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Residues at P2-P1 positions of ε- and ζ-cleavage sites are important in formation of beta-amyloid peptide

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Abstract

Most of the Alzheimer's disease (AD)-linked mutations in amyloid precursor protein (APP), which cause abnormal production of β-amyloid (Aβ), are localized at the major β-secretase- and γ-secretase cleavage sites. In this study, using an APP-knockout mouse neuronal cell line, our data demonstrated that at the P2-P1 positions of the ε-cleavage site at Aβ49 and the ζ-cleavage site at Aβ46, aromatic amino acids caused a strong reduction in total Aβ. On the other hand, residues with a long side chain caused a decrease in $Aβ_{40}$ and a concomitant increase in $Aβ_{42}$ and $Aβ_{38}$. These findings indicate that the structures of the substituting residues at these key positions strongly determine the efficiency and preference of γ-secretase-mediated APP processing, which determines the ratio of different secreted Aβ species, a crucial factor in the disease development. Our findings provide new insight into the mechanisms of γ-secretase-mediated APP processing and, specifically, into why most AD-linked APP mutations are localized at major γ-secretase cleavage sites. This information may contribute to the development of methods of prevention and treatment of Alzheimer's disease aimed at modulating γ-secretase activity.

Keywords

Alzheimer's disease; β-amyloid; γ-secretase; APP; Intramembrane processing

Introduction

The abnormal production and accumulation of β-amyloid peptide (Aβ) are believed to be the critical events in Alzheimer's disease (AD) pathogenesis. Aβ is proteolytically derived from a large amyloid precursor protein (APP) (Kang et al., 1987). In the amyloidogenic pathway, APP is first cleaved by β-secretase to generate the longer N-terminal soluble fragment sAPPβ and the short membrane-anchored C-terminal fragment C99 (CTFβ). Subsequently, CTFβ undergoes further processing by γ-secretase to release the APP intracellular C-

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terminal domain (AICD), also known as CTFε, resulting in the formation of Aβ (Selkoe and Schenk, 2003). γ-secretase cleaves APP at multiple sites in a sequential manner: first at the ε-cleavage site at Aβ49 rapidly followed by ζ-cleavage at Aβ46 and γ-cleavage at Aβ40/42, commencing at the site closest to the membrane boundary and proceeding toward the site in the middle of the transmembrane domain of APP (Zhao et al., 2005).

Molecular genetic studies have identified AD-linked mutations in three genes: *APP*, presenilin 1 *(PS1)* and presenilin 2 *(PS2)*. The *APP* gene encodes the APP protein, while the *PS1* and *PS2* genes encode PS1 and PS2 proteins, which share high homology and are believed to function as the catalytic subunit in γ-secretase. Molecular biochemical studies strongly suggest that these mutations cause disease by altering Aβ production, resulting in either elevating the level of total Aβ or specifically increasing the ratio of $A\beta_{42}/A\beta_{40}$ (Selkoe, 2001). Most of the AD-linked mutations were found in *PS1* and *PS2*. Several AD-linked mutations have been identified in *APP*. The well characterized Swedish mutation (Mullan et al., 1992) is located at the β-secretase cleavage site (where Asn-Leu were substituted for Lys-Met at the P2-P1 positions) and serves as a better substrate for β-secretase (Gruninger-Leitch et al., 2002; Tomasselli et al., 2003; Turner et al., 2001), leading to increased production of Aβ. The other well characterized APP mutation is the 717 mutation (Goate et al., 1991; Murrell et al., 1991), which causes an increase in the ratio of $A\beta_{42}/A\beta_{40}$ (Suzuki et al., 1994). It is noteworthy that this 717 mutation site happens to be the newly identified ζ-cleavage site at Aβ46 (Zhao et al., 2004). By individually replacing the membrane-domain residues of CTFβ outside the Aβ sequence with phenylalanine, Lichtenthaler et al. reported that mutations at Aβ45, Aβ46, Aβ49, and Aβ51 led to an increased $A\beta_{42}/A\beta_{40}$ ratio (Lichtenthaler et al., 1999). In a similar study, we recently reported that substitution of phenylalanine for the threonine at Aβ48 caused a dramatic decrease in Aβ formation and a concomitant accumulation in unprocessed CTFβ (Tan et al., 2008). The mutations at Aβ45 and Aβ46 are at the P2-P1 positions of the ζ-cleavage site. The mutations at Aβ48 and Aβ49 are at the P2-P1 positions of the ε-cleavage site. These observations suggest the residues at the P2-P1 positions of the upstream ε-cleavage and ζ-cleavage sites are important for γ-secretase-mediated APP processing. In the current study, we produced a series of mutations by replacing residues at these crucial positions with amino acids of different structures and sizes to determine the important roles of residues at these positions in APP processing and Aβ formation in an APP knockout mouse neuronal cell line.

Experimental procedures

General reagents

The γ-secretase inhibitors DAPM; compound E (CPDE); L-685, 458; and 31C (WPE-31C)-were from Calbiochem (San Diego, CA) and were dissolved in dimethylsulfoxide (DMSO). $A\beta_{40}$ and $A\beta_{42}$ were purchased from American Peptide (Sunnyvale, CA). $A\beta_{46}$ is a customized peptide. Monoclonal antibody 6E10 and polyclonal antibody anti-Aph-1α were from COVANCE (Dedham, MA). Polyclonal antibody anti-NCT was purchased from Sigma (St. Louis, MO). APP N-terminal-specific antibody 22C11 was from Research Diagnostics, INC (Concord MA). Polyclonal antibody C15 raised against the C-terminal 15 residues of human APP has been described previously (Zhao et al., 2004). HRP conjugated anti-rabbit and anti-mouse antibodies, Protein-A agarose beads, and ECL-Plus Western blotting reagents were all purchased from GE Healthcare, formerly Amersham Biosciences (Piscataway, NJ).

Plasmid construction and mutagenesis

The plasmid APPsw, which expresses Swedish mutant APP fused with myc-tag at its C-terminal (Thinakaran et al., 1996), was kindly provided by Dr. Gopal Thinakaran (University of Chicago). All other site-directed mutations were generated with this APPsw plasmid as a template using the Site-Directed mutaGenesis Kit (Stratagene, La Jolla, CA).

Cell culture, transfection and treatment

The mouse neuroblastoma N2a cell line (WT-7), which over-expresses both human *APPsw* and *PS1* (Kim et al., 2001) genes, and the mouse APP-knockout cell line ($APP^{-/-}$-1, (Tan, et al., 2008) were grown in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine. Using the liposome-mediated method, $APP^{-/-}$-1 cells were transfected with APPsw or its mutants using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Six hours after transfection, the medium was replaced, and the cells were cultured for an additional 30–42 h in the absence or presence of a γ-secretase inhibitor, as indicated for each specific experiment. Then the media were collected for analysis of secreted Aβ and sAPPα/β. The cells were harvested for analysis of the membrane-bound $Aβ_{46}$, full-length APP, and CTFα/β.

