Journal of Alzheimer's Disease 18 (2009) 167–176
DOI 10.3233/JAD-2009-1132
IOS Press

The GxxxG Motif in the Transmembrane Domain of AβPP Plays an Essential Role in the Interaction of CTFβ with the γ-secretase Complex and the Formation of Amyloid-β

Guozhang Mao[a], Jianxin Tan[a], Mei-Zhen Cui[a], Dehua Chui[b] and Xuemin Xu[a,*]
[a]*Department of Pathobiology College of Veterinary Medicine, The University of Tennessee, Knoxville, TN, USA*
[b]*Peking University Health Science Center, Hai Dian District, Beijing, China*

Accepted 11 March 2009

Abstract. γ-secretase-mediated processing of the amyloid-β protein precursor (AβPP) is a crucial step in the formation of the amyloid-β peptide (Aβ), but little is known about how the substrate AβPP interacts with the γ-secretase complex. To understand the molecular events involved in γ-secretase-mediated AβPP processing and Aβ formation, in the present study we determined the role of a well conserved GxxxG motif in the transmembrane domain of AβPP. Our data clearly demonstrate that substitution of aspartic acid for the key glycine residues in the GxxxG motif almost completely abolished the formation of Aβ. Furthermore, our data revealed that substitution of aspartic acid for the glycine in this GxxxG motif disrupts the interaction of AβPP with the γ-secretase complex. Thus, the present study revealed an essential role for the GxxxG motif in the interaction of AβPP with the γ-secretase complex and the formation of Aβ.

Keywords: Alzheimer's disease, amyloid-β peptide, amyloid-β precursor protein, GxxxG motif, γ-secretase

INTRODUCTION

Accumulating evidence supports the hypothesis that the abnormal accumulation of Aβ in the brain is a causative event in the development of Alzheimer's disease (AD) [1]. Aβ is proteolytically produced from a large amyloid-β precursor protein (AβPP), which can be processed via 2 alternative pathways, the non-amyloidogenic pathway and the amyloidogenic pathway. In the non-amyloidogenic pathway, AβPP is first cleaved by the α-secretase enzyme, resulting in the release of a soluble ectodomain, sAβPPα, and a membrane-associated C-terminal fragment, CTFα. Alternatively, in the amyloidogenic pathway, AβPP is first cleaved by the enzyme β-secretase to produce a soluble ectodomain, sAβPPβ, and a membrane-associated C-terminal fragment, CTFβ. Both CTFα and CTFβ are subsequently cleaved within the membrane domain by another enzyme, referred to as γ-secretase, to produce the short peptide p3 from CTFα or the full-length Aβ from CTFβ (for review see [2]). The α-cleavage occurs within the Aβ sequence and thus precludes the formation of full-length Aβ. On the other hand, intact Aβ is derived from AβPP by the sequential action of β-secretase and then γ-secretase. Therefore, β-secretase and γ-secretase have been therapeutic targets and, specifically, since the γ-cleavage is the decisive step in the formation of the C-terminal ends of Aβ, the

*Corresponding author: Xuemin Xu, PhD, Department of Pathobiology, College of Veterinary Medicine, The University of Tennessee, 2407 River Drive, Knoxville, TN 37996, USA. Tel.: +1 865 974 8206; Fax: +1 865 974 5616; E-mail: xmx@utk.edu.

ISSN 1387-2877/09/$17.00

mechanism of γ-secretase-mediated AβPP processing has been the subject of intensive investigation.

Substantial efforts have focused on how the γ-secretase complex is formed and the role of each component in the formation of the complex. Less attention has been paid to the question of how the substrate interacts with the γ-secretase complex. A recent study suggests that nicastrin (NCT) may function as a γ-secretase substrate receptor by binding to the N-terminal of CTFβ [2]. Studies have also suggested that the GxxxG motif in a transmembrane domain of a protein may play an important role in mediating the interaction of transmembrane proteins [3]. In this regard, it is notable that this GxxxG motif is present in the transmembrane domains of both AβPP, the substrate of γ-secretase, and Aph1α, a component of the γ-secretase complex. Specifically, a recent study showed that the substitution of aspartic acid for the glycine in the GxxxG motif of Aph1α disrupts the interaction of Aph1α with other components of the γ-secretase complex [4]. In the present study, we investigated whether this GxxxG motif in the transmembrane domain of AβPP plays a role in Aβ formation and in the interaction between substrate CTFβ and the γ-secretase complex. Significantly, our results demonstrate that the GxxxG motif is essential for CTFβ interaction with the γ-secretase complex and that substitution of aspartic acid for the critical glycine residue in the GxxxG motif almost completely abolishes the formation of Aβ.

MATERIALS AND METHODS

The γ-secretase inhibitors DAPT, DAPM, compound E, and L-685,458 were purchased from Calbiochem (San Diego, CA) and dissolved in dimethyl sulfoxide. $A\beta_{40}$ and $A\beta_{42}$ were purchased from American Peptide (Sunnyvale, CA). $A\beta_{38}$ and $A\beta_{46}$ are customized peptides. The proteasomal inhibitor MG132 was purchased from Peptides International (Louisville, KY). Antibody 6E10 and antibodies against Aph1α and Pen-2 were from Covance (Dedham, MA). The antibody against nicastrin was from Sigma (St. Louis, MO). The antibodies against the N- and C-termini of PS1 and the antibody against the C-terminus of AβPP were used as described previously [5].

Cell lines and plasmids

The N2a cell line stably expressing wild type presenilin 1 (PS1wt) was established and maintained as described previously [6]. Plasmids $A\beta PP_{29D}$, $A\beta PP_{33D}$, $A\beta PP_{37D}$, $A\beta PP_{29D/33D}$, and $A\beta PP_{29D/33D/37D}$ were constructed using cDNA for AβPPsw (kindly provided by Dr. Gopal Thinakaran, University of Chicago) as a template with the Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). These plasmids express mutant AβPP containing either a single or multiple substitution of aspartic acid for the glycine in the $G_{29}xxxG_{33}xxxG_{37}$ motif at the $A\beta_{29}$, $A\beta_{33}$, and $A\beta_{37}$ positions. As controls, we also generated the plasmids $A\beta PP_{29A}$ and $A\beta PP_{33A}$, which express mutant AβPP containing a single substitution of alanine for the glycine at the $A\beta_{29}$ or $A\beta_{33}$ position.

