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Major Carboxyl terminal fragments generated by Gamma-secretase processing of the Alzheimer's amyloid precursor are 50 and 51 aa in length

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Abstract

Objectives—To understand the cleavage of the amyloid β protein ($A\beta$) precursor by γ -secretase and determine its changes in a representative Familial (FAD) Alzheimer's disease (AD) mutation.

Methods—Transfected cells expressing wild type and FAD mutant APP were analyzed for changes in the levels of the major secreted $A\beta$ species and of the corresponding intracellular C-terminal APP fragments (APP intracellular domain, AICD) generated by γ -secretase whereas radiosequencing was used to precisely identify the resulting cleavage site(s).

Results—The AICD fragment(s) generated by γ -secretase cleavage co-migrated in gels with a 50 residue synthetic peptide used as control being, therefore, smaller than the expected 59 and 57 residues based on the classical $A\beta$ size ending at positions 40 ($A\beta$ 40) and 42 ($A\beta$ 42), respectively. In agreement with previous findings, an FAD mutant form of presenilin 1 (PS1-M139V) significantly increased the longer $A\beta$ 42 while showing trends towards reducing $A\beta$ 40. AICD levels were reduced by the mutation suggesting that γ -secretase activity may be actually impaired by the mutation. Radiosequence analysis in cells expressing wild type PS1 detected γ -secretase cleavage sites at the $A\beta$ peptide bond L⁴⁹-V⁵⁰ to generate a 50 aa AICD fragment (AICD50) and the $A\beta$ peptide bond T⁴⁸-L⁴⁹, generating an AICD of 51 aa (AICD51). No other cleavage sites were reliably detected.

Conclusions—Based on findings that the FAD mutation that increases $A\beta$ 42 also reduces AICD, we propose that γ -secretase activity is impaired by FAD mutations and predict that physiological and environmental agents that inhibit γ -secretase will actually induce AD pathogenesis rather than preventing it. Furthermore, we propose that the cleavage site to generate AICD is naturally ragged and occurs predominantly at two sites 48 and 49 aa from the start of the $A\beta$ sequence. Thus, end specific antibodies to these two sites will need to be generated to study the quantitative relationships between these two cleavages in sporadic AD and FAD.

Introduction

APP is a type I integral membrane protein with a short half-life due to its cleavage in the juxtamembrane region by an enzyme named α -secretase^{1–3}. This cleavage yields a secreted derivative, sAPP α (612–687 aa), which includes the first 16 aa of the $A\beta$ sequence and a