Immunoprecipitation and Western blot analysis

Immunoprecipitation and Western blot analysis were carried out as described previously (Zhao, Mao, Tan, Dong, Cui, Kim and Xu, 2004). Briefly, secreted Aβ was immunoprecipitated from conditioned medium using a monoclonal Aβ-specific antibody, 6E10. The immunoprecipitates were analyzed by 11% bicine/urea SDS-PAGE followed by Western blotting. For detection of the membrane-bound $Aβ_{46}$ and other APP derivatives, cells were lysed in Western blotting lysis buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitors) and separated by a 10–16% 2-step Tris-glycine SDS-PAGE system. After being transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Billerica, MA), the blots were probed with specific antibodies, and the immunoreactive bands were visualized using ECL-Plus. Protein-A agarose beads and ECL-Plus Western blotting reagents were purchased from Amersham Biosciences.

Co-immunoprecipitation

In order to determine the binding affinity between γ-secretase and its substrate, the following co-immunoprecipitation procedure, which was originally described in a previous study (Farmery et al., 2003), was employed with slight modification. Briefly, $APP^{-/-}$-1 cells, transiently transfected with APPsw or T48F mutant gene, cultured in the presence of 3 nM CPDE (or 500 nM L-685,458) for 12 h, were harvested and then homogenized in homogenization buffer A (20 mM HEPES, pH 7.4, 50 mM KCl, 2 mM EGTA, 10% glycerol and protease inhibitor mixture [Roche Applied Science, Indianapolis, IN]) containing 10 nM CPDE (or 2.5 μM L-685,458) by passing through a 20-gauge needle 30 times. The homogenized samples were centrifuged at 800 × *g* for 10 min to remove the unbroken cells and nuclei. The postnuclear supernatant was further centrifuged at 20,000 × *g* for 1 h resulting in the supernatant and pellet fractions. The resultant pellet, which contained $Aβ_{46}$, CTFα/β and γ-secretase components, was solubilized in buffer B (50mM PIPES, pH 7.0, 150mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and protease inhibitor mixture) (Li et al., 2000) containing 1% CHAPSO and 10 nM CPDE (or 2.5 μM L-685,458), for 1 h at 4°C and then centrifuged again at 20,000 × *g* for 30 min to remove the insoluble materials. The supernatant was diluted with an equal volume of solubilization buffer B to adjust CHAPSO to a final concentration of 0.5%. After pre-clearing with protein A-Sepharose beads for 3 h, the supernatant was incubated with each of the following antibodies (anti-Nicastrin, anti-APH1α, or C15) in the presence of 10 nM CPDE (or 2.5 μM L-685,458) with rotation at

4°C for 3–4 h, and then an appropriate amount of protein A-Sepharose beads was added, and incubation was continued overnight. After washing twice with solubilization buffer B containing 0.5% CHAPSO and γ-secretase inhibitors, and then twice with PBS, the immunocomplex was eluted with SDS-PAGE sample loading buffer and separated by 10–16% SDS-PAGE followed by Western blotting using 6E10 or C15 to detect the co-immunoprecipitated $A\beta_{46}$ or CTFα/β, separately.

Cell-free assay

The in vitro generation of CTFε (also known as AICD) by γ-secretase was assayed in a cell-free assay system (Pinnix et al., 2001), following the procedure described previously (Sastre et al., 2001) with minor modifications. Briefly, to determine the effect of mutations at T48 of the Aβ sequence on the generation of CTFε, $APP^{-/-}$-1 cells were transiently transfected with APPsw and its mutations. After being cultured for 24 h, the vehicle DMSO or γ-secretase inhibitor DAPM (100 nM) or L-685, 458 (0.5μM) was added, and cells were cultured for an additional 12 h. The cells were harvested in 9 volumes of homogenization buffer (10 mM MOPS, pH 7.0, 10 mM KCl) containing protease inhibitors (Complete, Roche) and homogenized by passing through a 20-gauge needle 30 times. After the removal of unbroken cells and nuclei by centrifugation at 800 × *g* at 4°C for 10 min, membranes were pelleted by centrifugation at 20,000 × *g* at 4°C for 30 min. The membranes were washed once with homogenization buffer and resuspended in assay buffer (150 mM sodium citrate pH 6.4, protease inhibitor cocktail). Aliquots of membranes were then incubated at either 0°C or 37°C for 2 h. Reactions were stopped by addition of SDS-loading buffer. After boiling for 5 min, the samples were analyzed with a 10–16% two-step, tris-glycine SDS-PAGE system followed by Western blotting probing with C15.

Results

γ-secretase-mediated processing of T48F mutant APP is normally inhibited by known γ-secretase inhibitors

In a recent study using APP knockout cells, we examined the effect of a series of F-mutations in the γ-secretase cleavage region on the formation of Aβ. We found that the mutant T48F, in which the threonine (T) at amino acid residue 48 of the Aβ sequence was replaced by phenylalanine (F), resulted in an dramatic reduction in the formation of secreted Aβ (Tan, et al., 2008). Concomitantly, significant accumulations of both CTFβ and CTFα were detected in cells expressing the T48F mutant. These results suggest that the reduction in secreted Aβ is a result of inefficient processing of CTFβ by γ-secretase (Tan, et al., 2008).

In the current study, we first examined the effects of γ-secretase inhibitors on the processing of this T48F mutant by examining expression levels of γ-secretase. We previously reported that the most commonly used commercially available γ-secretase inhibitors can be classified into two groups according to their effects on the formation and turnover of membrane-bound $A\beta_{46}$ (Zhao, et al., 2004; Zhao et al., 2007). One group includes the non-transition state inhibitors, such as CPDE, DAPT, and DAPM, which inhibit the downstream γ-cleavage site resulting in blockage of the formation of secreted $A\beta_{40}$ and $A\beta_{42}$, but cause an accumulation of their precursor, the membrane-bound $A\beta_{46}$; the other group of inhibitors, including the transition state analog inhibitors, such as L-685,458 and 31C, block the formation of Aβ by inhibiting upstream cleavage sites, i.e. ε- and ζ-cleavage sites, without the formation of intermediate $A\beta_{46}$. We next examined the effects of these inhibitors on the processing of the T48F mutant APP. $APP^{-/-}$-1 cells transfected with APPsw or T48F mutant were cultured in the absence or presence of γ-secretase inhibitors. As shown in Fig. 1, when cells were cultured in the absence of inhibitors, a large amount of secreted Aβ was detected in cells