Cell-free assay

In vitro generation of CTFε/AICD by γ-secretase activity was assayed in a cell-free assay system as described previously [5].

Immunoprecipitation and Western blotting

Regular immunoprecipitation and Western blotting were carried out as described previously [7]. For co-immunoprecipitation, the following procedure was employed. Briefly, cells cultured in the presence of 5 μM MG132 and either 3 nM compound E or 500 nM L-685, 458 for 10–12 h were harvested and homogenized in buffer A (30 mM HEPES, pH 7.5, 10 mM KCl, protease inhibitor cocktail [Roche, Indianapolis, IN]) containing 5 μM MG132 and 10 nM compound E (or 2.5 μM L-685, 458) by passing through a 20-gauge needle 30 times. The homogenized samples were centrifuged at 800 x g for 10 min to remove the unbroken cells and nuclei. The post-nuclear supernatants were centrifuged at 20,000 x g for 1 h, and the resulting membrane pellets were resuspended in 1 ml IP buffer (1% CHAPSO [8], 30 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA containing Cocktail protease inhibitors and appropriate γ-secretase inhibitor) and sonicated for 10 s on ice at 80% power employing a Fisher Sonic Dismembrator Model 300 with a 3.5 mm diameter tip. The lysates were cleared by centrifuging at 14,000 × g for 5 min at 4°C, and the supernatants were subjected to co-immunoprecipitation using appropriate antibodies, followed by Western blot analysis as described previously [5].

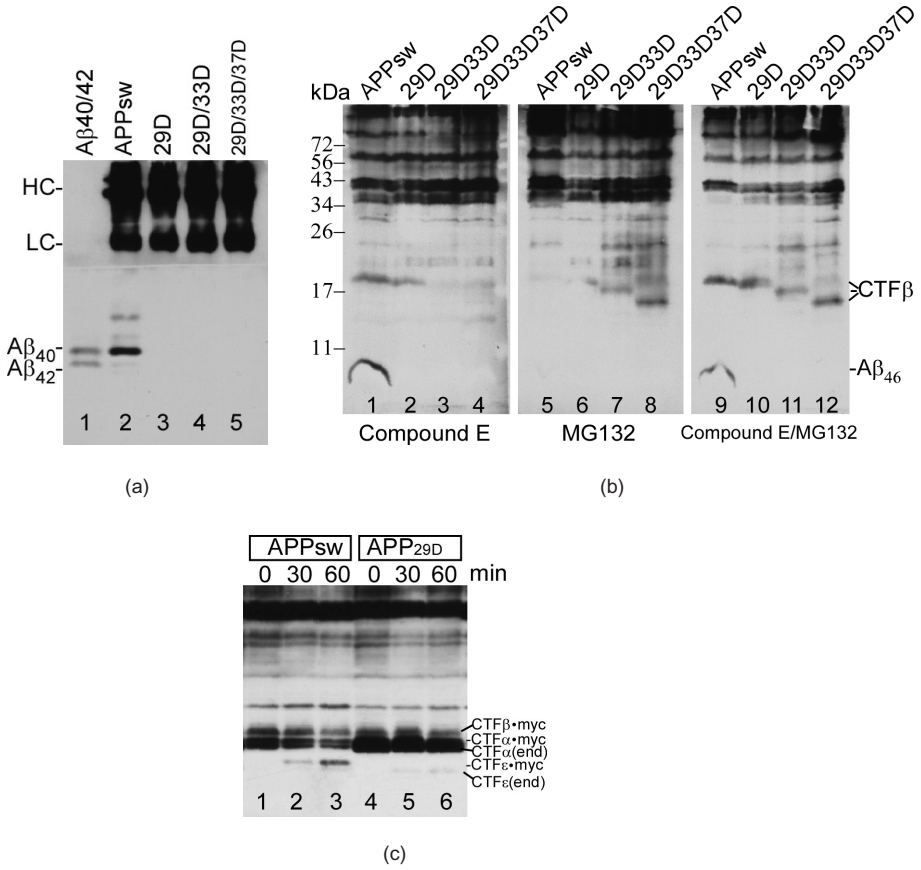

Fig. 1. Substitution of aspartic acid (D) for glycine (G) in the GxxxG motif had no effect on the formation of CTFβ, but abolished the formation of secreted Aβ and CTFε. **A**) Secreted A$\beta_{40/42}$ was immunoprecipitated from conditioned media (CM) of cells expressing Swedish mutant AβPPsw (lane 2); however, no secreted Aβ was detected in cells expressing AβPPsw containing the single G29D mutation (AβPP$_{29D}$, lane 3), double AβPP$_{29D/33D}$ mutation (lane 4), or triple AβPP$_{29D/33D/37D}$ mutation (lane 5). Secreted Aβ was immunoprecipitated from CM using 6E10, which is a monoclonal antibody raised against the residues 1–17 of the Aβ sequence [15], and analyzed by a Urea-gel system followed by Western blot analysis as described in a previous study [5]. Lane 1 is the mixture of synthetic Aβ_{40} and Aβ_{42}. Since the monoclonal antibody 6E10 was used for both immunoprecipitation and Western blotting, the heavy chain (HC) and the light chain (LC) of IgG were detected. As shown in the top band, equal amounts of soluble sAβPPα produced by α-secretase were detectable by 6E10 from these transfected cells. **B**) CTFβ produced from these aspartate mutants was degraded without undergoing γ-secretase-mediated processing. Left panel, lysates from cells treated with γ-secretase inhibitor compound E; middle panel, lysates from cells treated with proteasomal inhibitor MG132; right panel, lysates from cells treated with both compound E and MG132. **C**) No CTFε generated from AβPP$_{29D}$ was detected. Membranes were prepared from cells expressing AβPPsw and AβPP$_{29D}$, and the generation of CTFε in a cell-free system was analyzed by 10–18% regular SDS-PAGE and probed with C15, an AβPP C-terminal specific antibody, as described previously [5]. Note, CTFβ, CTFα, and CTFε generated from exogenous AβPP, which is expressed with a myc tag fused to its C-terminal, were designated as CTFβ·myc, CTFα·myc, and CTFε·myc, respectively; CTFα and CTFε generated from endogenous AβPP were designated as CTFα (end) and CTFε (end), as described in a previous studies [5,20].