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membrane-bound fragment, CTF α (83 aa), which is further cleaved by in the intramembrane protease, γ -secretase, to yield a 3 kDa fragment (P3 or A α) starting at A β position 17⁴. In contrast, BACE1 cleaves APP on the N-terminal side of A β (β site) to generate the secreted derivative, sAPP β (596–671 aa), and a membrane-bound fragment, CTF β (99 aa), which is cleaved to A β of 4 kDa by γ -secretase⁴. While most (>90%) secreted A β is 40 aa long, the accumulated deposit in the AD brain is mostly 42/43 aa long and these longer forms selectively increase in FAD mutations on APP or presenilins 1 (PS1) or 2 (PS2). A large body of literature has demonstrated that PS1 and PS2 are active subunits of γ -secretase, which is a complex of four integral membrane proteins: PS1/2, anterior pharynx 1 (Aph1), presenilin enhance 2 (Pen2) and Nicastrin (NCT) that can be coexpressed in yeast to generate the active enzyme^{5,6}. A β 42 tends to form multimeric complexes more readily than A β 40 and some of the oligomeric intermediates are neurotoxic in culture, supporting a hypothesis that a neurodegenerative cascade triggered by A β aggregates (amyloid hypothesis) causes AD^{7,8}. The longer A β 42 forms increases in FAD and provides the strongest support for the amyloid hypothesis. Approximately eight years after the discovery of A β , one group isolated the APP intracellular fragment, AICD, from brain⁹. At the same time, we developed an assay to show that γ -secretase cleavage yields substantial quantities of a cytoplasmic APP-CTF and named it CTF γ (a.k.a. AICD, C γ , CTF ϵ , AID) that is lost in cells due to rapid degradation^{10,11}. Our study triggered several follow up studies that identified the AICD cleavage site as the junction of A β 49–50 and the fragment size as 50 aa and called it the ϵ site^{12–14}. One group studied the membrane A β fragment more extensively and demonstrate the presence of substantial quantities of A β 46 and called it the ζ site¹⁵. Other studies described a novel serial cleavage pathway where the initial γ -secretase cleavage at A β 49 generates additional cleavages in 3 aa intervals to generate A β of 38–43 aa^{15,16}. Detection of an alternative minor cleavage site at A β 48 led to the hypothesis that A β 48 generates A β 45 and A β 42 while A β 49 is processed to A β 46, A β 43 and A β 40, suggesting that the misprocessing occurs early in the sequential γ -secretase cleavage^{17,18}. However, these studies failed to detect any A β 49 and suggest that A β 49 is rapidly converted to A β 46 but A β 48 is more stable and give rise to lower levels of A β 45 and A β 42^{17–19}. Moreover, it was proposed that A β 46 couldn't give rise to A β 42, based on inhibitor and kinetic analyses¹⁷. However, these studies are contradicted by site-directed mutagenesis studies providing strong evidence that A β 46 can generate A β 40, A β 42 and A β 38^{16,20}. As discussed earlier, mass spectroscopy studies showed that ϵ cleavage by γ -secretases generates mostly AICD50 and small quantities of AICD51²¹. Since matrix effects and differential ability to quantitatively detect fragments are known to affect the interpretation of mass spectroscopy data, we chose to detect the exact cleavage site of AICD by radiosequencing^{22,23}. Our data confirm that primary cleavage of APP by γ -secretase to generate AICD is predominantly at residues A β 48 and A β 49 providing independent evidence that there is no hint of cleavage at A β 40 or A β 42. We also describe a second cleavage that generates AICD51 and provide evidence that these are the only two major cleavage sites for AICD production. Further, we previously showed that inhibition of γ -secretase by an FAD mutation increases A β 42, and provides an alternate model for the increase in A β 42 generation based on the reduced rate of turnover of its immediate precursors using antisense PS1 RNA²⁴. These studies support a hypothesis that inhibition of γ secretase may be a cause of AD, a problem that needs to be evaluated in typical late onset AD as independently proposed by Jie Shen and us previously^{25,26}.

Materials and Methods

Cells and reagents

Chinese hamster ovary (CHO) transfected with the 695 aa neuronal variant of wild type APP (2B7) has been described previously²⁷. Transient transfections of wild type and mutant PS1

was carried out using HEK293H (Invitrogen (Carlsbad, CA), which is very efficiently transfected by lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described previously (29). Radioactive ^{35}S methionine was purchased from Perkin Elmer-New England Nuclear (Massachusetts, USA). Antibodies O443, BAN50, BCO5 and BA27 against the C-terminal 20 aa of APP, 1–16 aa of A β , A β 42 end and A β 40 end were described previously. Peptide standards were from rPeptide (Augusta, GA) for A β and Calbiochem (EMD, San Diego, CA) for AICD fragments of 50 and 57 aa. Mutant APP and presenilin (PS)-1 constructs were described previously ^{24, 28}. Precast Bis-Tris gels and related reagents were obtained from Invitrogen (Carlsbad, CA). Other reagents and cell culture media and assay kits were obtained from either VWR or Fisher Scientific as described previously.

