γ-Cleavage Is Dependent on ζ-Cleavage during the Proteolytic Processing of Amyloid Precursor Protein within Its Transmembrane Domain

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β-Amyloid precursor protein apparently undergoes at least three major cleavages, γ-, ε-, and the newly identified ζ-cleavage, within its transmembrane domain to produce secreted β-amyloid protein (Aβ). However, the roles of ε- and ζ-cleavages in the formation of secreted Aβ and the relationship among these three cleavages, namely ε-, ζ-, and γ-cleavages, remain elusive. We investigated these issues by attempting to determine the formation and turnover of the intermediate products generated by these cleavages, in the presence or absence of known γ-secretase inhibitors. By using a differential inhibition strategy, our data demonstrate that Aβ46 is an intermediate precursor of secreted Aβ. Our co-immunoprecipitation data also reveal that, as an intermediate, Aβ46 is tightly associated with presenilin in intact cells. Furthermore, we identified a long Aβ species that is most likely the long sought after intermediate product, Aβ46 generated by ε-cleavage, and this Aβ46 is further processed by ζ- and γ-cleavages to generate Aβ43 and ultimately the secreted Aβ40/42. More interestingly, our data demonstrate that γ-cleavage not only occurs last but also depends on ζ-cleavage occurring prior to it, indicating that ζ-cleavage is crucial for the formation of secreted Aβ. Thus, we conclude that the C terminus of secreted Aβ is most likely generated by a series of sequential cleavages, namely first ε-cleavage which is then followed by ζ- and γ-cleavages, and that Aβ46 produced by ζ-cleavage is the precursor of secreted Aβ40/42.

The mechanism of the formation of the β-amyloid protein (Aβ) is the central issue in Alzheimer disease research, not only because Aβ is the major constituent of senile plaques, one of the neuropathological hallmarks of Alzheimer disease, but also because Aβ formation may be a causative event in the disease (1). Aβ is proteolytically derived from a large single transmembrane protein, the β-amyloid precursor protein (APP), as a result of sequential cleavages by β- and γ-secretases (1). β-Secretase has been identified as a type I membrane aspartyl protease, and the relationship among these three cleavages, namely ε-, ζ-, and γ-cleavages, also remain elusive. To address these key issues, the objectives of this study were focused on the following: (a) determining precursor and product relationship between Aβ46 and Aβ40/42; and (b) establishing the roles of ε- and γ-cleavages in the formation of secreted Aβ40/42.

MATERIALS AND METHODS

γ-Secretase inhibitors, DAPT, DAPM, compound E, L-685,458, and WPE-III-31C (31C) were purchased from Calbiochem and dissolved in dimethyl sulfoxide. Aβ40 and Aβ42 were purchased from American Peptide Co. (Sunnyvale, CA). Aβ46, Aβ43, and Aβ40 are customized peptides.

Cell Lines and Plasmids—N2a cells stably expressing either wild type presenilin 1 (PS1wt) alone or both PS1wt and Swedish mutant APP (APPsw) were kindly provided by Drs. Sangram S. Sisodia and Seong-Hun Kim (University of Chicago) and were maintained as described previously (11). The plasmid APPsw645, which expresses a C-terminal truncated APP ending at the ε-cleavage site Aβ40, was constructed using

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2 The abbreviations used are: Aβ, amyloid β-peptide; APP, β-amyloid precursor protein; APPsw, Swedish mutant APP; AICD, APP intracellular domain; CTF, C-terminal fragment; CM, conditioned medium; PS, presenilin; DAPT, N-(N'-[3,5-difluorophenacetyl]-l-alanyl)-(S)-phenylglycine t-butyl ester; DAPM, N-(N'-[3,5-difluorophenacetyl]-l-alanyl)-(S)-phenylglycine methyl ester; 31C, WPE-III-31C; TGN, trans-Golgi network; CHAPSO, 3-[3-cholamidopropyl]dimethylammonio]-1-hydroxy-2-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine; MOPS, 4-morpholino propane sulfonic acid; IAPP, full-length APP.
the site-directed mutagenesis kit (Stratagene). APPsw (12), kindly provided by Dr. Gopal Thirakaran (University of Chicago), was used as a template. A pair of oligonucleotides (E49, CGTCTACAATGGTTA- GATGCTGAGAGGAG; E49-r, GATTCTCACATCTAGGTTGATGAGC) were necessary for each other and contained a stop codon at position 50 of the AB sequence, were used as primers. 

**Cell-free Assay—**In vitro turnover of Aβ46 by γ-secretase activity was assayed in a cell-free assay system established previously (13), following the procedure described previously (7) with minor modifications. Briefly, N2a cells were cultured in the presence of DAPM for 12 h and harvested in 9 volumes of homogenization buffer (10 mM MOPS, pH 7.0, 10 mM KCl) containing protease inhibitors (Complete, Roche Applied Science) and homogenized by passing through a 20-gauge needle 30 times. After removal of unbroken cells and nuclei by centrifugation at 800 × g for 10 min, membranes were pelleted by centrifugation at 20,000 × g 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 mixture). Aliquots of equal amounts of membranes were then incubated at either 0 or 37 °C. After 1 h of incubation, aliquots (25 μl) were removed for Western blotting, and the remaining reaction mixtures were subjected to centrifugation at 20,000 × g for 30 min at 4 °C to yield the supernatant and pellet fractions. After addition of an equal volume of IP buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, and protease inhibitor mixture), the supernatant was subjected to immunoprecipitation using 6E10. The pellet fraction was solubilized with 1% Nonidet P-40 in IP buffer. After centrifugation at 20,000 × g for 15 min, the supernatant was diluted with equal amounts of IP buffer to lower the concentration of Nonidet P-40 to 0.5% and then subjected to immunoprecipitation using 6E10. The intracellular AB species were immunoprecipitated using 6E10. Both immunoprecipitates were analyzed by 10% Bicine/urea-SDS-PAGE, followed by Western blot analysis using 6E10 as described below.

