THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 45, pp. 37689–37697, November 11, 2005

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

Received for publication, July 22, 2005, and in revised form, September 9, 2005 Published, JBC Papers in Press, September 12, 2005, DOI 10.1074/jbc.M507993200

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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 $Aβ_{46}$ is an intermediate precursor of secreted Aβ. Our co-immunoprecipitation data also reveal that, as an intermediate, $Aβ_{46}$ 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, $Aβ_{49}$, generated by ε-cleavage, and this $Aβ_{49}$ is further processed by ζ- and γ-cleavages to generate $Aβ_{46}$ and ultimately the secreted $Aβ_{40/42}$. 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 $Aβ_{46}$ produced by ζ-cleavage is the precursor of secreted $Aβ_{40/42}$.

The mechanism of the formation of the β-amyloid protein (Aβ)[2] 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 (2, 3). Although the exact nature of γ-secretase is still a matter of debate, accumulating evidence supports the idea that γ-secretase is a multiple molecular complex composed of, at least, presenilins, nicastrin, Aph-1, and Pen-2 and that presenilin may function as the catalytic subunit (4).

In understanding the mechanism by which the C termini of secreted Aβ are generated during the processing of APP, three major intramembranous cleavages have been established. The first one is the cleavage now specifically referred to as γ-cleavage (5), which produces the C termini of most of the secreted Aβ species that end at amino acids 40 (Aβ40) or 42 (Aβ42) of the Aβ sequence. The second one is the ε-cleavage occurring between Aβ residues 49 and 50, which produces the N terminus of most of the APP intracellular domain (AICD) (5–8). The identification of this ε-cleavage site raises a question as to whether this ε-cleavage is obligatory for the generation of the C terminus of Aβ, and this also raises a question as to the relationship between ε- and γ-cleavages, *i.e.* whether they are independent of each other or sequential. One of the obstacles in addressing these questions is that neither the intermediate Aβ peptide, which ends at the ε-cleavage site, nor the C-terminal fragment, which starts with an N terminus generated by γ-cleavage, has ever been detected. In a recent study, we reported the identification of an intracellular long Aβ species, namely $Aβ_{46}$, and this led to the discovery of the third major cleavage site, the ζ-cleavage site at $Aβ_{46}$ between the known γ- and ε-cleavage sites (9). The presence of ζ-cleavage site at Aβ46 is further supported by a very recent study showing that $Aβ_{46}$ is the predominant form among the longer Aβ species detected intracellularly (10). However, the finding that the known γ-secretase inhibitors, such as DAPT, DAPM, and compound E, inhibit the formation of secreted $Aβ_{40/42}$, and on the other hand cause the accumulation of $Aβ_{46}$, raises the question as to whether $Aβ_{40/42}$ and $Aβ_{46}$ are produced by the same enzyme or by different enzymes (9). Moreover, 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, 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 $Aβ_{46}$ and $Aβ_{40/42}$; and (*b*) establishing the roles of ε- and ζ-cleavages in the formation of secreted $Aβ_{40/42}$.

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. $Aβ_{40}$ and $Aβ_{42}$ were purchased from American Peptide Co. (Sunnyvale, CA). $Aβ_{46}$, $Aβ_{48}$, and $Aβ_{49}$ 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 $Aβ_{49}$, was constructed using

* This work was supported by National Institutes of Health Grant NS42314 (to X. X.) and by American Heart Association Grant 0355339B (to M.-Z. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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]-2-hydroxy-1-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; MOPS, 4-morpholinepropanesulfonic acid; fAPP, full-length APP.

the site-directed mutagenesis kit (Stratagene). APPsw (12), kindly provided by Dr. Gopal Thinakaran (University of Chicago), was used as a template. A pair of oligonucleotides (E49, CGTCATCACCTTGTAGATGCTGAAGAAG; E49-r, CTTCTTCAGCATCTACAAGGTGATGACG), which are complementary to each other and contain a stop codon at position 50 of the Aβ sequence, were used as primers.

Cell-free Assay—In vitro turnover of $A\beta_{46}$ 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* 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 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* at 4 °C 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 Aβ 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 $A\beta_{49}$—Note that in all of the experiments throughout this study, $A\beta_{46}$ was determined by directly analyzing the cell lysates without immunoprecipitation. To determine the presence of the possible $A\beta_{49}$, 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* at 4 °C for 15 min, the supernatant was diluted with an equal amount of IP buffer, and the $A\beta_{49}$ and other intracellular Aβ species were immunoprecipitated using 6E10 in the presence or absence of DAPT. Of note, based on our previous data (9) and unpublished data,[3] it was found that all the tested nontransition state inhibitors, such as DAPT, DAPM, and compound E, cause intracellular accumulation of $A\beta_{46}$ 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 $A\beta_{46}$ in cells that would be used for determining the turnover of $A\beta_{46}$ either in intact cells or in a cell-free system in which the inhibitor used for causing the accumulation of $A\beta_{46}$ 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 Aβ 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 $A\beta_{46}$ 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. 4*B*) 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 $A\beta_{46}$ and PS1 (Fig. 4*A*), was solubilized in buffer B (50 mM PIPES, pH 7.0, 150 mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 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 $A\beta_{46}$ and CTFβ.

