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

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Abstract

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

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 A β in the brain is a causative event in the development of Alzheimer's disease (AD) [1]. A β is proteolytically produced from a large amyloid- β precursor protein (A β PP), which can be processed via 2 alternative pathways, the non-amyloidogenic pathway and the amyloidogenic pathway. In the non-amyloidogenic pathway, A β PP is first cleaved by the α -secretase enzyme, resulting in the release of a soluble ectodomain, sA β PP α , and a membrane-associated C-terminal fragment, CTF α . Alternatively, in the amyloidogenic pathway, A β PP is first cleaved by the enzyme β -secretase to produce a soluble ectodomain, sA β PP β , and a membrane-associated C-terminal fragment, CTF β . Both CTF α and CTF β are

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subsequently cleaved within the membrane domain by another enzyme, referred to as γ -secretase, to produce the short peptide p3 from CTF α or the full-length A β from CTF β (for review see [2]). The α -cleavage occurs within the A β sequence and thus precludes the formation of full-length A β . On the other hand, intact A β is derived from A β PP 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 A β , the mechanism of γ -secretase-mediated A β PP 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 CTF β [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 A β PP, the substrate of γ -secretase, and Aph1 α , 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 Aph1 α disrupts the interaction of Aph1 α with other components of the γ -secretase complex [4]. In the present study, we investigated whether this GxxxG motif in the transmembrane domain of A β PP plays a role in A β formation and in the interaction between substrate CTF β and the γ -secretase complex. Significantly, our results demonstrate that the GxxxG motif is essential for CTF β 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 A β .

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 β ₄₀ and A β ₄₂ were purchased from American Peptide (Sunnyvale, CA). A β ₃₈ and A β ₄₆ are customized peptides. The proteasomal inhibitor MG132 was purchased from Peptides International (Louisville, KY). Antibody 6E10 and antibodies against Aph1 α 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 A β PP 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 β PP_{29D}, A β PP_{33D}, A β PP_{37D}, A β PP_{29D/33D}, and A β PP_{29D/33D/37D} were constructed using cDNA for A β PPsw (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 A β PP containing either a single or multiple substitution of aspartic acid for the glycine in the

G₂₉xxxG₃₃xxxG₃₇ motif at the A β ₂₉, A β ₃₃, and A β ₃₇ positions. As controls, we also generated the plasmids A β PP_{29A} and A β PP_{33A}, which express mutant A β PP containing a single substitution of alanine for the glycine at the A β ₂₉ or A β ₃₃ position.

Cell-free assay

In vitro generation of CTF ϵ /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 coimmunoprecipitation, the following procedure was employed. Briefly, cells cultured in the presence of 5 μ M 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 μ M MG132 and 10 nM compound E (or 2.5 μ M L-685, 458) by passing through a 20-gauge needle 30 times. The homogenized samples were centrifuged at 800 $\times$ γ for 10 min to remove the unbroken cells and nuclei. The post-nuclear supernatants were centrifuged at 20,000 $\times$ γ 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 $\times$ γ 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].

RESULTS

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

N2a cells stably expressing PS1, used in previous studies [5,6], were further transfected with A β PP_{sw} (Swedish mutant A β PP) or A β PP_{sw} containing the aspartate substitutions (A β PP_{29D}, A β PP_{29D33D}, or A β PP_{29D33D37D}) in which aspartic acid (D) was substituted for glycine (G) at positions A β ₂₉, A β ₂₉/A β ₃₃ and A β ₂₉/A β ₃₃/A β ₃₇, respectively. After culturing in fresh media for 24 h, secreted A β 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 β _{40/42} was immunoprecipitated from CM of cells expressing A β PP_{sw} (lane 2); however, almost no A β was detected in CM of cells expressing A β PP_{29D} (lane 3), A β PP_{29D33D} (lane 4), or A β PP_{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 A β ₄₆ generated by ζ -cleavage

Next, we determined whether the absence of secreted A β in these aspartate mutant-transfected cells is due to the inhibition of the turnover of its intermediate A β ₄₆. Transfected cells were cultured in the presence of compound E, which has been shown to cause