**Detection of Aβ46**—Note that in all of the experiments throughout this study, Aβ46 was determined by directly analyzing the cell lysates without immunoprecipitation. To determine the presence of the possible Aβ46 cells were cultured in the absence of any inhibitors and lysed with 1% Nonidet P-40 in IP buffer. After centrifugation at 20,000 × g for 4 °C for 15 min, the supernatant was diluted with an equal amount of IP buffer and the Aβ46 and other intracellular AB species were immunoprecipitated using 6E10 in the presence or absence of DAPT. Of note, based on our previous data (9) and unpublished data, it was found that all the tested nontransition state inhibitors, such as DAPT, DAPM, and compound E, caused intracellular accumulation of Aβ46 in the same fashion. However, in comparison with DAPM, the inhibitory effects of DAPT and compound E last longer, and this is probably because the enzyme binding activity of the latter two is stronger than that of DAPM. Therefore, DAPT and compound E were used in the in vitro assay and during the immunoprecipitation procedure. DAPM was used to cause the accumulation of Aβ46 in cells that would be used for determining the turnover of Aβ46 either in intact cells or in a cell-free system in which the inhibitor used for causing the accumulation of Aβ46 needs to be removed. 

**Immunoprecipitation and Western Blotting**—Immunoprecipitation and Western blotting were carried out as described previously (9) with the exception that in some cases the immunoprecipitation was carried out in the presence of 500 nM DAPT as indicated in the figure legends. Briefly, 24 h after splitting, cells were treated with inhibitors at various concentrations or with vehicle only as a control. Eight hours after treatment, cells were harvested and lysed in Western blot lysis buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitors). Secreted AB was immunoprecipitated from conditioned media using a monoclonal Aβ-specific antibody 6E10 (Senetek). The immunoprecipitates were analyzed by 10% Bicine/urea-SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). The membranes were then probed with 6E10, and the immunoreactivity bands were visualized using ECL-Plus (Amersham Biosciences).

**Fractionation and Co-immunoprecipitation**—In order to determine the formation of the possible complex of Aβ46 and presenilin, the following procedure, which was originally described in a previous study (14), was employed with slight modification. Briefly, N2a cells expressing APPsw695/PS1wt cultured in the presence of 3 nM compound E (or 500 nM L-685,458; see Fig. 4B) for 10–12 h were harvested and then homogenized in homogenization buffer A (20 mM HEPES, pH 7.4, 50 mM KCl, 2 mM EGTA, 10% glycerol, protease inhibitor mixture (Roche Applied Science)) containing 10 nM compound E (or 2.5 μM L-685,458) by passing through a 20-gauge needle 30 times. The homogenized samples were subjected to centrifugation 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 the pellet fractions. The resultant pellet, which contains both Aβ46 and PS1 (Fig. 4A), was solubilized in buffer B (50 mM PIPES, pH 7.0, 150 mM KCl, 5 mM MgCl2, 5 mM CaCl2, and protease inhibitor mixture) (15) containing 1% CHAPSO and 10 nM compound E (or 2.5 μM L-685,458) for 1 h at 4 °C and then subjected to centrifugation again at 20,000 × g for 25 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 anti-PS1N, a rabbit polyclonal antibody raised against the N terminus of PS1 (9) in the presence of compound E (or 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 incubated 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 10–18% SDS-PAGE followed by Western blotting using 6E10 to detect the co-immunoprecipitated Aβ46 and CTFβ.

**RESULTS**

**L-685,458 Inhibits the Formation of Aβ46**—In our recent study, we have shown that treatment of cells with nontransition state γ-secretase inhibitors, such as DAPT, DAPM, and compound E, caused an increase in the accumulation of intracellular Aβ46, indicating that these inhibitors have no effect, or little effect, on the newly identified γ-cleavage. On the other hand, when the cells were cultured in the presence of transition state analogs, such as L-685,458 and 31C, Aβ46 was not detectable, strongly suggesting that these inhibitors inhibit the γ-cleavage and block the formation of Aβ46 (9). However, it cannot be ruled out that the absence of Aβ46 in cells treated with L-685,458 may be due to the inability of this inhibitor to block the turnover of Aβ46. To address these issues, N2a cells expressing both PS1wt and APPsw were treated with DAPT, compound E, and L-685,458, either individually or in combination. Both the cell lysate and the secreted Aβ46 immunoprecipitated from conditioned medium (CM) were analyzed by 10% Bicine/urea-SDS-PAGE as described previously (16), followed by Western blotting using 6E10. As shown in Fig. 1, treatment with 0.5 μM DAPT (lane 2) or

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2 G. Zhao and X. Xu, unpublished data.

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were centrifuged at 20,000 g.

Treatment with L-685,458, at a range of concentrations from 0.5 to 5 nM compound E, both of which have been shown to block the turnover of Aβ46, is due solely to its inhibition of the formation of Aβ46, and the resulting supernatants and pellets were subjected to immunoprecipitation by using 6E10. As shown in Fig. 1, the membranes were then incubated in the absence or presence of inhibitors (lanes 6–8). The right panel is the overexposure of the Western blot, in order to visualize the Aβ46 band in cells cultured in the absence of any inhibitor (lane 11).

As shown in Fig. 1, the membranes were then incubated in the absence of any inhibitor. Lane 20 is the mixture of synthetic Aβ46 and Aβ42. The right panel is the overexposure of the Western blot, in order to visualize the Aβ46 band in cells cultured in the absence of any inhibitor (lane 11).