expressing APPsw (panel C, lane 2), and a low amount of secreted Aβ was also detected in cells expressing T48F mutant (panel C, lane 3). When the cell lysates were probed with C15, almost no unprocessed CTFβ and CTFα were left in cells expressing APPsw (panel A, lane 2). In contrast, large amounts of unprocessed CTFβ and CTFα were detected in cells expressing T48F mutant (panel A, lane 3). However, when the cells were cultured in the presence of γ-secretase inhibitors, CTFβ and CTFα were accumulated to a similar level in both APPsw- and T48F-expressing cells (panel A, lanes 4 to 11). As a result, almost no secreted Aβ was detected in cells expressing APPsw or T48F mutant (panel C, lanes 4 to 11). As shown in panel B of Fig. 1, when the cell lysates were probed with 6E10, an antibody specific to the N-terminal sequence of Aβ (COVANCE, Dedham, MA), in addition to CTFβ, $A\beta_{46}$ was also detected in both the cells expressing APPsw and cells expressing T48F (lanes 8 to 11, lower part of the panel B), cultured in the presence of non-transition state inhibitors (CPDE and DAPM). However, when cells were cultured in the presence of transition state analog L-685, 458 and 31C, no $A\beta_{46}$ was formed in either transfectant (lanes 4 to 7). It was also noted that the level of $A\beta_{46}$ detected in cells expressing T48F mutant was much lower than that in cells expressing APPsw (compare lane 9 with lane 8; lane 11 with lane 10, lower part of the panel B). The resulting low level of intermediate $A\beta_{46}$ produced from T48F mutant again suggests that T48F mutant is not efficiently processed by γ-secretase in comparison with APPsw. Nevertheless, these observations indicate that γ-secretase-mediated processing of T48F mutant is inhibited by γ-secretase inhibitors in the same manner as γ-secretase-mediated processing of APP without T48F mutation, i.e., transition state analog L-685, 458 and 31C inhibit the formation of the intermediate $A\beta_{46}$ and the secreted $A\beta_{40/42}$; on the other hand, the non-transition state inhibitor CPDE block the formation of secreted $A\beta_{40/42}$, but causes an accumulation of the intermediate $A\beta_{46}$.

T48F mutation has no effect on the interaction of CTFα/β with the γ-secretase complex

The data presented in Fig. 1 suggest that T48F mutant is not efficiently processed by γ-secretase. One possible reason for this is that this T48F mutant might not efficiently interact with the γ-secretase complex. To test this possibility, we performed co-immunoprecipitation. Cells were cultured in the presence of L-685,458, which blocks the processing of CTFβ and CTFα by γ-secretase and causes accumulation of CTFα/β (Zhao, et al., 2004), and the cell lysates were subjected to immunoprecipitation using different antibodies. As shown in panel A of Fig. 2, CTFβ and CTFα (mostly CTFα) produced from APPsw as well as from T48F mutant were immunoprecipitated with C15 (lanes 6 and 7) or co-immunoprecipitated with antibodies against the γ-secretase components, namely, nicastrin (NCT, lanes 2 and 3) and Aph-1α (Aph1α, lanes 4 and 5). Since T48F mutant is not efficiently processed by γ-secretase, relatively high amounts of CTFβ and CTFα were immunoprecipitated with C15 (panel A, compare lane 6 with lane 7). Similarly, relatively high levels of CTFβ and CTFα were co-immunoprecipitated with NCT (compare lanes 2 and 3) and Aph-1α (compare lanes 4 and 5) from T48F mutant cells. Relatively high levels of CTFβ and CTFα were also detected by directly probing the cell lysates with C15 (compare lanes 8 and 9). Lane 10 is the cell lysate from WT-7 cells stably expressing APPsw, which was used as a standard for CFTβ. As shown in panel B of Fig. 2, when cells were cultured in the presence of CPDE, which blocks the formation of secreted $A\beta_{40/42}$ and causes an accumulation of membrane-bound intermediate $A\beta_{46}$, $A\beta_{46}$ was detected in both T48F mutant-expressing cells (lane 8) and APPsw-expressing cells (lane 9). This $A\beta_{46}$ has been found to be tightly associated with γ-secretase complex (Zhao, et al., 2005). As shown in panel B of Fig. 2, $A\beta_{46}$ was co-immunoprecipitated with NCT (panel B, lanes 2 and 3) as well as with Aph1α (panel B, lanes 4 and 5). Since T48F mutant produced less $A\beta_{46}$ than APPsw did (compare lanes 8 and 9 of panel B of Fig. 2), in T48F mutant-expressing cells, a low level of $A\beta_{46}$ was co-immunoprecipitated with NCT (compare lane 2 with lane 3) as well as with Aph-1α (compare lane 4 with lane 5). Lanes 8 and 9 are cell lysates. Lane 10

represents the WT-7 cells stably expressing APPsw cultured in the presence of CPDE and used as an $A\beta_{46}$ standard. These results clearly indicate that the T48F mutation has no effect on the interaction of APP, or precisely CTFα/β and $A\beta_{46}$, with the γ-secretase complex.

T48F mutation caused inefficient processing at the ε-cleavage site

Data presented in Fig. 2 indicate that the inefficient processing of T48F mutant by γ-secretase is not due to any possible altered binding affinity of T48F mutant to the γ-secretase complex. The data presented in Fig. 1 and Fig. 2 demonstrate that the T48F mutation resulted in a reduction in the formation of $A\beta_{46}$, suggesting an inefficient processing of APP at either the ζ-cleavage site at Aβ46 or at the upstream ε-cleavage site at Aβ49. Next, we examined the effect of this mutation on the ε-cleavage at Aβ49, which produces CTFε (also known as APP intracellular domain, AICD) (Zhao, et al., 2007), by employing a cell-free assay. $APP^{-/-}$-1 cells transfected with APPsw or T48F mutant were cultured in the absence or presence of γ-secretase inhibitors, L-685,458 or DAPM for 12 h. The cell membranes were prepared and subjected to cell-free assay as described in the "Materials and Methods." As shown in Fig. 3, in the absence of any γ-secretase inhibitor, most of the CTFα/β was processed and only a small amount of CTFα/β (mostly CTFα) was detected in the membrane of cells expressing APPsw (lane 3). In contrast to APPsw, a large amount of CTFα/β was detected in cells expressing the T48F mutant (lane 1), indicating a low processing efficiency of the CTFα/β produced from T48F mutant. When the cells were cultured in the presence of L-685,458, which blocks the turnover of CTFα/β by inhibiting the initial ε-cleavage (Zhao, et al., 2005), a similar amount of CTFα/β was detected in both APPsw- and T48F mutant-expressing cells (lanes 9 and 11). This result further confirms that the low level of CTFα/β detected in lane 3 is due to its quick turnover in the absence of inhibitors. When the cells were cultured in the presence of DAPM, which blocks the turnover of the intermediate $A\beta_{46}$ by γ-cleavage, but only partially inhibits the upstream ε-cleavage and ζ-cleavage (Zhao, et al., 2005), the level of CTFα/β detected in cells expressing APPsw was lower than that in cells expressing T48F mutant (Fig. 3, compare lane 7 with lane 5). The low level of CTFα/β detected in cells expressing APPsw is because most of the CTFβ/α was processed by upstream ε-cleavage and ζ-cleavage. Interestingly, when these membranes were incubated at 37°C for 2 h, a significant amount of CTFε was detected in membranes prepared from cells expressing APPsw (Fig. 3, lane 8), whereas only a small amount of CTFε was detected in membranes prepared from cells expressing the T48F mutant (lane 6), though the level of the accumulated CTFβ/α was high in T48F cells. This result strongly suggests that CTFβ produced from T48F mutant is not efficiently processed by γ-secretase at the ε-cleavage site. Since L-685,458 blocks ε-cleavage (Zhao, et al., 2005) and the turnover of CTFα/β, no detectable CTFε was produced in membranes prepared from L-685,458-treated cells (lanes 10 and 12). These results indicate that the CTFβ produced from APPsw and T48F mutant was processed at different efficiencies. A very small amount of CTFε was also detected in membranes prepared from untreated cells expressing T48F mutant (lane 2). Since the level of CTFβ was too low, no detectable CTFε was detected in untreated cells expressing APPsw (lane 4).