accumulation of intracellular $A\beta_{46}$ [5,7]. As shown in Fig. 1B, both $A\beta_{46}$ and $CTF\beta$ were detected in cells expressing $A\beta PPsw$ (lane 1, left panel), but no $A\beta_{46}$ was detected in the aspartate mutant-transfected cells (lanes 2 to 4). Although it was noted that a small amount of $CTF\beta$ was detected in cells transfected with $A\beta PP_{29D}$ (lane 2), none was detected in the double ($A\beta PP_{29D33D}$) and triple ($A\beta PP_{29D33D37D}$) aspartate mutant-transfected cells (lanes 3 and 4). These observations raised a question as to whether $CTF\beta$ is produced from the double and triple aspartate mutant-transfected cells. In addition to γ -secretase-mediated processing, $CTF\beta$ also undergoes random degradation [9]; thus, the absence of the $CTF\beta$ 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, $CTF\beta$ was indeed detected in cells transfected with $A\beta PP_{29D33D}$ and $A\beta PP_{29D33D37D}$ mutants (lanes 7 and 8). A small amount of $CTF\beta$ was also detected in $A\beta PP_{29D}$ -transfected cells (lane 6), but almost no $CTF\beta$ was detected in cells expressing $A\beta PPsw$ (lane 5). Interestingly, when the cells were treated with both compound E and MG132, similar levels of $CTF\beta$ 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 $CTF\beta$ became faster, in a dose-dependent manner.

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

In addition to γ -cleavage at $A\beta_{40/42}$ and ζ -cleavage at $A\beta_{46}$, γ -secretase also catalyzes an ϵ -cleavage at $A\beta_{49}$, resulting in the release of $CTF\epsilon$, also known as the $A\beta PP$ 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 $A\beta_{29}$ in the GxxxG motif on the formation of $CTF\epsilon$. Both cells expressing $A\beta PPsw$ and cells expressing $A\beta PP_{29D}$ were cultured in the presence of DAPM, which causes an accumulation of $CTF\beta$ [5], and the cell membranes were prepared as described under “Materials and Methods.” As shown in Fig. 1C, $CTF\epsilon$ produced from exogenous $A\beta PPsw$, which is expressed with a myc tag fused to its C-terminal and run slower than endogenous $CTF\epsilon$ 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 $CTF\epsilon$ generated from endogenous $A\beta PP$ was also detected (lane 3), with a concomitant decrease in both $CTF\beta$ and $CTF\alpha$. Interestingly, in $A\beta PP_{29D}$ -transfected cells, only a low amount of $CTF\epsilon$ generated from endogenous $A\beta PP$ was detected (lanes 5 and 6); however, almost no $CTF\epsilon$ produced from exogenous $A\beta PP_{29D}$ was detected (lanes 4–6). Concurrently, the level of unprocessed exogenous $CTF\beta$ and $CTF\alpha$ remained largely unchanged during the incubation period. This result indicates that mutant $A\beta PP_{29D}$ was not processed at the ϵ -cleavage site at $A\beta_{49}$. It was noted that the amounts of $CTF\beta$ and $CTF\alpha$ produced from $A\beta PP_{29D}$ were slightly decreased during prolonged incubation (lane 6). As discussed below, this is very likely because $CTF\beta$ and $CTF\alpha$ produced from $A\beta PP_{29D}$ do not interact with the γ -secretase complex as tightly as wild type $A\beta PP$ 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 $CTF\epsilon$ was detected (data not shown).

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

To understand how the mutation in the GxxxG motif affects the formation of A β , we explored the possibility that these mutations may disturb the interaction of A β PP, or precisely CTF β , with the γ -secretase complex. To do so, cells were cultured in the presence of both proteasomal 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 CTF β , 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 A β_{46} [7]. As shown in Fig. 2A, when the A β PP^{sw}-expressing cells were cultured in the presence of MG132 and L-685, 458, CTF β was co-immunoprecipitated with all of the components of the γ -secretase complex, i.e., PS1 (lane 9), NCT (lane 10), Aph1 α (lane 11), and Pen-2 (lane 12). When the A β PP^{sw}-expressing cells were cultured in the presence of MG132 and compound E, A β_{46} was co-immunoprecipitated with PS1 (lane 2), NCT (lane 3), and Aph1 α (lane 4). Notably, the amount of A β_{46} co-immunoprecipitated with NCT and Aph1 α was significantly high. Interestingly, as shown in Fig. 2B, in contrast with A β PP^{sw}-expressing cells, neither CTF β nor A β_{46} was co-immunoprecipitated with any of these γ -secretase components in cells expressing the A β 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 CTF β in the cell lysates, we used 6E10, an A β -specific antibody, and C15, an antibody specific to the C-terminus of A β PP, to perform the immunoprecipitation. As shown in lanes 8, 9, 15, and 16, CTF β 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 CTF β produced from A β PP_{29D} cannot be co-immunoprecipitated with the γ -secretase complex. Similar results were observed in cells transfected with A β PP_{29D33D} and A β 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 Aph1 α disrupts the assembly of the γ -secretase complex [4]. Next we determined whether the mutation in the GxxxG motif in A β PP has any effect on the assembly of the γ -secretase complex per se. To do so, cells expressing A β PP_{29D} were cultured in the absence or presence of the γ -secretase inhibitors L-685, 458 and compound E. Cell membrane preparation and coimmunoprecipitation 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), Aph1 α (third panel, lanes 1, 5 and 9), and Pen-2 (bottom panel, lanes 1, 5 and 9) were coimmunoprecipitated 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 Aph1 α (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

coimmunoprecipitated 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 G₂₉ xxxG₃₃ xxxG₃₇ stretch are equally important in A β formation and the interaction of CTF β with the γ -secretase complex