This hypothesis is also supported by the facts that Aβ46 can also be detected in cells not treated with any inhibitor after prolonged exposure of the Western blot (Fig. 1, lane 11), as has been shown in our recent study (9). On the other hand, treatment with L-685,458, at a range of concentrations from 0.5 to 2.5 μM, completely abolished the formation of secreted Aβ40/42 and the concomitant accumulation of intracellular Aβ46, respectively. Of note, by directly analyzing the cell lysate, Aβ46 can also be detected in cells not treated with any inhibitor after prolonged exposure of the Western blot (Fig. 1, lane 11), as has been shown in our recent study (9). On the other hand, treatment with L-685,458, at a range of concentrations from 0.5 to 2.5 μM, completely abolished the formation of secreted Aβ40/42 in the CM (Fig. 1, lanes 4–6, lower panel), whereas it did not cause the accumulation of intracellular Aβ46 (upper panel). To determine whether the absence of Aβ46 was a result of the failure of L-685,458 to block the turnover of Aβ46, cells were treated with L-685,458 plus 0.5 μM DAPT or plus 5 nM compound E, both of which have been shown to block the turnover of Aβ46 (9), see also Fig. 1, lanes 2 and 3. As shown in Fig. 1, the addition of DAPT (lanes 7 and 8) or compound E (lanes 9 and 10) did not lead to the accumulation of Aβ46 in the presence of L-685,458. This result clearly indicates that the absence of Aβ46 in cells treated with L-685,458, is due solely to its inhibition of the formation of Aβ46, rather than its failure to block the turnover of Aβ46.

Aβ46 Is Processed into Aβ40/42 in Vitro—As reported in our recent study (9), at a low range of concentrations, DAPM, DAPT, and compound E cause a dose-dependent decrease in secreted Aβ40/42 and a concomitant increase in intracellular Aβ46 (see also Fig. 1), suggesting a possible precursor-product relationship between Aβ46 and Aβ40/42. This hypothesis is also supported by the facts that Aβ46 contains the γ-cleavage site at Aβ40/42 and that Aβ46 is detectable in living cells in the absence of any inhibitors (Fig. 1, lanes 1 and 11), which suggests that γ-cleavage occurs prior to γ-cleavage, otherwise the γ-cleavage product Aβ46 would not have had a chance of being formed. To explore the possible precursor-product relationship between Aβ46 and Aβ40/42 we first determined whether Aβ46 is processed into Aβ40/42. To address this issue, a system that contains pre-existing Aβ46 is required. For this purpose, a cell-free system, which has been established and used in many previous studies to assay the in vitro γ-secretase activity (7,13), was employed. Cells were cultured in the presence of 100 nM DAPM, which has been shown to cause the accumulation of Aβ46 (Fig. 1), and the membranes were prepared as described under “Materials and Methods.” As shown in Fig. 2, the membranes were then incubated in the absence of inhibitors (lanes 5 and 6), in the presence of DAPT (lane 7), and in the presence of L-685,458 (lane 8) at 37°C. The sample in Fig. 2, lane 5, was incubated at 0°C. After 1 h of incubation, reaction mixtures were centrifuged at 20,000 × g, and the resulting supernatants and pellets were subjected to immunoprecipitation by using 6E10. As shown in Fig. 2, top panel, Aβ46 was not immunoprecipitated from the pellet of membranes incubated at 0°C (lane 5). However, Aβ46 was indeed immunoprecipitated from the pellet of membranes incubated at 37°C with a concomitant decrease in Aβ46 (Fig. 2, compare lane 6 with lane...
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...its finding that the γ-cleavage and the new ζ-cleavage can be differentially inhibited by different inhibitors made it possible to determine the relationship between Aβ46 and Aβ40/42 and the role of the new ζ-cleavage in the formation of secreted Aβ40/42 in a living cell system. As shown in Fig. 3A, 24 h after splitting, cells were treated with either 100 nM DAPM (lanes 3–5 and 7) or 0.5 μM L-685,458 (lane 6) for 12 h. Fig. 3A, lanes 1 and 2, were the controls incubated with the vehicle dimethyl sulfoxide (Me3SO) only. As shown in Fig. 1, at the specified concentrations, DAPM completely blocked the formation of secreted Aβ40/42 and caused marked accumulation of Aβ46 (Fig. 1, lane 18), and L-685,458 completely blocked the formation of Aβ40/42 and Aβ46 (Fig. 1, lane 4). After 12 h of incubation, L-685,458 (0.5 μM) was added to the cells in Fig. 3A, lane 2. L-685,458 was also added to the cells in Fig. 3A, lanes 5 and 7, in addition to the existing DAPM, and was continuously incubated for 40 min to completely stop the generation of new Aβ46 in these cells, because at this concentration, L-685,458 blocked the formation of Aβ46 (Fig. 1, lane 4). Since L-685,458 has no effect on the turnover of Aβ46, the ζ-cleavage generated from endogenous APP was designated as CTβ(ζ) (end), as described in a previous study (27). Considering the low level of the endogenous CTβ(ζ) (3rd panel), the detected AICD (bottom panel) is possibly the one derived from endogenous APP by ζ-cleavage. A generation of Aβ40/42 from Aβ46 in the presence of L-685,458 in living cells, 8, time course of the generation of Aβ40/42 from Aβ46 in the presence of L-685,458 in living cells. 