The effects of substitution of different amino acids for threonine at Aβ48 on Aβ formation

The results presented above suggest that substitution of phenylalanine for threonine at the position of Aβ48 has a strong effect on ε-cleavage. Next, we examined the effects of substitution of other amino acids for this threonine at Aβ48. As shown in the top panel of Fig. 4A, when the original threonine (T) was replaced by either serine (S), alanine (A), and glycine (G), no significant effect on the accumulation of CTFα/β was detected (top panel, lanes 2 to 5). When this threonine was replaced by lysine (K) or glutamic acid (E), also no accumulation of CTFα/β was observed (lanes 9 and 10). In contrast, when this threonine was replaced by tyrosine (Y, lane 7) and tryptophan (W, lane 8), as well as by phenylalanine

(F, lane 6), marked accumulation of CTFα/β was observed. Concurrently, secreted Aβ levels were very low in cells expressing T48F, T48Y, and T48W mutants (middle panel, lanes 6, 7, and 8). As summarized in Fig. 4B, in addition to the effects on total Aβ levels, these mutations also exhibited different effects on the formation of different Aβ species. For example, T48F mutant caused a decrease in $Aβ_{40}$ and a slight increase in $Aβ_{42}$ and $Aβ_{38}$ (Fig. 4A, middle panel, lane 6). Likewise, T48Y and T48W mutants caused a further decrease in $Aβ_{40}$ and a concomitant increase in $Aβ_{42}$ and $Aβ_{38}$ (Fig. 4A, middle panel, lanes 7 and 8). Interestingly, when the threonine at the Aβ48 position was replaced by charged residues, the T48K and T48E mutants also caused a decrease in $Aβ_{40}$ and a concomitant increase in $Aβ_{42}$ and $Aβ_{38}$ (with the T48K mutant having a stronger effect), but the level of total Aβ only modestly decreased (Fig. 4A, middle panel, lanes 9 and 10). Substitution of serine (S), alanine (A) and glycine (G) for the threonine at Aβ48 caused a slight increase in total Aβ and $Aβ_{40}$ with a slight decrease in $Aβ_{42}$ and $Aβ_{38}$ (Fig. 4A, middle panel, lanes 3 to 5). Next, we examined the effects of these mutations on the production of CTFε. As shown in the bottom panel of Fig. 4A, T48S, T48A, and T48G mutants had no significant effect on the production of CTFε (lanes 3 to 5). T48K and T48E mutants showed a slight decrease in CTFε levels (bottom panel, lanes 9 and 10). However, a significant decrease in CTFε production was observed in cells expressing T48F, T48Y, and T48W mutants, with a concomitant accumulation of unprocessed CTFα/β (lanes 6 to 8). The CTFα/β produced from T48E mutant migrates at a faster rate than that produced from other APP variants; this is probably due to the negative charge of the glutamic acid residues.

The effects of substitution of glycine or phenylalanine for the residues around the ε-cleavage and ζ-cleavage sites on Aβ formation

The data presented in Fig. 4A and 6 suggest that the size and the structure of the amino acids at the Aβ48 position have a strong effect on the formation of secreted Aβ. Next, we examined the effects of substitution of either the small glycine residue or the large phenylalanine residue for the residues around these ε-cleavage and ζ-cleavage sites on the formation of secreted Aβ. As shown in Fig. 5, it was noted that, among the mutations, similar to T48F mutation, L49F mutation caused a strong decrease in secreted Aβ and a concomitant increase in unprocessed CTFα/β (panels A and B, lanes 6 and 8). V46F and I45F mutations also caused a significant reduction in secreted Aβ (panel B, lanes 12 and 16) and a concomitant increase in CTFβ/α (panel A, lanes 12 and 16). It is notable that, in comparison with the glycine mutations at other positions, which caused either no change or a slight decrease in $Aβ_{42}$ and a slight increase in $Aβ_{40}$ (panel B, lanes 3, 5, and 10), the glycine mutations at Aβ49 (the ε-cleavage site) and at Aβ45 and Aβ46 (the ζ-cleavage site) resulted in increases in $Aβ_{42}$ and $Aβ_{38}$ (panel B, lanes 7, 11, and 15). Note that due to the capacity of the gel, lanes 13 to 16 were run on a separated gel with a control APPsw; thus the densities of the bands in the two panels may be different, and thus should be compared with their own internal controls.

Discussion

Secreted Aβ is produced from its precursor APP by β-secretase and γ-secretase (Selkoe, 2001). To produce the N-terminus of Aβ, APP is cleaved by β-secretase once. However, to produce the C-terminus of Aβ, APP is processed by γ-secretase at multiple cleavage sites, and these cleavages likely occur in a sequential manner: first, ε-cleavage at Aβ49, followed by ζ-cleavage at Aβ46 and finally by γ-cleavage at Aβ40/42, commencing from the membrane boundary to the middle of the APP membrane domain (Zhao, et al., 2005; Zhao, et al., 2004). There are two well characterized, AD-linked APP mutations that have been shown to either increase the level of total Aβ or specifically increase the ratio of $Aβ_{42}$ vs. $Aβ_{40}$ (Selkoe, 2001). The Swedish mutation occurs at the β-cleavage site, in which the

residues Lys-Met at the P2-P1 positions of the β-secretase cleavage site have been replaced by Asn-Leu. The P positions on the substrate are counted from the point of cleavage; P1 is the first residue towards the N-terminal, and the P1′ position is the first residue towards the C-terminal side of a cleavage site. For P nomenclature, see (Schechter and Berger, 1967). Studies have revealed that this Swedish mutation serves as a better substrate for β-secretase cleavage (Gruninger-Leitch, et al., 2002; Tomasselli, et al., 2003; Turner, et al., 2001), leading to increased production of Aβ. In this regard, it is noteworthy that the other well characterized, AD-linked 717 mutation site happens to be the newly identified γ-secretase-mediated ζ-cleavage site at Aβ46 (Zhao, et al., 2004). These findings suggest that even though these APP processing secretases, namely β-secretase and γ-secretase, cleave their substrate with a loose specificity, the primary sequences at the cleavage sites still have significant impact on the efficiency and preference of these cleavages. Thus, identification of the factors that determine the efficiency and preference of these cleavages should provide information important for understanding the mechanism of abnormal Aβ production.