RESULTS

Substitution of aspartic acid for the critical glycine residue in the GxxxG motif almost completely abolished the formation of A$\beta_{40/42}$

N2a cells stably expressing PS1, used in previous studies [5,6], were further transfected with AβPPsw (Swedish mutant AβPP) or AβPPsw containing the aspartate substitutions (AβPP$_{29D}$, AβPP$_{29D33D}$, or AβPP$_{29D33D37D}$) in which aspartic acid (D) was substituted for glycine (G) at positions Aβ_{29}, Aβ_{29}/Aβ_{33} and Aβ_{29}/Aβ_{33}/Aβ_{37}, respectively. After culturing in fresh media for 24 h, secreted Aβ was immunoprecipitated from conditioned media (CM) and analyzed using urea-gel as described in previous studies [5,7]. As shown in Fig. 1A, a significant amount of A$\beta_{40/42}$ was immunoprecipitated from CM of cells expressing AβPPsw (lane 2); however, almost no Aβ was detected in CM of cells expressing AβPP$_{29D}$ (lane 3),

AβPP$_{29D33D}$ (lane 4), or AβPP$_{29D33D37D}$ (lane 5) under the experimental conditions used in the present study.

Substitution of aspartic acid for the critical glycine residues in the GxxxG motif abolished the formation of Aβ_{46} generated by ζ-cleavage

Next, we determined whether the absence of secreted Aβ in these aspartate mutant-transfected cells is due to the inhibition of the turnover of its intermediate Aβ_{46}. Transfected cells were cultured in the presence of compound E, which has been shown to cause accumulation of intracellular Aβ_{46} [5,7]. As shown in Fig. 1B, both Aβ_{46} and CTFβ were detected in cells expressing AβPPsw (lane 1, left panel), but no Aβ_{46} was detected in the aspartate mutant-transfected cells (lanes 2 to 4). Although it was noted that a small amount of CTFβ was detected in cells transfected with AβPP$_{29D}$ (lane 2), none was detected in the double (AβPP$_{29D33D}$) and triple (AβPP$_{29D33D37D}$) aspartate mutant-transfected cells (lanes 3 and 4). These observations raised a question as to whether CTFβ is produced from the double and triple aspartate mutant-transfected cells. In addition to γ-secretase-mediated processing, CTFβ also undergoes random degradation [9]; thus, the absence of the CTFβ produced from these mutants is a result of random degradation. To address these questions, we treated the cells with the proteosomal inhibitor MG132. As shown in the middle panel of Fig. 1B, in the presence of MG132, CTFβ was indeed detected in cells transfected with AβPP$_{29D33D}$ and AβPP$_{29D33D37D}$ mutants (lanes 7 and 8). A small amount of CTFβ was also detected in AβPP$_{29D}$-transfected cells (lane 6), but almost no CTFβ was detected in cells expressing AβPPsw (lane 5). Interestingly, when the cells were treated with both compound E and MG132, similar levels of CTFβ were detected in all cells (Fig. 1B, right panel, lanes 9–12). Note that with the substitution of aspartic acid (D) for glycine (G), the migration rate of CTFβ became faster, in a dose-dependent manner.

Substitution of aspartic acid for the critical glycine residue in the GxxxG motif abolished the formation of CTFε/AICD generated by ε-cleavage

In addition to γ-cleavage at A$\beta_{40/42}$ and ζ-cleavage at Aβ_{46}, γ-secretase also catalyzes an ε-cleavage at Aβ_{49}, resulting in the release of CTFε, also known as the AβPP intracellular domain (AICD) [10–13]. Next, we performed a cell-free assay to determine the effect of substitution of aspartic acid for the glycine at Aβ_{29} in the GxxxG motif on the formation of CTFε. Both cells expressing AβPPsw and cells expressing AβPP$_{29D}$ were cultured in the presence of DAPM, which causes an accumulation of CTFβ [5], and the cell membranes were prepared as described under "Materials and Methods." As shown in Fig. 1C, CTFε produced from exogenous AβPPsw, which is expressed with a myc tag fused to its C-terminal and run slower than endogenous CTFε does, was detected when the membrane was incubated at 37°C for 30 min (lane 2) and increased in a time-dependent manner (lane 3). A low amount of CTFε generated from endogenous AβPP was also detected (lane 3), with a concomitant decrease in both CTFβ and CTFα. Interestingly, in AβPP$_{29D}$-transfected cells, only a low amount of CTFε generated from endogenous AβPP was detected (lanes 5 and 6); however, almost no CTFε produced from exogenous AβPP$_{29D}$ was detected (lanes 4–6). Concurrently, the level of unprocessed exogenous CTFβ and CTFα remained largely unchanged during the incubation period. This result indicates that mutant AβPP$_{29D}$ was not processed at the ε-cleavage site at Aβ_{49}. It was noted that the amounts of CTFβ and CTFα produced from AβPP$_{29D}$ were slightly decreased during prolonged incubation (lane 6). As discussed below, this is very likely because CTFβ and CTFα produced from AβPP$_{29D}$ do not interact with the γ-secretase complex as tightly as wild type AβPP and are not protected from random degradation. When the same cell-free assay was performed using cells expressing double and triple aspartate mutants, similarly, no exogenous CTFε was detected (data not shown).

Substitution of aspartic acid for the critical glycine residue in the GxxxG motif disturbed the interaction between CTFβ and the γ-secretase complex

To understand how the mutation in the GxxxG motif affects the formation of Aβ, we explored the possibility that these mutations may disturb the interaction of AβPP, or precisely CTFβ, with the γ-secretase complex. To do so, cells were cultured in the presence of both protosomal inhibitor MG132 and either the presence of the transition state γ-secretase inhibitor L-685, 458, which has been shown to inhibit the formation of A$\beta_{40/42}$ and result in the accumulation of CTFβ, or the presence of non-transition state inhibitor compound E, which has been shown to inhibit the formation of A$\beta_{40/42}$ but causes accumulation of the intermediate Aβ_{46} [7]. As shown