FIGURE 3. Generation of secreted Aβ40/42 from Aβ46 in the presence of L-685,458 in living cells. Lane 1 indicates the cells cultured in the presence of L-685,458 (0.5 μM) alone; DM indicates the cells cultured in the presence of DAPM (100 nM) alone; DM/L indicates the cells cultured in the presence of both DAPM and L-685,458; − indicates the cells cultured in the absence of any inhibitor. Cells were first cultured in the absence of any inhibitor or the presence of one kind of inhibitor as indicated for 12 h. The cells were then cultured in the absence of any inhibitor or in the presence of one kind of inhibitor or a combination of two kinds of inhibitors, as indicated for 40 min. The cells were washed with cold medium and cultured in fresh media containing no inhibitor (−) or one kind of inhibitor for 1 h (A and lanes 1–5 of B) or 2 h (lanes 6–10 of B) as indicated. Lane 8 in A and lane 11 in B are the mixtures of synthetic Aβ46 and Aβ40/42, A and B, the top panels are cell lysates, and the 2nd panels are Aβ-immunoprecipitated from CM. Both were analyzed by 10% Bicine/urea-SDS-PAGE. A, lanes 5 and 7 are duplicate experiments. A, 3rd panel shows the cell lysates analyzed by 10–18% regular SDS-PAGE and probed with C15. The bottom panel is the longer exposure of the same Western blot of the 3rd panel. Note, CTβ(ζ) and CTβ(ζ) generated from exogenous APP, which is expressed with a Myc tag fused to its C terminus, were designated as CTβ(ζ) and CTβ(ζ), respectively; CT(ζ) generated from endogenous APP were designated as CTβ(ζ) (end), as described in a previous study (27). Considering the low level of the endogenous CTβ(ζ) (3rd panel), the detected AICD (bottom panel) is possibly the one derived from endogenous APP by ζ-cleavage. A generation of Aβ40/42 from Aβ46 in the presence of L-685,458 in living cells, 8, time course of the generation of Aβ40/42 from Aβ46 in the presence of L-685,458 in living cells. 

3A, 24 h after splitting, cells were treated with either 100 nM DAPM (lanes 3–5 and 7) or 0.5 μM L-685,458 (lane 6) for 12 h. Fig. 3A, lanes 1 and 2, were the controls incubated with the vehicle dimethyl sulfoxide (Me3SO) only. As shown in Fig. 1, at the specified concentrations, DAPM completely blocked the formation of secreted Aβ40/42 and caused marked accumulation of Aβ46 (Fig. 1, lane 18), and L-685,458 completely blocked the formation of Aβ40/42 and Aβ46 (Fig. 1, lane 4). After 12 h of incubation, L-685,458 (0.5 μM) was added to the cells in Fig. 3A, lane 2. L-685,458 was also added to the cells in Fig. 3A, lanes 5 and 7, in addition to the existing DAPM, and was continuously incubated for 40 min to completely stop the generation of new Aβ46 in these cells, because at this concentration, L-685,458 blocked the formation of Aβ46 (Fig. 1, lane 4). Since L-685,458 has no effect on the turnover of Aβ46 (Fig. 2), this treatment also allows the complete turnover of the Aβ46 possibly existing in the cells of lane 2 of Fig. 3A. As a control, cells in Fig. 3A, lane 1, were cultured in the presence of Me3SO throughout...
the course of the experiment. All cells were then washed twice with fresh medium containing the appropriate inhibitor, which was to be used in the next incubation step, and cultured for an additional 2 h either in the presence or absence of inhibitors as indicated. Aβ_{40/42} was immunoprecipitated using 6E10 from CM of the last 2-h cultures. Both cell lysates and Aβ_{40/42} immunoprecipitated from CM were analyzed by 10% Bicine/urea-SDS-PAGE followed by Western blotting using 6E10.