In a recent study, our data revealed that substitution of phenylalanine for the threonine residue at Aβ48, the P2 position of ε-cleavage, resulted in a dramatic decrease in secreted Aβ (Tan, et al., 2008). Secreted $Aβ_{40/42}$ is produced from the intermediate $Aβ_{46}$, which is produced by ζ-cleavage (Zhao, et al., 2005). To further determine which cleavage was affected by the introduction of this T48F mutation, we examined the effect of this mutation on the formation of the intermediate $Aβ_{46}$. As shown in Fig. 1, very low $Aβ_{46}$ was detected in cells expressing T48F mutant. This result suggests that the decrease in secreted Aβ is due to an inefficient generation of the intermediate $Aβ_{46}$ by the upstream cleavages, the ζ-cleavage at Aβ46 or the ε-cleavage at Aβ49. Next, we determined the effect of this T48F mutation on the formation of CTFε produced by ε-cleavage. As shown in Fig. 3, in spite of the high level of accumulated CTFβ/α, the amount of CTFε produced from T48F-expressing cells was lower than that produced from APPsw-expressing cells, indicating that the T48F mutant was not efficiently cleaved by ε-cleavage. Taken together, these results clearly indicate that the decrease in the secreted Aβ caused by the T48F mutation is due to inefficient processing at the ε-cleavage site, the initial cleavage in the series of sequential ε-, ζ-, and γ-cleavages catalyzed by γ-secretase. These findings further confirmed the notion that downstream cleavages depend on the upstream cleavages occurring first, and that the initial ε-cleavage is a rate limiting step in the γ-secretase-mediated processing of APP (Zhao, et al., 2005).

To further determine the importance of the residue at the Aβ48 position, we made a series of mutations by replacing the threonine residue at Aβ48 (T48) with different amino acids of various sizes and polarities. It was interesting to find that the effects on the levels of total Aβ are mostly determined by the structures of the amino acids, while the effects on the ratio of different Aβ species vs. total Aβ are largely determined by the sizes of the side chain of the substituting residues. Regardless of the polarity, replacing T48 with the aliphatic amino acids: glycine (G), alanine (A), and serine (S), threonine (T, native), glutamic acid (E), and lysine (K) (Fig. 6) resulted in only a slight gradient descending in total Aβ along with the increase in length of the side chain of the substituting residues. However, in spite of the slight changes in the level of total Aβ, there was a dramatic decrease in the ratio of $Aβ_{40}/Aβ_{total}$, and a concomitant increase in the ratios of $Aβ_{42}/Aβ_{total}$ and $Aβ_{38}/Aβ_{total}$ proportional with the length of the side chains of the substituting residues. When T48 was replaced with aromatic amino acids, phenylalanine (F), tyrosine (Y), and tryptophan (W), the level of total Aβ was significantly decreased in comparison with the aliphatic acid substitutions, but only modest differences in the total Aβ were observed among these aromatic amino acid substitutions. Interestingly, similar to the case of aliphatic acid substitutions, a dramatic decrease in the ratio of $Aβ_{40}/Aβ_{total}$ and a concomitant increase in the ratio of $Aβ_{42}/Aβ_{total}$ (specifically, the ratio of $Aβ_{38}/Aβ_{total}$) were observed proportional

with the increase in size of the side chain of the substituting aromatic amino acids. Since there is almost no detectable $A\beta_{40}$ in cells expressing T48Y and T48W mutants, the increase in the ratio of $A\beta_{38}/A\beta_{total}$ corresponds with a decrease in $A\beta_{42}/A\beta_{total}$. This result provides further support for the notion that $A\beta_{38}$ is produced from $A\beta_{42}$ (Zhao, et al., 2007). It should be pointed out that, in this study, we used the concepts of the ratios of $A\beta_{40}/A\beta_{total}$, $\beta_{42}/A\beta_{total}$ and $A\beta_{38}/A\beta_{total}$ instead of the ratio of $A\beta_{42}/A\beta_{40}$ because the most effective mutations, such as the T48K, T48Y and T48W mutations, caused a dramatic decrease in $A\beta_{40}$, which was too low to be used to make a calculation of a meaningful ratio of $A\beta_{42}/A\beta_{40}$.

In addition to the residue at Aβ48, which is at the P2 position of the ε-cleavage site, we also examined the effects on the formation of Aβ of substitution of the smallest glycine and the large amino acid phenylalanine for the residue at Aβ49, the P1 position of ε-cleavage, and the residues at Aβ45 and Aβ46, which are the P2-P1 positions of the ζ-cleavage site. Our data clearly demonstrated that, in comparison with mutations at Aβ47 and Aβ50, which are the P1′ positions of ζ-cleavage and ε-cleavage, respectively, mutations at the P2-P1 positions of these cleavage sites had the strongest effects on the formation of Aβ. Specifically, F-mutation at these positions had the strongest effect on both the efficiency and the preference of Aβ formation, resulting in a decrease in the level of total Aβ, an increase in the ratio of $A\beta_{42}/A\beta_{total}$ and a decrease in $A\beta_{40}/A\beta_{total}$. G-mutation at the P1 position of both the ε-cleavage site and ζ-cleavage site caused a modest decrease in total Aβ, but a significant increase in the ratio of $A\beta_{42}/A\beta_{total}$ and a decrease in $A\beta_{40}/A\beta_{total}$. G-mutation at the P2 position (Aβ48) of the ε-cleavage site caused a slight increase in $A\beta_{40}/A\beta_{total}$ (Fig. 4B). However, G-mutation at the P2 position (Aβ45) of the ζ-cleavage site caused a significant decrease in $A\beta_{40}/A\beta_{total}$ and a concomitant increase in $A\beta_{42}/A\beta_{total}$ and $A\beta_{38}/A\beta_{total}$ (Fig. 5, compare lane 15 with 14).

In summary, the data presented in the current study clearly indicate that the residues at the P2-P1 positions of the ε-cleavage site and ζ-cleavage site play an important role in γ-secretase-mediated APP processing, specifically with ε-cleavage, the initial cleavage in the series of sequential ε-, ζ-, and γ-cleavages. Regardless of the polarity, the effects on the level of total Aβ were determined by the structure of residues at Aβ48, the P2 position of the ε-cleavage site. Aliphatic amino acids have little effect on the level of total Aβ, while the aromatic amino acids cause a strong reduction in total Aβ. On the other hand, among the same group of aliphatic amino acids or the same group of aromatic amino acids, residues with a long side chain caused a decrease in $A\beta_{40}$ and a concomitant increase in $A\beta_{42}$ and $A\beta_{38}$. Thus, the structures of the substituting residues at these key positions strongly determine the cleavage efficiency, and the sizes of substituting residues determine the cleavage preference of γ-secretase-mediated APP processing.