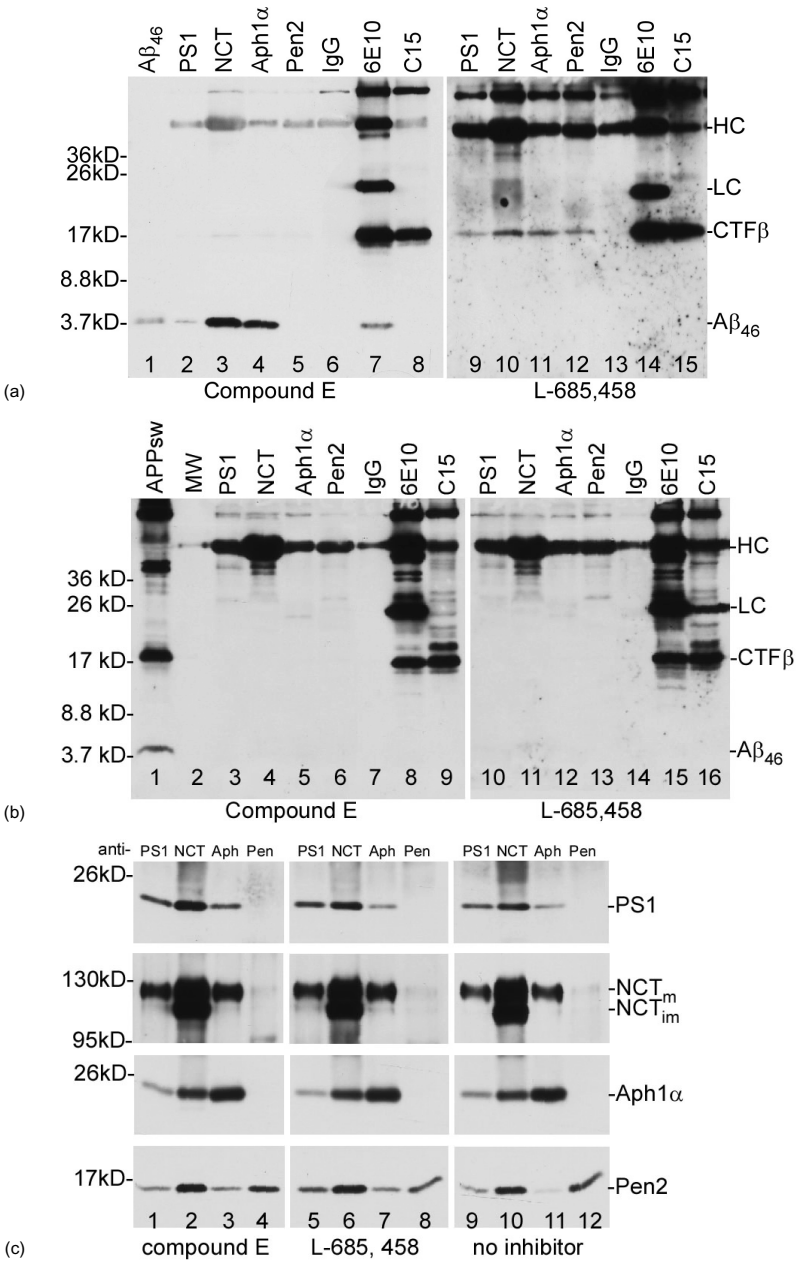

Fig. 2. AβPP$_{29D}$ mutant did not interact with the γ-secretase complex. Cells expressing AβPPsw (2A) and cells expressing AβPP$_{29D}$ (2B) were cultured in the presence of MG132 plus compound E (left panel) or in the presence of L-685, 458 (right panel). Co-immunoprecipitation was carried out using antibodies against components of γ-secretase as indicated on the top of the blot. 6E10, which is specific to Aβ, and C15, which is specific to the C-terminus of AβPP, were also used as controls. 6E10 was used to probe CTFβ and any peptide containing an Aβ sequence. Since 6E10 was used for both immunoprecipitation and Western blot, the light chain (LC) of mouse IgG was detected in lanes 7 and 14 in 2A and lanes 8 and 15 in 2B. In 2A, lane 1 is the Aβ_{46} standard. In 2B, lane 1 is the cell lysate from cells expressing AβPPsw; lane 2 is the mix of regular protein markers. 2C. AβPP$_{29D}$ mutant does not affect the assembly of the γ-secretase complex per se. Cells expressing AβPP$_{29D}$ mutant were cultured either in the presence of compound E (lanes 1 to 4), L-685, 458 (lanes 5 to 8), or no inhibitors (lanes 9 to 12). Co-immunoprecipitation was carried out using antibodies against components of γ-secretase as indicated on the top of the blot. The immunocomplexes were analyzed by regular SDS-PAGE (10–15% for PS1 and Aph1α; 8% for NCT; and 10–18% for Pen-2) and each of the γ-secretase components probed as indicated on the right of the blot. PS1 was immunoprecipitated with anti-PS1N and detected using an anti-PS1C antibody as described previously [5].

in Fig. 2A, when the AβPPsw-expressing cells were cultured in the presence of MG132 and L-685, 458, CTFβ was co-immunoprecipitated with all of the components of the γ-secretase complex, i.e., PS1 (lane 9), NCT (lane 10), Aph1α (lane 11), and Pen-2 (lane 12). When the AβPPsw-expressing cells were cultured in the presence of MG132 and compound E, $A\beta_{46}$ was co-immunoprecipitated with PS1 (lane 2), NCT (lane 3), and Aph1α (lane 4). Notably, the amount of $A\beta_{46}$ co-immunoprecipitated with NCT and Aph1α was significantly high. Interestingly, as shown in Fig. 2B, in contrast with AβPPsw-expressing cells, neither CTFβ nor $A\beta_{46}$ was co-immunoprecipitated with any of these γ-secretase components in cells expressing the $A\beta PP_{29D}$ mutant cultured in the presence of MG132 plus compound E (lanes 3–6) or L-685, 458 (lanes 10–13). To confirm the presence of CTFβ in the cell lysates, we used 6E10, an Aβ-specific antibody, and C15, an antibody specific to the C-terminus of AβPP, to perform the immunoprecipitation. As shown in lanes 8, 9, 15, and 16, CTFβ was indeed immunoprecipitated from the cell lysates of cells cultured in the presence of either compound E (lanes 8 and 9) or L-685, 458 (lanes 15 and 16). These results indicate that the CTFβ produced from $A\beta PP_{29D}$ cannot be co-immunoprecipitated with the γ-secretase complex. Similar results were observed in cells transfected with $A\beta PP_{29D33D}$ and $A\beta PP_{29D33D37D}$ aspartate mutants (Fig. 3).