As shown in Fig. 3A, 2nd panel, secreted Aβ_{40/42} was detected in cells cultured in the absence of any inhibitors throughout the course of the experiment (lane 1, 2nd panel). Secreted Aβ_{40/42} was also detected in cells cultured in the absence of inhibitors during the last 2-h incubation period, with a concomitant decrease in both CTFβ and Aβ_{40/42} (Fig. 3A, lane 4). As expected, secreted Aβ_{40/42} was not detected in cells cultured in the presence of L-685,458 either throughout the course of the experiment (Fig. 3A, lane 6) or during the last two incubation periods (40 min and 2 h) (lane 2). Also, secreted Aβ_{40/42} was not detected in cells cultured in the presence of DAPM throughout the course of the experiment (Fig. 3A, lane 3). However, when the DAPM was replaced by L-685,458 during the last 2-h incubation period, secreted Aβ_{40/42} was detected in the media, and concomitantly, the pre-accumulated Aβ_{46} disappeared (Fig. 3A, compare lanes 5 and 7 with lane 3, top panel). Note that the level of CTFβ in Fig. 3A, lanes 5 and 7, remains unchanged (compare lanes 5 and 7 with lane 3, top panel). In order to confirm further that CTFβ in Fig. 3A, lanes 5 and 7, was not processed by γ-secretase in the presence of L-685,458 during the last 2-h incubation period, we also examined the formation of AICD in these cells. As shown in the bottom panel of Fig. 3A, a significant amount of AICD was detected in cells in lane 4 (bottom panel) with a concomitant decrease in both CTFβ and CTFα (lane 4, top and 3rd panels). The cells in Fig. 3A, lane 4, were cultured in the presence of DAPM, which causes accumulation of both Aβ_{40} and CTFβ, during the first two (12 h and 40 min) incubation periods and then were cultured in the absence of inhibitors during the last 2-h incubation period. However, AICD was not detected in cells cultured in the presence of L-685,458 either throughout the course of the experiment (Fig. 3A, lane 6, bottom panel) or during the last two incubation periods (40 min and 2 h) (lanes 2 and 5, bottom panel), indicating that L-685,458 prevented CTFβ from turnover. Therefore, the Aβ_{40/42} detected in Fig. 3A, lanes 5 and 7, should have been produced solely from the pre-accumulated Aβ_{46} by DAPM during the prior 12 h of culture in the presence of DAPM. This is also supported by the fact that without pre-accumulation of Aβ_{46} during the first 12 h of culture, Aβ_{40/42} was not detected in cells (Fig. 3A, lane 2) cultured in the presence of L-685,458 during the last two incubation periods (40 min and 2 h). Since both Aβ_{46} and CTFβ decreased, the secreted Aβ_{40/42} detected in Fig. 3A, lane 4, is apparently the sum of the Aβ_{40/42} produced from both pre-accumulated Aβ_{46} and CTFβ, and the CTFβ was most likely first converted to Aβ_{46} and the resulting Aβ_{46} was further processed to Aβ_{40/42}. A small amount of AICD was also detected in cells cultured in the presence of DAPM throughout the course of the experiment (Fig. 3A, lane 3, bottom panel). This result further confirmed that the nontransition state inhibitor DAPM has less effect on the turnover of CTFβ by e- and γ-cleavages. As discussed below, the accumulation of CTFβ in the presence of DAPM is possibly the result of the partial inhibitory effect of DAPM on e-cleavage or, alternatively, results from the accumulation of Aβ_{46}, which remains tightly associated with PS1 (Fig. 4) and which prevents CTFβ from accessing the γ-secretase. It was noted that the amount of AICD detected was smaller than expected, compared with the decrease in CTFβ and CTFα in Fig. 4, lanes 1 and 4. This is likely because of the rapid degradation of this CTF fragment in living cells, as reported previously (18). Nevertheless, the detection of
As shown in Fig. 3B, lanes 1 and 6 (lower panel), in the absence of inhibitors, Aβ_{40/42} is apparently produced in a time-dependent manner during the last 1 (lane 1) and 2 h (lane 6) of culture. As shown in Fig. 3B, lanes 2 and 7, CTFβ accumulated in a time-dependent manner, but neither secreted Aβ_{40/42} nor intracellular Aβ was detected in the cells treated with L-685,458 throughout the course of the experiment (lower panel). Instead, an accumulation of intracellular Aβ_{46} and CTFβ was observed in these cells (Fig. 3B, upper panel). In contrast, when DAPM was removed during the last 1 and 2 h of incubation, the accumulated Aβ_{46} and CTFβ, with concomitant increase in secreted Aβ_{40/42}, were decreased in a time-dependent manner (Fig. 3B, compare lane 9 with lane 4 of both upper and lower panels). More interestingly, when DAPM was replaced with L-685,458 during the last 1 and 2 h of incubation, the time-dependent decrease in pre-accumulated Aβ_{46} (Fig. 3B, compare lane 10 with lane 5, upper panel) and the concomitant increase in secreted Aβ_{40/42} (compare lane 10 with lane 5, lower panel) was also observed. It is notable that the accumulated CTFβ remained unchanged (Fig. 3B, compare lane 10 with lane 5, upper panel) during this time course. These results clearly indicate that the secreted Aβ_{40/42} detected in these cells was solely produced from the pre-accumulated Aβ_{46}. This conclusion is also supported by the observation that without the pre-accumulating Aβ_{46}, no secreted Aβ_{40/42} was detected in CM of cells cultured in the presence of L-685,458 during the last 1 and 2 h (Fig. 3B, lanes 2 and 7). The secreted Aβ_{40/42} detected in Fig. 3B, lane 9, is the sum of the Aβ_{40/42} produced from both accumulated Aβ_{46} and CTFβ.

The fact that the secreted Aβ_{40/42} is produced from Aβ_{46} in the presence of L-685,458, in both cell-free and living cell systems, clearly indicates that L-685,458 has no direct inhibitory effect on the γ-cleavage. Therefore, the absence of secreted Aβ_{40/42} in cells treated with L-685,458, which blocks the formation of Aβ_{46} from CTFβ by γ-cleavage, indicates that Aβ_{40/42} cannot be generated directly from CTFβ by γ-cleavage. In other words, formation of Aβ_{46} by γ-cleavage is an indispensible step during the course of γ-secretase-mediated processing of CTFβ to produce Aβ_{40/42}.

Aβ_{46} is associated with PS1—A previous study has shown that as a substrate of γ-secretase, CTFβ forms a complex with PS1, which is the putative catalytic subunit of the γ-secretase complex, at the sites of Aβ formation (19). If Aβ_{46} is the precursor of Aβ_{40/42}, then Aβ_{46} as an intermediate, may still be associated with PS1. To determine whether Aβ_{46} is still associated with PS1, the co-immunoprecipitation experiment was performed. As described under “Materials and Methods,” lysates of cells treated with compound E, which causes the accumulation of Aβ_{46}, were first subjected to centrifugation at 20,000 g to remove the unbroken cells and nuclei. The resulting postnuclear supernatant was subjected to further centrifugation at 20,000 g resulting in the supernatant, which contains the low density microsomal and cytosolic fractions (20), and the pellet, the crude membrane fraction containing the trans-Golgi network (TGN) and plasma membrane (21). As shown in the upper panel of Fig. 4A, Aβ_{46} was detected in the whole cell lysate (lane 2) and the crude membrane fraction of 20,000 × g (lane 3), but not in the supernatant fraction of 20,000 × g (lane 4). Most interestingly, as shown in the lower panel of Fig. 4A, PS1 was also detected in the whole cell lysate (lane 2) and the fraction of pellet at 20,000 × g (lane 3), but not in supernatant at 20,000 × g (lane 4), indicating that Aβ_{46} co-fractionates with PS1 into the crude membrane fraction. Therefore, as described under “Materials and Methods,” after solubilization of the pellet fraction of 20,000 × g, co-immunoprecipitation was carried out by using anti-PS1N, an antibody specific to the N terminus of PS1 (9). As shown in Fig. 4B, Aβ_{46} was indeed co-immunoprecipitated with PS1 from the crude membranes prepared from cells treated with compound E (lane 3) but not in cells treated with L-685,458 (lane 5), which inhibits the formation of Aβ_{46} from CTFβ and causes accumulation of CTFβ (Fig. 1). In agreement with the previous study (19), CTFβ was co-immunoprecipitated with PS1 in the L-685,458-treated cells (Fig. 4B, lane 5, lower panel). As controls, neither Aβ_{46} nor CTFβ was immunoprecipitated by pre-immune rabbit IgG (Fig. 4B, lanes 2 and 4). The observation that Aβ_{46} is tightly associated with PS1 in the TGN-containing membrane fraction is in agreement with the previous report that TGN is the major site for Aβ formation (22).