Western blot analysis for AICD

CHO-2B7 cells were cultured in 100 mm dishes in Opti-MEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum to ~ 70% saturation. The cells from two plates were scraped with a rubber policeman and resuspended in ice cold 50 mM HEPES, 0.15M NaCl, pH 7.0 containing the Roche protease inhibitor cocktail (HBS) and differentially centrifuged at 2500 rpm ($600 \times g$) for 10 min and the supernatant (S1) was centrifuged at 10,000 rpm ($12,000 \times g$) for 15 min to collect the pellet (P2). The pellet was resuspended in ice cold HBS, washed and resuspended in the same buffer. The washed P2 was incubated at 37°C for 2 h and centrifuged once again at 14,000 rpm ($20,000 \times g$) for 15 min to remove the membrane and recover the supernatant containing AICD as described previously ¹¹. Supernatants were separated on precast 10% Bis-Tris gels and electrophoresed using the MES buffer system to ensure separation of low molecular weight fragments in the 4–6 kDa range. The gels were transferred onto nitrocellulose membranes (VWR) in a transfer buffer consisting of 25 mM Tris, 191 mM glycine and 20% methanol using a Bio-Rad wet transfer apparatus for 90 min at 90 V. The blots were dried overnight, washed extensively in distilled water for 15 min, soaked in boiling phosphate buffered saline (Fisher Scientific) for 10 min, rinsed in distilled water and blocked for 1–2 h in 10% newborn bovine serum (NBS) in 50 mM Tris pH 8.0, 0.15 M NaCl (TBS) containing 0.05% Tween-20 (TBST). We find that this step improves signal of nearly all Western blots and therefore extends beyond antigen retrieval. The blocked membranes were washed extensively with TBST (6×10 min) before incubation with O443 (10,000 fold dilution) in TBS containing 2% NBS for 1–2 h. Following extensive wash in TBST as described earlier, the blots were probed with horseradish peroxidase-labeled goat anti-rabbit antibody (Jackson Immunoresearch, 1:7500) for 1 h and washed extensively again in TBST. The blots were then incubated in super signal west pico chemiluminescent substrate (Fisher Scientific) and exposed to x-ray film to capture the signal. The blots were scanned using an Umax scanner with transparency attachment and quantified by the Molecular Dynamics Storm software-image quant. The procedure detects as little as 0.1 fmole of synthetic AICD-C50 and AICD-C57 peptides.

Radiolabeling and enzyme assay

Ten dishes of 100mm were seeded with CHO-2B7 cultures and grown to 70% confluence. Cells were washed once with Hank's balanced salt solution (Gibco), equilibrated in methionine-deficient medium (Gibco) for 30 min and subjected to metabolic labeling for 16 h in methionine-deficient medium containing 1 mCi of ^{35}S methionine (ICN), as previously reported ²². AICDs were generated as described above and immunoprecipitated with the O443 antibody against the 20 C-terminal residues of APP, separated on 10% Bis-Tris gels with MES buffer and transferred to Immobilon P (Millipore) using a Bio-Rad (Hercules, CA) Western blotting apparatus, as described by the manufacturer. The membrane was exposed to X-ray film overnight and the radioactive band was identified, marked and excised along with surrounding regions for Edman degradation using a protein sequencer (Applied Biosystems; Foster City, CA). The released amino acid from each sequencing

cycle was recovered and ^{35}S Methionine radioactivity assessed in a Beckman LS-250 liquid scintillation counter.

Statistical Analysis

A β and AICD from wild type and mutant PS1-expressing cells were compared by paired two tailed Student's t test using GraphPad Prism version 6.

Results

Most AICD released comigrates with C50 rather than C57

CHO-2B7 cells membranes were fractionated to obtain the P2 fragment and incubated in vitro for γ -secretase to release the cytoplasmic C-terminal domain, AICD or CTF γ . Western blot analysis of the supernatants from the in vitro assay was carried out as described in Materials and Methods. It shows that most of the C-terminal fragment obtained in the in vitro γ -secretase assay comigrates with C50 suggesting that it is the major peptide generated by γ -secretase cleavage (Fig 1). A high level of MG132 (50 and 200 nM), a proteasome inhibitor that also inhibits γ -secretase²⁹, reduces production of the AICD fragment (Fig 1). These studies indicate that little, if any AICD of 57 or 59 residues that complements A β 40 or A β 42 is generated by γ -secretase cleavage.