Substitution of aspartic acid for the critical glycine residue in the GxxxG motif did not affect the assembly of the γ-secretase complex

It has been reported that substitution of aspartic acid for glycine in the GxxxG motif in Aph1α disrupts the assembly of the γ-secretase complex [4]. Next we determined whether the mutation in the GxxxG motif in AβPP has any effect on the assembly of the γ-secretase complex per se. To do so, cells expressing $A\beta PP_{29D}$ were cultured in the absence or presence of the γ-secretase inhibitors L-685, 458 and compound E. Cell membrane preparation and co-immunoprecipitation were performed as described under "Materials and Methods." As shown in Fig. 2C, when cells are cultured in the presence of γ-secretase inhibitors (compound E, first column; L-685, 458, second column) or absence of inhibitor (third column), similar levels of NCT (second panel, lanes 1, 5 and 9), Aph1α (third panel, lanes 1, 5 and 9), and Pen-2 (bottom panel, lanes 1, 5 and 9) were co-immunoprecipitated with PS1 by an anti-PS1 antibody. Similarly, all the components of the γ-secretase complex were co-immunoprecipitated by antibodies against NCT (lanes 2, 6, and 10) and Aph1α (lanes 3, 7, and 11). Anti-Pen-2 antibody immunoprecipitated Pen-2 itself (bottom panel, lanes 4, 8, and 12), but only trace amounts of other components of γ-secretase were co-immunoprecipitated with Pen-2 (lanes 4, 8, and 12, first, second, and third panels). This is possibly because the anti-Pen-2 antibody used in this study, upon binding to an epitope, results in a disruption of the γ-secretase complex. In addition, mainly the mature form of nicastrin was co-immunoprecipitated with other γ-secretase components (second panel across the columns).

The glycine residues in the G_{29} xxxG_{33} xxxG_{37} stretch are equally important in Aβ formation and the interaction of CTFβ with the γ-secretase complex

To further determine the importance of the glycine residue in the $G_{29}xxxG_{33}xxxG_{37}$ stretch, in addition to the $A\beta_{29}$, we have created the other two single aspartate mutations at $A\beta_{33}$ and $A\beta_{37}$. As controls, we have also created alanine mutations at $A\beta_{29}$ and $A\beta_{33}$ and determined the effects of these mutations on the formation of Aβ and the interaction of CTFβ with the γ-secretase complex. As shown in Fig. 3A, secreted Aβ was immunoprecipitated from cells expressing AβPPsw (lane 2). However, no Aβ was detected in cells expressing $A\beta PP_{29D}$ (lane 5), $A\beta PP_{33D}$ (lane 6), and $A\beta PP_{37D}$ (lane 7). These results indicate that substitution of aspartic acid for each of the three glycine residues in the $G_{29}xxxG_{33}xxxG_{37}$ stretch has a similar negative effect on the formation of Aβ. Interestingly, when the glycine residues at $A\beta_{29}$ and $A\beta_{33}$ were substituted by alanine, the resulting mutants $A\beta PP_{29A}$ (lane 3) and $A\beta PP_{33A}$ (lane 4) produced amounts of Aβ equal to those produced by AβPPsw. It was noted that the Aβ peptides produced from alanine mutant $A\beta PP_{29A}$ and $A\beta PP_{33A}$ have faster migration rates than do the Aβ peptides produced from AβPPsw. This is probably because the alanine residue is more hydrophobic (hydropathy index $= 1.8$) than glycine (hydropathy index $= -0.4$) and caused an increase in the hydrophobicity of the Aβ peptide, resulting in a faster migration of these peptides in a urea SDS-PAGE.

Next, we cultured the cells in the presence of both compound E and MG132 and performed co-immunoprecipitation using antibodies against the γ-secretase components NCT and PS1. As shown in Fig. 3B, both CTFβ and $A\beta_{46}$ were co-immunoprecipit-