To further determine the importance of the glycine residue in the G₂₉xxxG₃₃xxxG₃₇ stretch, in addition to the A β ₂₉, we have created the other two single aspartate mutations at A β ₃₃ and A β ₃₇. As controls, we have also created alanine mutations at A β ₂₉ and A β ₃₃ and determined the effects of these mutations on the formation of A β and the interaction of CTF β with the γ -secretase complex. As shown in Fig. 3A, secreted A β was immunoprecipitated from cells expressing A β PP_{sw} (lane 2). However, no A β was detected in cells expressing A β PP_{29D} (lane 5), A β PP_{33D} (lane 6), and A β PP_{37D} (lane 7). These results indicate that substitution of aspartic acid for each of the three glycine residues in the G₂₉xxxG₃₃xxxG₃₇ stretch has a similar negative effect on the formation of A β . Interestingly, when the glycine residues at A β ₂₉ and A β ₃₃ were substituted by alanine, the resulting mutants A β PP_{29A} (lane 3) and A β PP_{33A} (lane 4) produced amounts of A β equal to those produced by A β PP_{sw}. It was noted that the A β peptides produced from alanine mutant A β PP_{29A} and A β PP_{33A} have faster migration rates than do the A β peptides produced from A β PP_{sw}. 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 A β 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 coimmunoprecipitation using antibodies against the γ -secretase components NCT and PS1. As shown in Fig. 3B, both CTF β and A β ₄₆ were co-immunoprecipitated with γ -secretase components in cells expressing A β PP_{sw} (lanes 1 and 2) and cells expressing alanine mutant A β PP (lanes 3 to 6). However, neither CTF β nor A β ₄₆ were co-immunoprecipitated with γ -secretase components in cells expressing single or double and triple aspartate mutant A β PP (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-Aph1 α antibody, as shown in the bottom panel of Fig. 3B, a similar level of Aph1 α 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 G₂₉ xxxG₃₃ xxxG₃₇ motif by either aspartic acid or alanine has no effect on the formation of CTF β

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 CTF β were detected by the 6E10 antibody, which is specific to the N-terminal of the A β sequence, in cells expressing A β PP_{sw} and cells expressing aspartic acid and alanine mutant A β PP. As mentioned above, the substitution of aspartic acid (D) for glycine (G) resulted in a faster migration rate of CTF β 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, Aph1 α , and Pen-2. These components are essential for the catalysis and proteolysis of substrates, such as A β PP and Notch, within their transmembrane domains [14]. A β PP undergoes multiple intramembranous cleavages, including at least three major cleavages, namely ϵ -cleavage at A β ₄₉, ζ -cleavage at A β ₄₆, and γ -cleavage at A β _{40/42} to produce secreted A β _{40/42} [5]. These cleavages apparently occur in a sequential manner, i.e., after β -cleavage of A β PP, the resulting CTF β 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 A β PP 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 A β PP in the interaction of A β PP 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 A β _{40/42} by γ -cleavage. Our data also demonstrate that CTF β is produced from these aspartate mutants, indicating that these mutations have no effect on the β -secretase-mediated processing of A β PP. However, our data clearly demonstrate that CTF β produced from these aspartate mutations are neither processed by ζ -cleavage at A β ₄₆ nor processed by ϵ -cleavage at A β ₄₉ by γ -secretase. One may wonder whether the fact that A β is undetectable in aspartate mutant A β PP-expressing cells is because of the failure of the antibody used to recognize the A β species produced from these aspartate mutants. This is very unlikely, first, because the monoclonal antibody 6E10 used to detect A β in this study was raised against residues 1–17 of the A β sequence [15]. The aspartate mutations created in the GxxxGxxxG motif are within sequence A β ₂₉ to A β 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 CTF β from random degradation, the antibody 6E10 was able to detect CTF β , 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 CTF ϵ was produced from these aspartate mutants (Fig. 1C), indicating that the CTF β produced from the aspartate mutant A β PP was not processed by γ -secretase. Therefore, the fact that no secreted A β was detected in cells expressing aspartate mutant A β PP is because no A β 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