Radiosequencing identifies two cleavage sites for AICD generation

Although the Western blot data in Fig 1 are definitive in not showing larger AICD fragment, a problem with this approach is that the method has a limited resolution in its ability to identify the exact cleavage sites and sizing on gel can be affected by conformation and post-translational modification. Therefore, we used Edman degradation to radio-sequence the AICD fragment generated from ^{35}S -methionine-labeled 2B7 cells. The products of Edman degradation were analyzed by scintillation counting; the number of cycles necessary to detect the radioactive methionine indicates the length of the peptide that extends towards the N-terminus of the radioactive residue. Fig 2 and Table 1 show the distribution of the radioactive amino acid peaks obtained in the first 12 cycles of Edman degradation analysis in a representative experiment. Two radioactive peaks were detected at cycles 2 and 3 whereas the rest of the cycles consistently rendered background levels of radioactivity, indicating the presence of two fragments—one containing methionine at position 2 and the other at position 3—identifying the cleavage sites at A β peptide bonds L⁴⁹-V⁵⁰ and T⁴⁸-L⁴⁹ and the generation of AICD50 and AICD51 (Fig 3). No fragments complementing A β 40 (AICD59) or A β 42 (AICD57) were detected.

These studies confirm previous mass spectroscopy findings regarding γ -secretase cleavage generating AICD fragments of 50 and 51 aa¹⁸. The study detects no AICD fragment other than the 50 and 51 aa forms suggesting that we need to study the biological effects of these two forms instead of using AICD57 and AICD59 predicted from the A β 40 and A β 42 cleavage sites.

An FAD mutant PS1 reduces AICD levels

PS1 M139V is an FAD mutation that increases the levels of A β 42 and represents an example of the major hypothesis in the AD field that toxic amyloid oligomers are responsible for the characteristic neurodegeneration and dementia³⁰. To determine whether AICD is affected by this FAD mutation, HEK293H cells were transiently transfected with either APPwt + PAG3 vector or APPwt and PS1-M139V in PAG3. Both these transiently transfected cells showed detectable A β 40 and A β 42 levels by a standard ELISA assays (Fig 4). The data (Fig 4, Left panel) show that the relative A β 42 levels increase in cells transfected with the FAD mutant PS1 as reported by several groups previously³¹. In

addition, the studies show a significant drop in the levels of AICD levels in the cells expressing mutant PS1 (Fig 4, Right panel). However, there was no significant change in A β 40 levels. These results suggest that FAD mutations may lead to partial inhibition of γ -secretase rather than modify its specificity and thereby act as dominant-negative mutations.