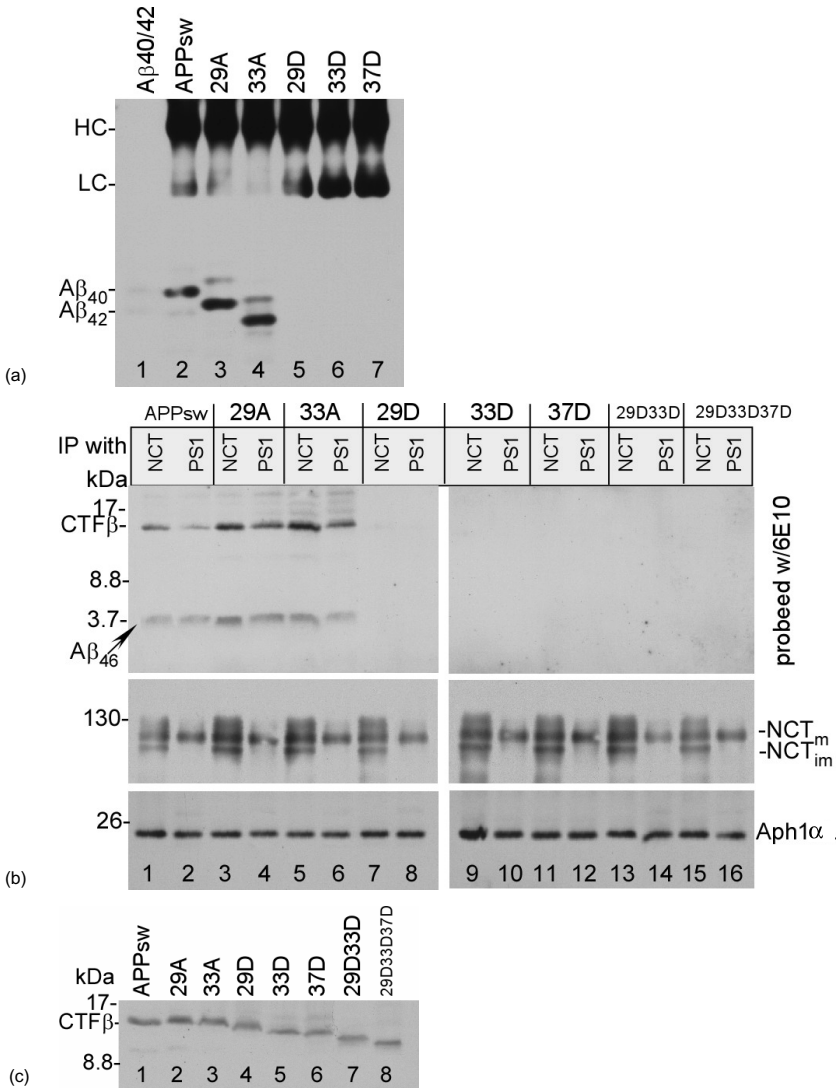

Fig. 3. Substitution of alanine for glycine in the GxxxGxxxG motif had no effect on the formation of Aβ and the interaction of CTFβ with the γ-secretase complex. **A)** Substitution of alanine for glycine in the GxxxGxxxG motif had no effect on the formation of Aβ. Secreted A$\beta_{40/42}$ was immunoprecipitated from CM of cells expressing Swedish mutant AβPPsw (lane 2), mutant AβPP29A (lane 3), and AβPP33A (lane 4), which are the AβPPsw-containing alanine mutations at Aβ_{29} and Aβ_{33}. However, no secreted Aβ was detected in cells expressing AβPPsw containing the single aspartate mutation, G29D (lane 5), G33D (lane 6), and G37D (lane 7). The fast migration rates of Aβ produced from AβPPsw containing alanine mutations is probably due to high hydrophobicity of alanine. Note that the secreted Aβ was analyzed using urea-SDS-PAGE as described in the Materials and Methods. **B)** Substitution of alanine for glycine in the GxxxGxxxG motif had no effect on the interaction of CTFβ with the γ-secretase complex. Cells expressing AβPPsw and cells expressing AβPPsw containing various alanine and aspartate mutations were cultured in the presence of compound E and MG132. Co-immunoprecipitation was carried out using antibodies against nicastrin (NCT) and presenilin 1 (PS1), the components of γ-secretase, as indicated on top of the blot. The plot was probed with 6E10 (top panel), antibodies against NCT (middle panel), and Aph1α (bottom panel). Both CTFβ and Aβ_{46} were co-immunoprecipitated with the γ-secretase components NCT and PS1 from AβPPsw-expressing cells (lanes 1 and 2) and from alanine mutant-expressing cells (top panel, lanes 3 to 6). Neither CTFβ nor Aβ_{46} were co-immunoprecipitated from single and multiple aspartate mutants AβPP (top panel, lanes 7 to 16). Both the mature and immature forms of NCT were immunoprecipitated by anti-NCT antibody (middle panel, lanes 1, 3, 5, 7, 9, 11, 13, and 15). Only the mature form of NCT was co-immunoprecipitated by anti-PS1 antibody (middle panel, lanes 2, 4, 6, 8, 10, 12, 14, and 16). Aph1α was co-immunoprecipitated by both anti-NCT and anti-PS1 antibodies (bottom panel, lanes 1 to 16). Note, the immunoprecipitate complexes were were analyzed by regular SDS-PAGE. C, In the presence of proteasomal inhibitor MG132 and γ-secretase inhibitor L-685, 458, CTFβ was detected in all cells expressing AβPPsw and all the alanine and aspartate mutants. The faster migration of the CTFβ-containing aspartate mutation is probably because of the high hydrophilicity of the aspartate residue.

ated with γ-secretase components in cells expressing AβPPsw (lanes 1 and 2) and cells expressing alanine mutant AβPP (lanes 3 to 6). However, neither CTFβ nor $Aβ_{46}$ were co-immunoprecipitated with γ-secretase components in cells expressing single or double and triple aspartate mutant AβPP (lanes 7 to 16).

When the blot was probed with anti-NCT antibody, as shown in the middle panel of Fig. 3B, both mature and immature forms of NCT were immunoprecipitated by the anti-NCT antibody (lanes 1, 3, 5, 7, 9, 11, 13, and 15), but only the mature form of NCT was immunoprecipitated by the anti-PS1 antibody (lanes 2, 4, 6, 8, 10, 12, 14, and 16). When the plot was probed with anti-Aph1α antibody, as shown in the bottom panel of Fig. 3B, a similar level of Aph1α was immunoprecipitated by both the anti-NCT antibody and anti-PS1 antibody. These results indicate that neither alanine mutations nor aspartate mutations in the GxxxG motif have any effect on the assembly of the γ-secretase complex.

Substitution of the critical glycine residue in the G_{29} xxxG_{33} xxxG_{37} motif by either aspartic acid or alanine has no effect on the formation of CTFβ

When the lysates of cells, which were cultured in the presence of both MG132 and L-685, 458, were directly analyzed by Western blotting, as shown in Fig. 3C, similar levels of CTFβ were detected by the 6E10 antibody, which is specific to the N-terminal of the Aβ sequence, in cells expressing AβPPsw and cells expressing aspartic acid and alanine mutant AβPP. As mentioned above, the substitution of aspartic acid (D) for glycine (G) resulted in a faster migration rate of CTFβ in a dose-dependent manner.