Detection of the possible Aβ_{49}—The data presented above clearly demonstrate that Aβ_{46} is an intermediate precursor of secreted Aβ_{40/42}. We next attempted to determine the possible presence of Aβ_{49} generated by e-cleavage. Lysates of cells cultured in the absence of inhibitor were subjected to immunoprecipitation followed by Western blotting.
using 6E10. As shown in Fig. 5A, Aβ46 was immunoprecipitated from untreated cells (lane 2). Most interestingly, when the immunoprecipitation was carried out in the presence of DAPT, in addition to the band of Aβ46, a band with a slower migration rate was detected (Fig. 5A, lane 3). Possibly due to the lower concentration and the hydrophobicity, mass spectrometric analysis of this Aβ species was unsuccessful. To estimate its molecular size, we synthesized three Aβ peptides, Aβ40, Aβ46, and Aβ49. As the new Aβ species migrates at the same rate as that of the synthetic Aβ49 (Fig. 5A, lane 4), it is most likely the long sought after intermediate, Aβ46, generated by e-cleavage. This conclusion is also supported by the fact that the majority of AICD starts from Aβ46 as reported by previous studies (5–8). It should be noted that under normal conditions, Aβ46 can only be detected after enrichment by immunoprecipitation carried out in the presence of DAPT, indicating its rapid turnover.

**Aβ46 Is the Precursor of Aβ49.**—Data presented in Fig. 3A clearly indicate that L-685,458 inhibits e-cleavage that produces Aβ40. To determine further the effect of L-685,458 on the γ-cleavage, which produces Aβ40, and the relationship between Aβ46 and Aβ49, we created a construct, APPsw645, that expresses a C-terminal truncated APPsw ending at the e-cleavage site Aβ46. N2a cells, which stably express wild type PS1, were stably transfected with APPsw645. As shown in Fig. 5B, secreted Aβ40/42 was detected in the medium of cells cultured in the absence of any inhibitors (lane 2, middle panel). However, when the cells were treated with compound E, no secreted Aβ40/42 was detected in the medium with a concomitant accumulation of intracellular Aβ46 (Fig. 5B, lane 3, top panel). This result clearly indicates that formation of secreted Aβ40/42 from Aβ46 is also mediated by the formation of the intermediate Aβ46. Most interestingly, when cells were treated with L-685,458, neither Aβ40/42 nor Aβ46 was detected (Fig. 5B, lane 4, top and middle panels), with concomitant accumulation of intracellular Aβ46 (lane 4, top panel). Given the fact that L-685,458 has no effect on the γ-cleavage that produces Aβ40/42 (Figs. 2 and 3), this result indicates that the blockage of the formation of Aβ46 from Aβ49 by L-685,458, is not due to inhibition of γ-cleavage but rather due to inhibition of ζ-cleavage that produces Aβ46, which can be further processed into Aβ40/42 even in the presence of L-685,458.

It was noted that a low amount of Aβ49 was also detected in cells cultured in the absence of inhibitors (Fig. 5B, lane 2, top panel). This may be a result of the lower efficiency of γ-secretase processing because of the lack of AICD, which may be required for APP to efficiently initiate the interaction with the γ-secretase complex. This inefficient interaction of Aβ40 with γ-secretase complex may also account for the detection of the unprocessed Aβ49 secreted into the medium (Fig. 5B, lane 2, middle and bottom panels). In this regard, it was also noted that in the presence of inhibitors, specifically in the presence of L-685,458, a significant amount of unprocessed Aβ49 was detected in the media (Fig. 5B, lanes 3 and 4, middle and bottom panels). One possibility is that in the presence of these inhibitors, the γ-secretase-bound Aβ49, or the intermediate Aβ46, occupies the binding site of γ-secretase complex and prevents other unprocessed Aβ49 from binding to γ-secretase complex, resulting in the secretion of these unprocessed Aβ49 into the media. Fig. 5B, bottom panel, is the result of reanalysis of the same samples in the medium (urea gel), using 10–18% regular SDS-PAGE to determine the Aβ49.

**DISCUSSION**

By using the combination of L-685,458 with compound E or L-685,458 with DAPT, we clearly demonstrated that the absence of Aβ46, in cells treated with L-685,458 is not due to its failure to block the turnover of Aβ46, but instead is due exclusively to its inhibition of the formation of Aβ46. Our data further demonstrate that in both the cell-free system and in living cells, L-685,458 has no detectable effect on the turnover of Aβ46 under the current experimental conditions. A similar inhibition profile was also observed for 31C (data not shown), indicating that these inhibitors, known as transition state analogs, share the same inhibitory specificity, i.e. they specifically inhibit the formation of AICD by e-cleavage and the formation of Aβ46 by ζ-cleavage, but have no effect on γ-cleavage which produces secreted Aβ40/42 from Aβ46. The observation that L-685,458 has no effect on the turnover of Aβ46 is important because this made it possible to determine the following: 1) the precursor and product relationship between Aβ46 and Aβ40/42; 2) the key role of ζ-cleavage in the formation of Aβ; and 3) the sequential relationship among the three major intramembrane cleavages, namely the γ-cleavage, the e-cleavage, and the newly identified ζ-cleavage.