Discussion

The finding that FAD mutations subtly alter APP processing to generate increased levels of A β 42 has been a major foundation of the amyloid hypothesis and has been a widely repeated finding⁷. However, only a few studies have examined the effects of PS1 mutations on AICD with varying results^{21, 32}. In this study, we show that an FAD mutant form of PS1 increases A β 42 and reduces AICD levels. The drop is significant (Fig 4) and suggests that FAD mutations can reduce γ -secretase activity, at least with respect to AICD generation. We have previously shown that antisense RNA against PS1 increases A β 42 like some inhibitors of γ -secretase, which also leads to the same conclusion²⁴. Furthermore, we have previously shown that overexpression of all γ -secretase subunits together in HEK293H cells increases A β and AICD, but actually reduces the A β 42/A β 40 ratio conversely showing that increasing γ -secretase reduces processing to the longer A β 42 form and therefore reinforces the conclusion that the FAD-associated increase in A β 42 may be due to reduction of γ -secretase³³. However, it is important to note that AICD production may be independent of A β given that γ -secretase has two activities, the first named ϵ -cleavage cuts CTF β to A β 45–49 and the second γ -cleavage to generate secreted A β of 39–43 residues (Fig 5)^{19, 21}. In addition to A β 48/49, one study identified a critical intermediate, A β 46, in A β 40 and A β 42 generation and named this as ζ cleavage without attempts to detect the corresponding AICD³⁴. The current study suggests that there is no AICD corresponding in size to forms other than A β 48/49, supporting the view that they are intermediates derived from further processing of these longer A β forms^{16, 19, 20, 34–36}. Given a sequential cleavage pattern from A β 48/49 to A β 45/46 to A β 42/43 to A β 39/40, a slow or impaired γ -secretase will likely release the longer A β 42/43 forms into the media rather than convert it to the shorter 39/40 forms. We have also proposed that accumulation of the immediate substrate, CTF β , may lead to increased release of A β in spurts, which may facilitate aggregation³. However, if the proximal substrate for secreted A β 42 is A β 45–49, the phenomenon may be fostered by accumulating these intermediates rather than CTF β . Indeed, we find that partial γ -secretase inhibition can yield very high amounts of A β 40 and A β 42 presumably by accumulation CTF β and longer A β intermediates (Barnwell et al, manuscript in preparation).

The model that inhibition of γ -secretase to explain the generation of differential amounts of A β 40 and A β 42 by simple enzyme kinetics rather than by invoking a change in enzyme specificity is more parsimonious and provides a simple kinetic mechanism to explain the three observed phenomena: 1. Multiple FAD mutations spanning the entire sequence of PS1 increase A β 42/A β 40³¹; 2. Treatment with antisense RNA against PS1 or γ -secretase inhibitors increases A β 42/A β 40 ratios²⁴; and 3. Overexpression of all γ -secretase subunits reduces A β 42/A β 40 ratios²⁸.

Another implication of our model is that γ -secretase inhibition may lead to increases in secreted A β levels simply by accumulating substrate (CTF β and longer A β forms) and releasing them in spurts. This type of accumulation-release mechanism should result in a high local A β concentration and facilitate A β aggregation to its toxic oligomers³⁷. Thus, even if A β oligomers are the only cause of AD-related neurodegeneration, one of the major triggering events may be the inhibition of γ -secretase. Since γ -secretase is an essential enzyme in the whole organism complete inhibition may lead to rapid death. Even complete inhibition limited to some cell populations and may lead to accumulation of high levels of membrane-bound fragments: CTF α and CTF β , which may trigger inflammatory pathways

to clear the dysfunctional cells as recently noted in the γ -secretase deficiency associated with acne inversa^{25, 38}. Although partial γ -secretase inhibition may be less damaging, it can clearly affect critical processes such as endothelial tight junction barrier by facilitating vascular endothelial growth factor^{39, 40}. Thus, partial γ -secretase inhibition may increase A β and be damaging by multiple mechanisms that include impairment of signaling by other regulatory substrates, inflammation in response to accumulation of misfolded proteins in the membrane, and impaired membrane function due to congestion in the protein trafficking pathways caused by accumulated peptides^{26, 41}. A β is certainly one of the major accumulated peptides and could be the major toxin in initiating the disease process. However, A β 40 and A β 42 represent metabolites that are generated by normal processing of APP that is highly conserved in higher vertebrates. Under normal conditions, it is well tolerated and does not accumulate. The finding that FAD mutations inhibit γ -secretase but increase A β 42 suggests that partial inhibition of γ -secretase can be the cause of AD pathogenesis^{3, 26}. Prevention of AD must therefore involve preservation of γ -secretase rather than its inhibition.

Conclusion

Due to extensive efforts spanning behavior, imaging, neuropathology, genetics and toxicology, A β accumulation is recognized as an important trigger in dementia of the Alzheimer type. This study presents a slightly different twist to the hypothesis by presenting evidence that FAD mutations may actually impair γ -secretase, the final step in A β biogenesis, suggesting that even if A β toxicity is the trigger in AD, γ -secretase inhibition may be an incorrect approach for its treatment. In addition, the study presents details on intracellular γ -secretase cleavage products of APP and identifies fragments whose function and toxicity need to be evaluated with respect to their role in neurodegeneration and dementia.