DISCUSSION

γ−secretase is a unique enzyme complex that is composed of at least four transmembrane proteins, including PS1, NCT, Aph1α, and Pen-2. These components are essential for the catalysis and proteolysis of substrates, such as AβPP and Notch, within their transmembrane domains [14]. AβPP undergoes multiple intramembranous cleavages, including at least three major cleavages, namely ε-cleavage at $Aβ^{49}$, ζ-cleavage at $Aβ_{46}$, and γ-cleavage at $Aβ_{40/42}$ to produce secreted $Aβ_{40/42}$ [5]. These cleavages apparently occur in a sequential manner, i.e., after β-cleavage of AβPP, the resulting CTFβ first undergoes ε-cleavage followed by a rapid ζ-cleavage and then γ-cleavage, commencing at the site closest to the membrane boundary and proceeding toward the site within the middle of the transmembrane domain [5]. However, little is still known about how the AβPP substrate interacts with this γ-secretase complex within the membrane domain. In the present study, we determined the role of the conserved GxxxG motifs in the transmembrane domain of AβPP in the interaction of AβPP with the γ-secretase complex. Our data clearly demonstrate that substitution of aspartic acid for glycine in the GxxxG motif, singly or doubly or triply, almost abolishes the formation of $Aβ_{40/42}$ by γ-cleavage. Our data also demonstrate that CTFβ is produced from these aspartate mutants, indicating that these mutations have no effect on the β-secretase-mediated processing of AβPP. However, our data clearly demonstrate that CTFβ produced from these aspartate mutations are neither processed by ζ-cleavage at $Aβ_{46}$ nor processed by ε-cleavage at $Aβ_{49}$ by γ-secretase. One may wonder whether the fact that Aβ is undetectable in aspartate mutant AβPP-expressing cells is because of the failure of the antibody used to recognize the Aβ species produced from these aspartate mutants. This is very unlikely, first, because the monoclonal antibody 6E10 used to detect Aβ in this study was raised against residues 1–17 of the Aβ sequence [15]. The aspartate mutations created in the GxxxGxxxG motif are within sequence $Aβ_{29}$ to $Aβ_{37}$ and are far away from the epitope recognized by 6E10. In addition, as shown in Fig. 1B, in the presence of proteasomal inhibitor, which protects CTFβ from random degradation, the antibody 6E10 was able to detect CTFβ, which contains the aspartate mutations. This result clearly indicates that these aspartate mutations have no effect on the recognition of the aspartate mutant peptides by the 6E10 antibody. Second, our data clearly showed that no CTFε was produced from these aspartate mutants (Fig. 1C), indicating that the CTFβ produced from the aspartate mutant AβPP was not processed by γ-secretase. Therefore, the fact that no secreted Aβ was detected in cells expressing aspartate mutant AβPP is because no Aβ is produced from these aspartate mutants rather than because of the inability of 6E10 to recognize these mutant peptides. To address the question of why these aspartate mutant CTFβ could be processed by γ-secretase, our data further demonstrate that the CTFβ produced from these aspartate mutants cannot be co-immunoprecipitated with the γ-secretase complex. These results strongly indicate that any one of the glycine residues in the G_{29}xxxG_{33}xxxG_{37} stretch is equally important in Aβ formation and in the interaction of CTFβ with the γ-

secretase complex. The observation that substitution of aspartic acid for the critical glycine residues in the $G_{29}xxxG_{33}xxxG_{37}$ stretch strikingly disturbs the interaction of CTFβ with the γ-secretase complex may provide an explanation for the fact that almost no CTFβ could be detected when the aspartate mutant-expressing cells were cultured in the absence of the proteasomal inhibitor MG132. The observation that CTFβ produced from these aspartate mutants was detected in cells cultured in the presence of proteasomal inhibitor MG132 strongly suggests that the CTFβ produced from these aspartate mutant AβPP was degraded by a proteasomal inhibitor MG132-sensitive enzyme without undergoing γ-secretase-mediated processing, and thus, no Aβ could be produced. In other words, these results strongly suggest that the GxxxG motif in the transmembrane domain of CTFβ are crucial for interaction of CTFβ with, and its subsequent processing by, γ-secretase. It was noted that, in the presence of γ-secretase inhibitor compound E, a low level of CTFβ was detected in cells expressing AβPP$_{29D}$ mutant, possibly because this single aspartate mutant is still able to loosely interact with the γ-secretase complex and, thus, be partially protected from random degradation. This speculation is in agreement with the observation that MG132 only partially protected CTFβ produced from AβPP$_{29D}$ and a full protection was observed by addition of both compound E and MG132 (Fig. 1B), indicating that a portion of CTFβ produced from AβPP$_{29D}$ underwent γ-secretase processing. The fact that no Aβ was detected in AβPP$_{29D}$-expressing cells may be because the level of Aβ was too low to be detected under current experimental conditions.

The GxxxG motif has been established as a framework for the helix-helix interaction of transmembrane domains [3] and has been shown to be involved in mediating both homomeric and heteromeric associations of integrin transmembrane helices [16]. Interestingly, this GxxxG motif was found in transmembrane domains of both CTFβ, the substrate of γ-secretase, and Aph1α, a component of γ-secretase. This raises a possibility that the GxxxG motifs in CTFβ and Aph1α may contribute to the interaction between CTFβ and the γ-secretase complex. This possibility is strongly supported by our data that substitution of aspartic acid for the critical glycine residues in the GxxxG motif strikingly disturbs the interaction of CTFβ with the γ-secretase complex. A recent study has suggested that nicastrin, the other component of the γ-secretase complex, may function as a receptor for γ-secretase to recruit substrate CTFβ by binding to its N-terminus [2]. Thus, the simultaneously binding by both nicastrin and Aph1α may account for the tight association of CTFβ and, specifically, intermediate Aβ_{46} with the γ-secretase complex, as reported previously [5]. This dual binding may also provide an explanation for the fact that higher amounts of CTF and, specifically, the intermediate Aβ_{46}, were co-immunoprecipitated with nicastrin and Aph1α than those with PS1 and Pen-2 (Figs 2A and 3B).

A recent study showed that the substitution of aspartic acid for glycine in the GxxxG motif of Aph1α disrupts the interaction of Aph1α with other components of the γ-secretase complex [4]. We also determined the effect of the aspartate mutations in the GxxxG motif of the AβPP transmembrane domain on the assembly of the γ-secretase complex. Our data clearly demonstrate that, in contrast to the aspartate mutations in the GxxxG motif in Aph1α, the aspartate mutations in the GxxxG motif in AβPP have no effect on the assembly of the γ-secretase complex. There are two possible explanations for this result: one is that AβPP is not required for the assembly of γ-secretase per se; the other is that the role of AβPP in the γ-secretase assembly is compensated for by other substrates of γ-secretase, such as Notch-1 or Erb-B4.

Recently it has been reported that the GxxxG motif in the transmembrane domain of AβPP may play a role in the dimerization of AβPP [17]. Substitution of alanine (A) for the key glycine (G) residues, such as G29A and G33A mutations, in the GxxxG motifs was found to alter the generation of Aβ. It has been thought that this was due to the impairment of the dimerization of AβPP [17]. However, our study demonstrated that the G29A and G33A alanine mutations have no effects on Aβ formation (Fig. 3). The observation that alanine mutation has no effect on Aβ formation was also reported by another group in their recent study [18]. Interestingly, this study also reported that the mutations, which strongly reduced Aβ formation, have no effect on the dimerization of AβPP [18]. The observation that substitution of alanine for glycine in the GxxxG motif has no effect on Aβ formation is in agreement with the fact that the GxxxG motif and 'GxxxG-like' motifs (in which one or both glycine residues are substituted by other small residues, such as alanine or serine) function similarly in mediating the interaction of transmembrane helices [19].