CTF β could be processed by γ -secretase, our data further demonstrate that the CTF β 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 G₂₉xxxG₃₃xxxG₃₇ stretch is equally important in A β formation and in the interaction of CTF β with the γ -secretase complex. The observation that substitution of aspartic acid for the critical glycine residues in the G₂₉xxxG₃₃xxxG₃₇ stretch strikingly disturbs the interaction of CTF β with the γ -secretase complex may provide an explanation for the fact that almost no CTF β could be detected when the aspartate mutant-expressing cells were cultured in the absence of the proteasomal inhibitor MG132. The observation that CTF β produced from these aspartate mutants was detected in cells cultured in the presence of proteasomal inhibitor MG132 strongly suggests that the CTF β produced from these aspartate mutant A β PP was degraded by a proteasomal inhibitor MG132-sensitive enzyme without undergoing γ -secretase-mediated processing, and thus, no A β could be produced. In other words, these results strongly suggest that the GxxxG motif in the transmembrane domain of CTF β are crucial for interaction of CTF β 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 CTF β was detected in cells expressing A β PP_{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 CTF β produced from A β PP_{29D} and a full protection was observed by addition of both compound E and MG132 (Fig. 1B), indicating that a portion of CTF β produced from A β PP_{29D} underwent γ -secretase processing. The fact that no A β was detected in A β PP_{29D}-expressing cells may be because the level of A β 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 CTF β , the substrate of γ -secretase, and Aph1 α , a component of γ -secretase. This raises a possibility that the GxxxG motifs in CTF β and Aph1 α may contribute to the interaction between CTF β 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 CTF β 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 CTF β by binding to its N-terminus [2]. Thus, the simultaneously binding by both nicastrin and Aph1 α may account for the tight association of CTF β and, specifically, intermediate A β 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 A β ₄₆, were co-immunoprecipitated with nicastrin and Aph1 α 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 Aph1 α disrupts the interaction of Aph1 α with other components of the γ -secretase complex [4]. We also determined the effect of the aspartate mutations in the GxxxG motif of

the A β PP 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 Aph1 α , the aspartate mutations in the GxxxG motif in A β PP have no effect on the assembly of the γ -secretase complex. There are two possible explanations for this result: one is that A β PP is not required for the assembly of γ -secretase per se; the other is that the role of A β PP 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 A β PP may play a role in the dimerization of A β PP [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 A β . It has been thought that this was due to the impairment of the dimerization of A β PP [17]. However, our study demonstrated that the G29A and G33A alanine mutations have no effects on A β formation (Fig. 3). The observation that alanine mutation has no effect on A β formation was also reported by another group in their recent study [18]. Interestingly, this study also reported that the mutations, which strongly reduced A β formation, have no effect on the dimerization of A β PP [18]. The observation that substitution of alanine for glycine in the GxxxG motif has no effect on A β 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 A β PP is essential for its interaction with the γ -secretase complex and A β formation. Taken together, these observations suggest that the GxxxG motif plays a crucial role in the interaction of CTF β 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 A β PP and A β formation, but may also provide a therapeutic target aimed at design of inhibitors that disturb the substrate and enzyme interaction.

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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 Aβ_{40/42} was immunoprecipitated from conditioned media (CM) of cells expressing Swedish mutant AβPP_{sw} (lane 2); however, no secreted Aβ was detected in cells expressing AβPP_{sw} 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 Aβ₄₀ and Aβ₄₂. 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 AβPP_{sw} 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 A β PP, which is expressed with a myc tag fused to its C-terminal, were designated as CTF β myc, CTF α ·myc, and CTF ϵ ·myc, respectively; CTF α and CTF ϵ generated from endogenous A β PP were designated as CTF α (end) and CTF ϵ (end), as described in a previous studies [5,20].

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Fig. 2.

AβPP_{29D} mutant did not interact with the γ-secretase complex. Cells expressing AβPP_{sw} (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 Aβ₄₆ standard. In 2B, lane 1 is the cell lysate from

cells expressing A β PPsw; lane 2 is the mix of regular protein markers. 2C. A β PP_{29D} mutant does not affect the assembly of the γ -secretase complex per se. Cells expressing A β PP_{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 Aph1 α ; 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].

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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 Aβ_{40/42} was immunoprecipitated from CM of cells expressing Swedish mutant AβPP^{sw} (lane 2), mutant AβPP^{29A} (lane 3), and AβPP^{33A} (lane 4), which are the AβPP^{sw}-containing alanine mutations at Aβ₂₉ and Aβ₃₃. However, no secreted Aβ was detected in cells expressing AβPP^{sw} containing the single aspartate mutation, G29D (lane 5), G33D (lane 6), and G37D (lane 7). The fast migration rates of Aβ produced from AβPP^{sw} 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 AβPP^{sw} and cells expressing AβPP^{sw} containing various alanine and aspartate mutations were cultured in the presence of compound E and MG132. Co-immunoprecipitation was carried out using antibodies against