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Figure 1.

AICD derived from CHO-2B7 cells migrate with the APP-C50 synthetic peptide. CHO-2B7 cells were cultured, membranes isolated for an in vitro γ -secretase assay, separated on Tris-Tricine 10–20% gels (Life Technologies) along with synthetic APP-C50 and APP-C57 standards and analyzed by Western blotting with the O443 antibody as described in Materials and Methods and reported previously¹¹. MG132 was used to inhibit γ -secretase cleavage as described previously. 15 and 10 kDa markers are indicated and AICD migrates at ~ 7 kDa.

Figure 2. Distribution of ^{35}S -methionine by cycles of Edman degradation. CHO-2B7 cells were cultured and radiolabeled and AICD generated from the membranes were immunoprecipitated with O443 as described in Materials and Methods. The quantity of ^{35}S -methionine released in each cycle after Edman degradation was determined by scintillation counting and the percentage of counts per minute versus total were plotted as described in Materials and Methods.

Figure 3.

Target sequence for γ -secretase cleavage showing the major cleavage sites that generate AICD.

The sequence of the C-terminal APP 101 residues starting 2 aa before A β to A β 99 is shown. The known cleavage sites are indicated by “|” and major cleavage sites identified by Edman degradation analysis are shown below the target sequence and labeled AICD50 and AICD51. The reference radioactive methionine detected by radiosequencing is indicated in bold by an asterisk **M***.

Figure 4.

FAD mutant PS1 M139V increases Aβ42 and reduces AICD levels.

APP and either wild type or mutant PS1 were transiently transfected into HEK293H cells or CHO cells and the secreted Aβ40 (pM) and Aβ42 (pM) detected by a standard sandwich ELISA assay with means and standard deviations plotted (Left panel). Membranes from the cells were isolated and assayed for changes in AICD levels by Western blotting quantified by densitometry showing means and standard deviations. Unpaired two-tailed Student's t test for Aβ42 ($t=3.496$, $df=6$, $P=0.0129^*$) and AICD ($t=2.789$, $df=4$, $P=0.0494^*$) showed significant differences between wild type and mutant cells, but Aβ40 ($t=1.748$, $df=6$, $P=0.131$) did not show statistically significant difference.

Figure 5.

Model showing APP processing by γ -secretase at the γ - and ϵ - sites

APP is cleaved to CTF α and CTF β , which are cleaved by γ -secretase at the ϵ site to release AICD of the indicated sizes with relative levels. The most common cleavage site confirmed in this study based on AICD length (Table 1) should give rise to A β 49 but this fragment has not been reported, whereas considerable amounts of A β 48 are efficiently detected¹⁹. This study detects substantial amounts of AICD of 51 aa consistent with the reports showing A β 48. The final step consists of γ -site cleavages, which occur at residues 42 and 43. Although A β 43 is readily detected in lysates, unlike A β 42, it is not detected in media¹⁹. In contrast a second γ -site cleavage at residues 38–40 release secreted A β primarily of 40 residues. However, it is possible that most of these γ -site cleavages are independent of each other even though they may be dependent on the ϵ -site cleavage.

Table 1

Yield of ^{35}S -methionine from AICD radiosequencing indicates that the cleavage occurs after A β 48 and A β 49

Cycle	% yield	Predicted AICD	Predicted A β
1	3.4	C49	AB50
2	55.0	C50	AB49
3	37.8	C51	AB48
4	0.0	C52	AB47
5	3.4	C53	AB46
6	0.0	C54	AB45
7	-0.7	C55	AB44
8	-0.7	C56	AB43
9	1.7	C57	AB42
10	0.0	C58	AB41
11	0.0	C59	AB40
12	0.0	C60	AB39