In summary, our data revealed that the GxxxG motif in AβPP is essential for its interaction with the γ-secretase complex and Aβ formation. Taken together, these observations suggest that the GxxxG motif plays a crucial role in the interaction of CTFβ with,

and the subsequent processing by, the γ-secretase complex. This finding is not only important for the understanding of the enzymatic mechanism of γ-secretase-mediated processing of AβPP and Aβ formation, but may also provide a therapeutic target aimed at design of inhibitors that disturb the substrate and enzyme interaction.

ACKNOWLEDGMENTS

We thank Dr. Seong-Hun Kim, Dr. Sangram S. Sisodia, and Dr. Gopal Thinakaran (University of Chicago) for providing the N2a cell line and AβPPsw plasmid. We thank Ms. Misty R. Bailey, College of Veterinary Medicine, The University of Tennessee, for her critical reading of the manuscript. This work was supported by grants from the National Institutes of Health (NS42314) and the Alzheimer's Association (IIRG) to XX and by a grant from the National Institutes of Health (HL074341) to M-ZC.

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=17).

REFERENCES

[1] Hardy J and Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297,** 353-356.

[2] Shah S, Lee SF, Tabuchi K, Hao YH, Yu C, LaPlant Q, Ball H, Dann CE, 3rd, Sudhof T, and Yu G (2005) Nicastrin functions as a gamma-secretase-substrate receptor. *Cell* **122,** 435-447.

[3] Russ WP and Engelman DM (2000) The GxxxG motif: a framework for transmembrane helix-helix association. *J Mol Biol* **296,** 911-919.

[4] Lee SF, Shah S, Yu C, Wigley WC, Li H, Lim M, Pedersen K, Han W, Thomas P, Lundkvist J, Hao YH, and Yu G (2004) A conserved GXXXG motif in APH-1 is critical for assembly and activity of the gamma-secretase complex. *J Biol Chem* **279,** 4144-4152.

[5] Zhao G, Cui MZ, Mao G, Dong Y, Tan J, Sun L, and Xu X (2005) g-Cleavage is dependent on z-cleavage during the proteolytic processing of amyloid precursor protein within its transmembrane domain. *J Biol Chem* **280,** 37689-37697.

[6] Kim SH, Leem JY, Lah JJ, Slunt HH, Levey AI, Thinakaran G, and Sisodia SS (2001) Multiple effects of aspartate mutant presenilin 1 on the processing and trafficking of amyloid precursor protein. *J Biol Chem* **276,** 43343-43350.

[7] Zhao G, Mao G, Tan J, Dong Y, Cui M-Z, Kim S-H, and Xu X (2004) Identification of a New Presenilin-dependent z-Cleavage Site within the Transmembrane Domain of Amyloid Precursor Protein. *J Biol Chem* **279,** 50647-50650.

[8] Pierce JC, Sauer B, and Sternberg N (1992) A positive selection vector for cloning high molecular weight DNA by the bacteriophage P1 system: improved cloning efficacy. *Proc Natl Acad Sci U S A* **89,** 2056-2060.

[9] Pitsi D and Octave JN (2004) Presenilin 1 stabilizes the C-terminal fragment of the amyloid precursor protein independently of gamma-secretase activity. *J Biol Chem* **279,** 25333-25338.

[10] Gu Y, Misonou H, Sato T, Dohmae N, Takio K, and Ihara Y (2001) Distinct intramembrane cleavage of the beta-amyloid precursor protein family resembling gamma-secretase-like cleavage of Notch. *J Biol Chem* **276,** 35235-35238.

[11] Sastre M, Steiner H, Fuchs K, Capell A, Multhaup G, Condron MM, Teplow DB, and Haass C (2001) Presenilin-dependent gamma-secretase processing of beta-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. *EMBO Rep* **2,** 835-841.

[12] Yu C, Kim SH, Ikeuchi T, Xu H, Gasparini L, Wang R, and Sisodia SS (2001) Characterization of a presenilin-mediated amyloid precursor protein carboxyl-terminal fragment gamma. Evidence for distinct mechanisms involved in gamma -secretase processing of the APP and Notch1 transmembrane domains. *J Biol Chem* **276,** 43756-43760.

[13] Weidemann A, Eggert S, Reinhard FB, Vogel M, Paliga K, Baier G, Masters CL, Beyreuther K, and Evin G (2002) A novel epsilon-cleavage within the transmembrane domain of the Alzheimer amyloid precursor protein demonstrates homology with Notch processing. *Biochemistry* (*Mosc*) **41,** 2825-2835.

[14] Sisodia SS and St George-Hyslop PH (2002) γ-secretase, Notch, Aβ and Alzheimer's disease: where do the presenilins fit in? *Nat Rev Neurosci* **3,** 281-290.

[15] Kim KSea (1988) Production and characterization of monoclonal antibodies reactive to synthetic cerebrovascular amyloid peptide. *Neurosci Res Comm* **2,** 121-130.

[16] Schneider D and Engelman DM (2004) Involvement of transmembrane domain interactions in signal transduction by α/β integrins. *J Biol Chem* **279,** 9840-9846.

[17] Munter LM, Voigt P, Harmeier A, Kaden D, Gottschalk KE, Weise C, Pipkorn R, Schaefer M, Langosch D, and Multhaup G (2007) GxxxG motifs within the amyloid precursor protein transmembrane sequence are critical for the etiology of Abeta42. *EMBO J* **26,** 1702-1712.

[18] Kienlen-Campard P, Tasiaux B, Van Hees J, Li M, Huysseune S, Sato T, Fei JZ, Aimoto S, Courtoy PJ, Smith SO, Constantinescu SN, and Octave J-N (2008) Amyloidogenic Processing but Not Amyloid Precursor Protein (APP) Intracellular C-terminal Domain Production Requires a Precisely Oriented APP Dimer Assembled by Transmembrane GXXXG Motifs. *J Biol Chem* **283,** 7733-7744.

[19] Senes A, Engel DE, and DeGrado WF (2004) Folding of helical membrane proteins: the role of polar, GxxxG-like and proline motifs. *Curr Opin Struct Biol* **14,** 465-479.

[20] Ikeuchi T, Dolios G, Kim SH, Wang R, and Sisodia SS (2003) Familial Alzheimer disease-linked presenilin 1 variants enhance production of both Abeta 1-40 and Abeta 1-42 peptides that are only partially sensitive to a potent aspartyl protease transition state inhibitor of "gamma-secretase". *J Biol Chem* **278,** 7010-7018.