By using the differential inhibition approach, our data presented in Figs. 2 and 3 clearly reveal the important findings that in the presence of L-685,458, Aβ46, undergoes further γ-cleavage to produce secreted Aβ40/42, both in a cell-free system and in living cells. These results indicate that L-685,458 does not directly inhibit γ-cleavage. Therefore, the fact that inhibition of the formation of Aβ46 by L-685,458 also blocks the formation of Aβ40/42 from CTFβ (Fig. 3) and Aβ49 (Fig. 5B, lane 4) indicates that without the formation of the intermediate Aβ46, by γ-cleavage, Aβ40/42 cannot be directly generated from CTFβ or Aβ46 by γ-secretase, i.e. Aβ46 is the intermediate precursor of Aβ40/42. However, it cannot be totally ruled out that Aβ40/42 can be generated directly from CTFβ or Aβ46 by a distinct γ-cleavage, which is inhibited by L-685,458.

To confirm further the notion that Aβ46 is the intermediate precursor of Aβ40/42 or, in other words, Aβ46 is the intermediate product of the γ-secretase-mediated proteolytic processing of CTFβ, we performed co-immunoprecipitation experiments and found that, as an intermediate product during the intramembranous processing by γ-secretase, Aβ46 is indeed tightly associated with PS1. In agreement with the previous study (19), CTFβ was also co-immunoprecipitated with PS1 (Fig. 4B, lanes 3 and 5). It was noted that in compound E-treated cells, only a small amount of CTFβ was detected in the co-immunoprecipitate (Fig. 4B, lane 3). One possibility is that, in the presence of compound E, which prevents the turnover of Aβ46 into Aβ40/42, the accumulated intermediate Aβ46 occupies the binding site of the γ-secretase complex and thus prevents the further binding of CTFβ to the γ-secretase complex and results in less CTFβ co-immunoprecipitating with PS1. This possibility is also supported by the observation that most of the accumulated CTFβ is not associated with PS1 but is detected in the subcellular fraction that is free of PS1 (Fig. 4A, lane 4). In contrast to the low amount of CTFβ co-immunoprecipitating with PS1 in L-685,458-treated cells, the observation that a significantly high amount of Aβ46 was co-immunoprecipitating with PS1 in compound E-treated cells indicates that the complex formed between PS1 and Aβ46 is more stable than that formed between PS1 and CTFβ. The tight association of Aβ46 with PS1 in the TGN-containing membrane fraction, which has been reported to be the major site of Aβ generation (22), provides further strong support for the notion that Aβ46 is an intermediate product formed during the γ-secretase processing and that further turnover of Aβ46 must be dependent on a PS1-containing enzyme, i.e. most likely the same γ-secretase. Taken together, these results indicate that once bound to presenilin, the initial substrate, CTFβ, and, specifically, the intermediate product, Aβ46, are closely associated with presenilin until the release of the final product of secreted Aβ40/42. Thus, all the data presented support our hypotheses that Aβ46 is the precursor of
secreted $\beta_{40/42}$, and that the $\zeta$-cleavage, which produces $\beta_{40}$, plays a key role in $\beta\beta$ formation.

The fact that L-685,458 has no effect on the turnover of $\beta_{46}$ (Figs. 2 and 3) and that L-685,458 blocks the formation of $\beta_{46}$ from $\beta_{40}$ by $\zeta$-cleavage (Fig. 5B), and also blocks the formation of AICD by $\epsilon$-cleavage (Fig. 3), indicates that the transition state analog L-685,458 specifically inhibits both $\epsilon$- and $\zeta$-cleavages but has no effect on $\gamma$-cleavage. Moreover, the finding that L-685,458 does not directly inhibit $\gamma$-cleavage strongly supports an important notion that this inhibitor inhibits the formation of $\beta_{40/42}$ in living cells by a mechanism other than directly inhibiting the $\gamma$-cleavage, namely by indirectly inhibiting the formation of $\beta_{46}$ by $\zeta$-cleavage. This idea is supported by the fact that $\beta_{46}$ is detectable in cells cultured in the absence of any inhibitor, indicating that $\zeta$-cleavage must occur prior to $\gamma$-cleavage, i.e. $\zeta$-cleavage is upstream of $\gamma$-cleavage. Therefore, the finding that inhibition of upstream $\zeta$-cleavage by L-685,458, which does not directly inhibit $\gamma$-cleavage, completely prevents the downstream $\gamma$-cleavage from taking place strongly suggests that in living cells $\gamma$-cleavage only occurs secondarily but is also dependent on $\zeta$-cleavage occurring first. In this regard, the fact that the putative $\beta_{46}$, which contains the $\zeta$-cleavage site at A$\beta_{46}$, is detectable in living cells (Fig. 5A, lane 3) strongly suggests that $\epsilon$-cleavage occurs prior to $\zeta$-cleavage, otherwise the $\epsilon$-cleavage product $\beta_{40}$ would not have had a chance of being formed. Moreover, our data clearly demonstrate that $\beta_{40}$ cannot be processed directly into $\beta_{40/42}$ by $\gamma$-cleavage. It has to be first processed into $\beta_{46}$ by $\zeta$-cleavage and then the $\beta_{46}$ undergoes further processing by $\gamma$-cleavage to produce $\beta_{40/42}$ (Fig. 5B). Taken together, as illustrated in Fig. 6, our data strongly suggest the possibility that under normal conditions, after $\beta$- or $\alpha$-cleavage of APP, the resulting CTF$\beta$ and CTF$\alpha$ first undergo $\epsilon$-cleavage, followed by a sequential but rapid $\zeta$-cleavage, and then by a $\gamma$-cleavage, commencing at the site closest to the membrane boundary and proceeding toward the site in the middle of the transmembrane domain of APP. Support for this sequential action model also comes from the notion that water molecules play an important role in the peptide bond hydrolysis catalyzed by a protease, and $\gamma$-secretase has been proposed to be an aspartyl protease (4). According to the catalytic mechanism of aspartyl protease, in order to hydrolyze the peptide bond of the substrate, one of the two aspartate residues in the enzyme active site, disposed on opposite faces of the peptide bond to be cleaved, needs to first act as general base to activate the water molecule. The activated water molecule then attacks and breaks the peptide bond, in cooperation with the second aspartate, which acts as a general acid to protonate the departing amine product. The $\epsilon$-cleavage site is close to the membrane boundary and is easily accessed by water molecules in the cytosol. The initial $\epsilon$-cleavage may not only release the AICD but may also create a path for the water molecule to have access to the next cleavage site, namely $\zeta$-cleavage site and then $\gamma$-cleavage site. Accordingly, without removal of the three C-terminal residues from $\beta_{46}$ by $\zeta$-cleavage, water molecules may not be able to access the $\gamma$-cleavage site, resulting in the prevention of $\gamma$-cleavage from taking place. Thus, the blockage of water access may account, at least in part, for the fact that $\gamma$-cleavage depends on $\zeta$-cleavage occurring first.

