins: (brown) presenilin, composed of nine transmembrane domains, (dark blue) nicastrin, composed of a single transmembrane, (orange-red) Aph-1, composed of seven trans-membrane domains, and (light blue) Pen-2, composed of two trans-membrane domains. The substrate CTF β (yellow) is also present. During maturation, presenilin undergoes endoproteolysis, and the resulting N-terminal fragment and C-terminal fragment remain associated. The two conserved aspartates (D) in the adjacent 6th trans-membrane domain and 7th transmembrane domain are essential for γ -secretase activity. Mature nicastrin is a highly glycosylated at its N-terminal ectodomain.

Fig. 3.

γ -secretase-mediated major intramembrane cleavages of A β PP. Now it is clear that γ -secretase catalyzes three major cleavages within the membrane domain of A β PP, namely the γ -cleavage at A $\beta_{40/42}$, the ϵ -cleavage at A β_{49} , and the ζ -cleavage at A β_{46} . Studies also strongly suggest that cleavage at A β_{43} is also a necessary intermediate step. It is notable that the two well-characterized AD-linked mutations, the Swedish mutation and the A β PP717 mutation, happen to occur at two major cleavage sites vital for the generation of the N-terminus and the C-termini of the A β peptides, suggesting the possibility that these mutations cause abnormal A β formation by influencing these major cleavages.

Fig. 4.

Schematic illustration of γ -secretase-mediated sequential cleavages of $A\beta$ PP. After β -secretase cleavage, the resulting C-terminal fragment of $A\beta$ PP ($CTF\beta$) undergoes a series of sequential cleavages catalyzed by γ -secretase to produce $A\beta$. As a major path, $CTF\beta$ is first scissored by ϵ -cleavage at $A\beta_{49}$, followed by ζ -cleavage at $A\beta_{46}$ to produce the major intermediate $A\beta_{46}$. $A\beta_{46}$ is mainly processed at $A\beta_{43}$ and the resulting $A\beta_{43}$ is further processed into $A\beta_{40}$, which can be further processed into $A\beta_{37}$, but is principally released from the γ -secretase complex and becomes the major form of secreted $A\beta$ species. Alternatively, $A\beta_{46}$ can also be processed at $A\beta_{42}$ at a low efficiency, and the resulting $A\beta_{42}$ can be either further processed into $A\beta_{38(39)}$ or released at a low rate.

Fig. 5.

Schematic illustration of γ -secretase-mediated A β PP processing and A β formation. Using electronic microscopy and single-particle image analysis on the purified active γ -secretase complex, a 3D structure of this enzyme has been recently proposed [98]. Based on this 3D structure, the γ -secretase complex contains a cylindrical interior chamber, in which the catalytic site is located, with two channels facing the two sides of membrane. It has been assumed that the two channels would allow the entry of water molecules, their access to the γ -secretase-active site, and the release of the cleavage products. Based on this electronic microscopic 3D structure, the γ -secretase-mediated sequential cleavages and A β formation are dynamically illustrated. Since the ϵ -cleavage site is close to the membrane boundary and is easily accessed by water molecules in the cytosol, ϵ -cleavage occurs first (step 1). The initial ϵ -cleavage may not only release CTF ϵ (AICD), but may also create a path for the water molecules to have access to the next cleavage site, the ζ -cleavage site, and trigger ζ -cleavage to occur. The result is the release of an additional three amino acids (red dot, step 2), thus, allowing the water molecules further access to the γ -cleavage site and allowing the final cleavage step (step 3) to occur, leading to the formation of secreted A $\beta_{40/42}$. Then the γ -secretase complex may be disassembled and subjected to recycling or disposal.

Fig. 6. α -helical wheel arrangement of amino acids 37 to 52 of CTF β , a view from the cytosol side. The C-terminal residue of each detectable major A β species produced by γ -secretase-mediated cleavages is shown in gray with a bold outline. Note, under normal conditions (Fig. 6A), the peptide bond between residues 46 and 47 and the peptide bond between residues 43 and 44 are aligned to the center of the lower half of the α -helical wheel, which may be the enzyme attacking site. Hydrolyses of these peptide bonds produce the major intermediate A β_{46} and A β_{43} . It is noted that after each cleavage, the α -helical structure may be slightly loosened, unfolded and stretched, resulting in a counterclockwise turn of the α -helix and this makes the peptide bond between residues 40 and 41 move toward the enzyme-attacking site; this may partially explain why the majority of the secreted A β species is A β_{40} rather than A β_{42} . In contrast, under some circumstances, such as environmental or genetic abnormalities, specifically, in the presence of AD-linked mutations in presenilin or in A β PP, the relative position between the γ -secretase active site and its substrate may be altered. As shown in Fig. 6B, if these changes in the position of CTF β relative to the γ -secretase active site can be mimicked by rotating the CTF β α -helical wheel clockwise 60°, the peptide bond between A β_{42} and A β_{43} will move toward the center of the lower half wheel and become much more susceptible to γ -secretase activity, resulting in an increase in the formation of A β_{42} .