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References

Farmery MR, Tjernberg LO, Pursglove SE, Bergman A, Winblad B, Naslund J. Partial purification and characterization of gamma-secretase from post-mortem human brain. J Biol Chem. 2003; 278:24277–24284. [PubMed: 12697771]

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, Mant R, Newton P, Rooke K, Roques P, Talbot C, Pericak-Vance M, Roses A, Williamson R, Rossor M, Owen M, Hardy J. Segregation of a missense mutation in the amyloid

precursor protein gene with familial Alzheimer's disease. Nature. 1991; 349:704–706. [PubMed: 1671712]

Gruninger-Leitch F, Schlatter D, Kung E, Nelbock P, Dobeli H. Substrate and inhibitor profile of BACE (beta-secretase) and comparison with other mammalian aspartic proteases. J Biol Chem. 2002; 277:4687–4693. [PubMed: 11741910]

Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Muller-Hill B. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature. 1987; 325:733–736. [PubMed: 2881207]

Kim SH, Leem JY, Lah JJ, Slunt HH, Levey AI, Thinakaran G, Sisodia SS. Multiple effects of aspartate mutant presenilin 1 on the processing and trafficking of amyloid precursor protein. J Biol Chem. 2001; 276:43343–43350. [PubMed: 11564743]

Li YM, Lai MT, Xu M, Huang Q, DiMuzio-Mower J, Sardana MK, Shi XP, Yin KC, Shafer JA, Gardell SJ. Presenilin 1 is linked with gamma-secretase activity in the detergent solubilized state. Proc Natl Acad Sci U S A. 2000; 97:6138–6143. [PubMed: 10801983]

Lichtenthaler SF, Wang R, Grimm H, Uljon SN, Masters CL, Beyreuther K. Mechanism of the cleavage specificity of Alzheimer's disease gamma-secretase identified by phenylalanine-scanning mutagenesis of the transmembrane domain of the amyloid precursor protein. Proc Natl Acad Sci U S A. 1999; 96:3053–3058. [PubMed: 10077635]

Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, Lannfelt L. A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat Genet. 1992; 1:345–347. [PubMed: 1302033]

Murrell J, Farlow M, Ghetti B, Benson MD. A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. Science. 1991; 254:97–99. [PubMed: 1925564]

Pinnix I, Musunuru U, Tun H, Sridharan A, Golde T, Eckman C, Ziani-Cherif C, Onstead L, Sambamurti K. A novel gamma-secretase assay based on detection of the putative C-terminal fragment-gamma of amyloid beta protein precursor. J Biol Chem. 2001; 276:481–487. [PubMed: 11035007]

Sastre M, Steiner H, Fuchs K, Capell A, Multhaup G, Condron MM, Teplow DB, Haass C. Presenilin-dependent gamma-secretase processing of beta-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. EMBO Rep. 2001; 2:835–841. [PubMed: 11520861]

Schechter I, Berger A. On the size of the active site in proteases. I Papain. Biochem Biophys Res Commun. 1967; 27:157–162. [PubMed: 6035483]

Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev. 2001; 81:741–766. [PubMed: 11274343]

Selkoe DJ, Schenk D. Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics. Annu Rev Pharmacol Toxicol. 2003; 43:545–584. [PubMed: 12415125]

Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos L Jr, Eckman C, Golde TE, Younkin SG. An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. Science. 1994; 264:1336–1340. [PubMed: 8191290]

Tan J, Mao G, Cui MZ, Kang SC, Lamb B, Wong BS, Sy MS, Xu X. Effects of gamma-secretase cleavage-region mutations on APP processing and Abeta formation: interpretation with sequential cleavage and alpha-helical model. J Neurochem. 2008; 107:722–733. [PubMed: 18752644]

Thinakaran G, Teplow DB, Siman R, Greenberg B, Sisodia SS. Metabolism of the "Swedish" amyloid precursor protein variant in neuro2a (N2a) cells. Evidence that cleavage at the "beta-secretase" site occurs in the golgi apparatus. J Biol Chem. 1996; 271:9390–9397. [PubMed: 8621605]

Tomasselli AG, Qahwash I, Emmons TL, Lu Y, Leone JW, Lull JM, Fok KF, Bannow CA, Smith CW, Bienkowski MJ, Heinrikson RL, Yan R. Employing a superior BACE1 cleavage sequence to probe cellular APP processing. J Neurochem. 2003; 84:1006–1017. [PubMed: 12603825]

Turner RT 3rd, Koelsch G, Hong L, Castanheira P, Ermolieff J, Ghosh AK, Tang J. Subsite specificity of memapsin 2 (beta-secretase): implications for inhibitor design. Biochemistry. 2001; 40:10001–10006. [PubMed: 11513577]

Zhao G, Cui MZ, Mao G, Dong Y, Tan J, Sun L, Xu X. γ-Cleavage is dependent on ζ-cleavage during the proteolytic processing of amyloid precursor protein within its transmembrane domain. J Biol Chem. 2005; 280:37689–37697. [PubMed: 16157587]

Zhao G, Mao G, Tan J, Dong Y, Cui MZ, Kim SH, Xu X. Identification of a new presenilin-dependent ζ-cleavage site within the transmembrane domain of amyloid precursor protein. J Biol Chem. 2004; 279:50647–50650. [PubMed: 15485850]

Zhao G, Tan J, Mao G, Cui MZ, Xu X. The same gamma-secretase accounts for the multiple intramembrane cleavages of APP. J Neurochem. 2007; 100:1234–1246. [PubMed: 17241131]

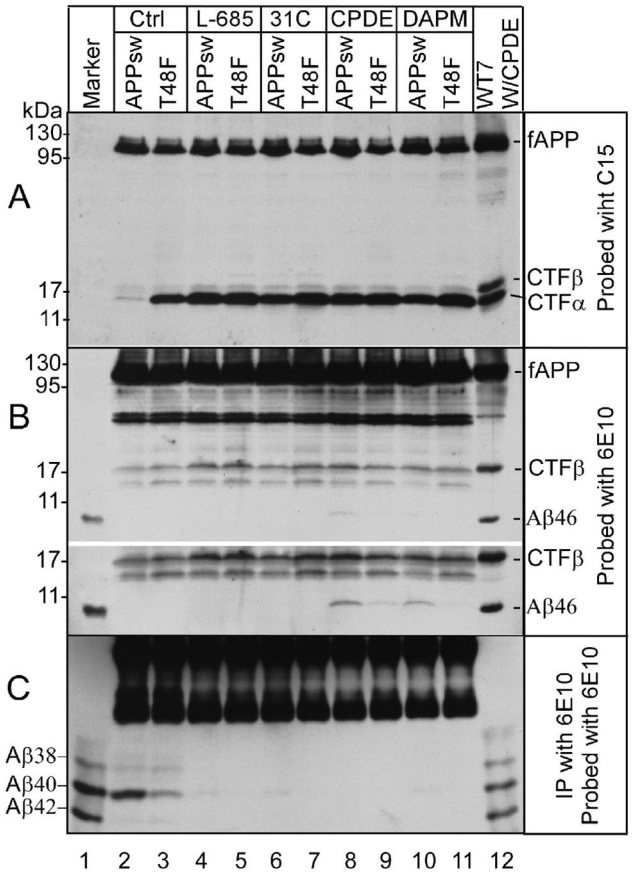

Fig. 1. γ-secretase-mediated processing of T48F mutant APP is normally inhibited by known γ-secretase inhibitors