Regarding the relationship between $\epsilon$- and $\zeta$-cleavages, as discussed above, one possibility is that $\epsilon$-cleavage occurs before $\zeta$-cleavage. However, since L-685,458 blocks both $\epsilon$- and $\zeta$-cleavages and the inhibitor, which specifically inhibits $\epsilon$-cleavage or $\zeta$-cleavage, has not yet been identified, it cannot be ruled out that $\epsilon$-cleavage and $\zeta$-cleavage may also occur simultaneously. Nevertheless, even though it is currently not clear whether $\zeta$-cleavage is dependent on $\epsilon$-cleavage, the finding that the generation of $\beta_{40/42}$ from $\beta_{46}$ has to be mediated by the formation of $\beta_{46}$ by $\zeta$-cleavage indicates that once $\epsilon$-cleavage occurs it has to be followed by $\zeta$-cleavage to produce $\beta_{40/42}$ which is then further processed into $\beta_{40/42}$ by $\gamma$-cleavage.

Regarding the catalytic mechanism of the $\gamma$-secretase, the finding that $\epsilon$- and $\zeta$-cleavages and $\gamma$-cleavage can be differentially inhibited by transition state analogs and nontransition state inhibitors, respectively, suggests several possibilities. First, these cleavages may be catalyzed by two enzymes, and second, these cleavages may be catalyzed by one enzyme that has two inhibitor binding sites, one for the transition state analogs, such as L-685,458, and the other for the nontransition state inhibitors, such as compound E, as suggested by a recent inhibitor binding kinetic study (23). The sequential relationship of these cleavages and specifically the finding that $\gamma$-cleavage is dependent on $\epsilon$- and $\zeta$-cleavages occurring first, suggest that $\gamma$, $\zeta$, and $\epsilon$-cleavages are catalyzed by a single enzyme. The single enzyme model is further strongly supported by the fact that the intermediate $\beta_{46}$ is tightly associated with P51, the putative catalytic subunit of the $\gamma$-secretase complex. The one enzyme model is also supported by the fact that both groups of the inhibitors have been shown to bind to presenilins (24–26). According to this model and the hypothesis that transition state analogs and nontransition state inhibitors bind to different sites (23), the transition state analogs may inhibit the initial cleavage, namely the $\epsilon$-cleavage, by directly binding to the catalytic site. As a result, the downstream $\zeta$- and $\gamma$-cleavages are also prevented from taking place. On the other hand, the nontransition state inhibitors may bind to a remote site of the enzyme and induce conformational changes in the enzyme, resulting in the preferential inhibition of $\gamma$-cleavage, with less effect on the $\epsilon$- and $\zeta$-cleavages, by preventing the $\gamma$-cleavage site from having access to the catalytic site of the enzyme. However, the one catalytic site model fails to account for the fact that $\beta_{46}$ is still processed to $\beta_{40/42}$ by $\gamma$-cleavage in the presence of the transition state analog (Figs. 2 and 3), which is assumed to bind the catalytic site (23). Therefore, the third possibility is likely that these sequential cleavages may be catalyzed by an enzyme that has two catalytic sites, one engaged in carrying out the $\epsilon$- and $\zeta$-cleavages and the other engaged in carrying out the $\gamma$-cleavage. Regardless of whether there is one or two catalytic sites, according to the one enzyme model, at high concentrations the nontransition state inhibitors, which preferentially inhibit $\gamma$-cleavage, may also inhibit $\epsilon$- and $\zeta$-cleavages by an allosteric mechanism, i.e. these compounds may induce conformational changes into the $\gamma$-secretase complex, resulting in partial inhibition of $\epsilon$- and $\zeta$-cleavages. The other possibility that may account for the accumulation of CTF$\beta$ in cells treated with nontransition state inhibitors is that in the presence of nontransition state inhibitors, which inhibit the turnover of $\beta_{46}$ into $\beta_{40/42}$, the accumulated intermediate $\beta_{46}$
occupies the binding site of the γ-secretase complex and prevents the further binding of CTFβ to the γ-secretase complex, resulting in accumulation of unprocessed CTFβ.

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