The $APP^{-/-}$-1 cells transiently transfected with APPsw or T48F mutant APP were cultured for 24 h and treated with vehicle reagent DMSO or one of the following γ-secretase inhibitors for another 12 h: 0.5 μmol/L L-685 458, 5 μmol/L 31C, 3 n mol/L CPDE and 100 n mol/L DAPM. Cell lysates were analyzed by 10–16% SDS-PAGE and probed with C15 (A) or 6E10 (B). Lane 1 shows molecular weight standards (A), the standard $Aβ_{46}$ (B), and a mix of $Aβ_{38}$/$Aβ_{40}$/$Aβ_{42}$ (C). Lane 12 is the cell lysate of WT7 cells, which stably express a high level of APPsw and were used to indicate the positions of CTFα/β (A) and $Aβ_{46}$ (B). The bottom section of panel B is the longer exposure of the lower half of the top section to visualize the membrane-bound $Aβ_{46}$. The secreted Aβs immunoprecipitated from conditioned media were separated on 11% bicine/urea SDS-PAGE and probed with 6E10 (panel C). Lanes 1 and 12 are the mixture of standard $Aβ_{38}$, $Aβ_{40}$ and $A_{β42}$ markers.

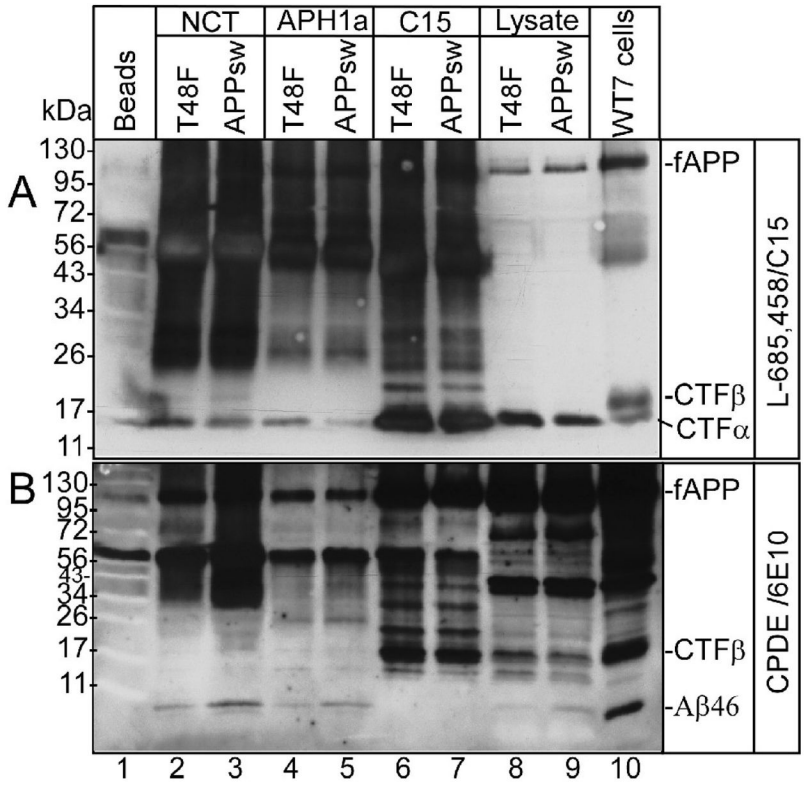

Fig. 2. T48F mutation has no effect on the interaction of CTFβ with the γ-secretase complex

The $APP^{-/-}$-1 cells transiently transfected with APPsw or T48F mutant APP were cultured for 24 h and then treated with the following γ-secretase inhibitors for an additional 12 h: 0.5 μmol L-685 458 (A) or 3 n mol CPDE (CPDE, panel B). Co-immunoprecipitation was performed as described under "Experimental procedures." The cell lysates and the resulting immunoprecipitates were analyzed by 10–18% SDS-PAGE followed by Western blotting using C15 (A) or 6E10 (B). Lane 1 shows molecular weight standards. Lanes 2 and 3, 4 and 5, and 6 and 7 are immunoprecipitates with antibodies anti-NCT, anti-Aph1α and C15, respectively. Lanes 8 and 9 are lysates from the cells transfected with APPsw or T48F mutant APP. Lane 10 is the cell lysate of WT7 treated with CPDE.

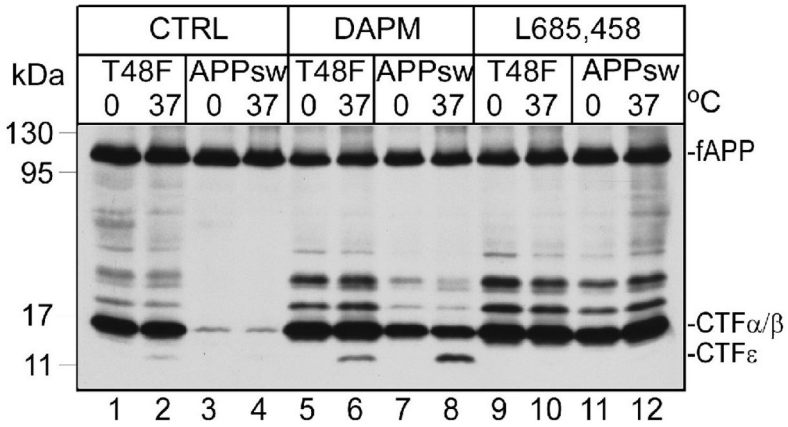

Fig. 3. Mutation T48F caused a decrease in the efficiency of ε-cleavage

The $APP^{-/-}$-1 cells transiently transfected with APPsw or T48F mutant APP were cultured for 24 h and then treated with either the γ-secretase inhibitor DAPM (100 n mol) or L-685,458 (0.5 μmol) for another 12 h. Cells were collected and subjected to cell-free assay as described in the "Experimental procedures." The resultant samples were separated by 10–16% SDS-PAGE and probed with C15.

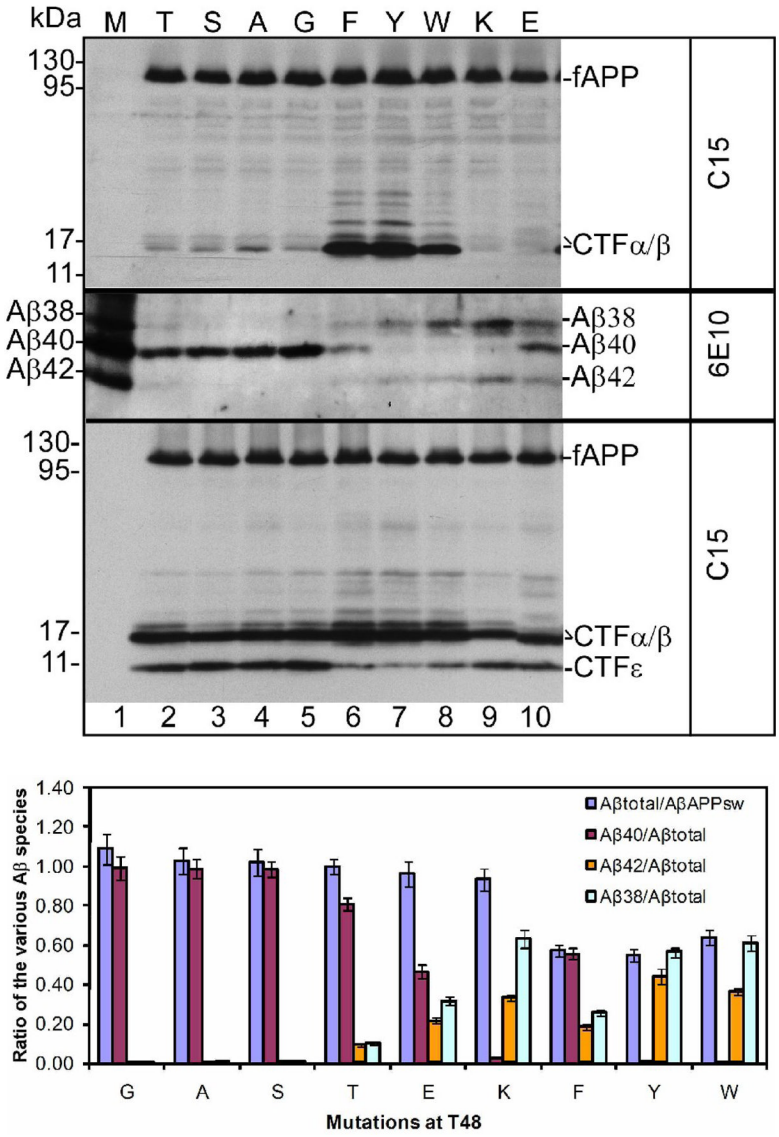

Fig. 4. The effects of substitution of different amino acids for threonine at Aβ48 on APP processing and Aβ formation

(A) $APP^{-/-}$-1 cells transiently transfected with APPsw and its different mutants (T48S, T48A, T48G, T48F, T48Y, T48W, T48K and T48E) were cultured for 24 h and then treated with solvent DMSO (top and middle panels) or DAPM (100 n mol, bottom panel) for 12 h. Cell lysates were separated by 10–16% SDS–PAGE and probed with the antibody C15 (top panel). The secreted Aβs immunoprecipitated from conditioned media were separated on 11% bicine/urea SDS-PAGE and probed with 6E10 (middle panel). The cell free assay samples were analyzed by 10–16% SDS-PAGE and detected with C15 (panel C). Lane 1 is either molecular weight standards (top and bottom panels) or the mixture of $Aβ_{38}$, $Aβ_{40}$ and $Aβ_{42}$ standards (middle panel). (B) Average of the results of densitometic analysis of three repeated Western blots shown in the middle panel of (A). The density of each band was measured, and the ratios were calculated using the Gel Digitizing Software UN-SCAN-IT (Silk Scientific, Orem, UT). Data from the ratio of total $Aβ_{total}/Aβ_{APP}$ (bars in blue), $Aβ_{40}/Aβ_{total}$ (bars in purple), $Aβ_{42}/Aβ_{total}$ (bars in brown), and $Aβ_{38}/Aβ_{total}$ (bars in light blue)

are expressed as the mean ± SEM, n = 3. The amount of Aβ generated was normalized to the expression level of each particular APP variant.

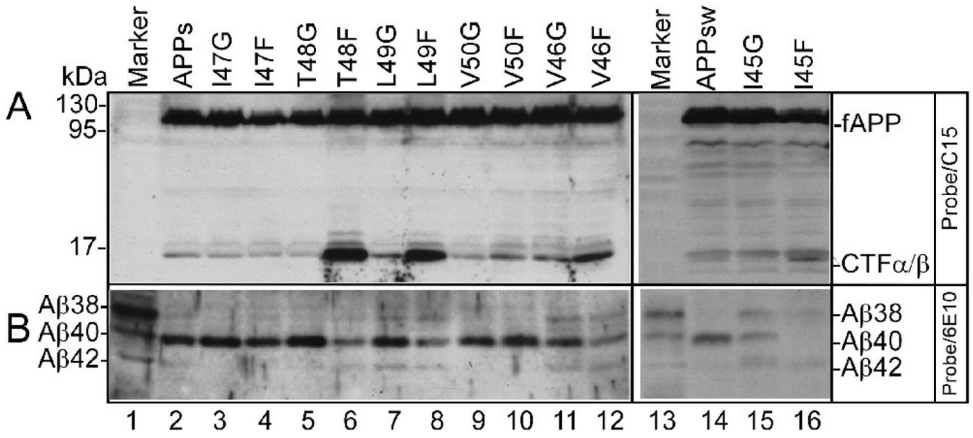

Fig. 5. The effects of substitution by glycine or phenylalanine for the residues around the ε-cleavage and ζ-cleavage sites on Aβ formation

The APP$^{-/-}$-1 cells transiently transfected with APPsw or its different mutants (I47G/F, T48G/F, L49G/F, V50G/F, V46G/F, or I45G/F) were cultured for 36 h. Cell lysates were separated by 10–16% SDS-PAGE and probed with C15 (panel A). Lanes 1 and 13 are the molecular weight standard. The secreted Aβs immunoprecipitated from conditioned media were analyzed on 11% bicine/urea SDS-PAGE and probed with 6E10 (panel B). Lanes 1 and 13 are the mixture of $Aβ_{38}$, $Aβ_{40}$ and $Aβ_{42}$ standards.

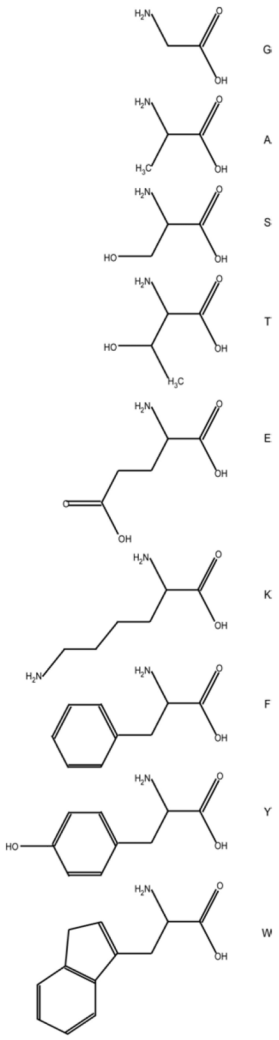

Fig. 6. The molecular structure of amino acids

glycine (G), alanine (A), serine (S), threonine (T), glutamate (E), lysine (K), phenylalanine (F), tyrosine (Y), and tryptophan (W). These amino acids are aligned by the length and structure of their side chains.