RESULTS

L-685,458 Inhibits the Formation of $A\beta_{46}$—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 $A\beta_{46}$, indicating that these inhibitors have no effect, or little effect, on the newly identified ζ-cleavage. On the other hand, when the cells were cultured in the presence of transition state analogs, such as L-685,458 and 31C, $A\beta_{46}$ was not detectable, strongly suggesting that these inhibitors inhibit the ζ-cleavage and block the formation of $A\beta_{46}$ (9). However, it cannot be ruled out that the absence of $A\beta_{46}$ in cells treated with L-685,458 may be due to the inability of this inhibitor to block the turnover of $A\beta_{46}$. 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 $A\beta_{40/42}$ 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

[3] G. Zhao and X. Xu, unpublished data.

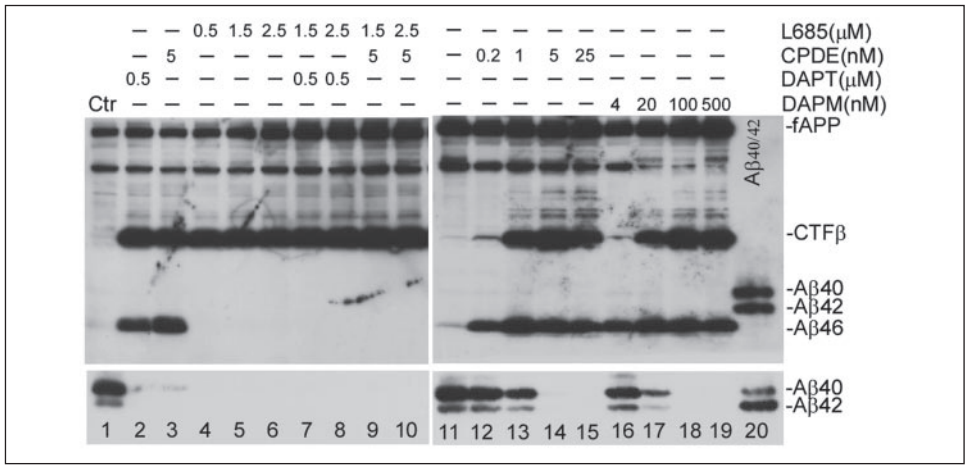

FIGURE 1. **The absence of Aβ_{46} in cells treated with L-685,458 is solely due to inhibition of the formation of Aβ_{46}.** Both cell lysates (*upper panel*) and the secreted A$\beta_{40/42}$ immunoprecipitated from conditioned medium (*lower panel*) were analyzed by 10% Bicine/urea-SDS-PAGE. *Lane 1* is the control (*Ctr*), *i.e.* cells cultured in the absence of any inhibitor. *Lane 20* is the mixture of synthetic Aβ_{40} and Aβ_{42}. The *right panel* is the overexposure of the Western blot, in order to visualize the Aβ_{46} band in cells cultured in the absence of any inhibitor (*lane 11*).

5 nM compound E (*lane 3*) caused a marked decrease in secreted A$\beta_{40/42}$ (*lower panel*) and an accumulation of intracellular Aβ_{46} (*upper panel*). Fig. 1, *lanes 12–15* and *lanes 16–19,* shows the dose-dependent effects of compound E and DAPM on the reduction of secreted A$\beta_{40/42}$ and the concomitant accumulation of intracellular Aβ_{46}, respectively. Of note, by directly analyzing the cell lysate, Aβ_{46} 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 A$\beta_{40/42}$ in the CM (Fig. 1, *lanes 4–6, lower panel*), whereas it did not cause the accumulation of intracellular Aβ_{46} (*upper panel*). To determine whether the absence of Aβ_{46} was a result of the failure of L-685,458 to block the turnover of Aβ_{46}, 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 Aβ_{46} (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 Aβ_{46} in the presence of L-685,458. This result clearly indicates that the absence of Aβ_{46}, in cells treated with L-685,458, is due solely to its inhibition of the formation of Aβ_{46}, rather than its failure to block the turnover of Aβ_{46}.

Aβ_{46} Is Processed into A$\beta_{40/42}$ 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 A$\beta_{40/42}$ and a concomitant increase in intracellular Aβ_{46} (see also Fig. 1), suggesting a possible precursor-product relationship between Aβ_{46} and A$\beta_{40/42}$. This hypothesis is also supported by the facts that Aβ_{46} contains the γ-cleavage site at Aβ40/42 and that Aβ_{46} is detectable in living cells in the absence of any inhibitors (Fig. 1, *lanes 1* and *11*), which suggests that ζ-cleavage occurs prior to γ-cleavage, otherwise the ζ-cleavage product Aβ_{46} would not have had a chance of being formed. To explore the possible precursor-product relationship between Aβ_{46} and A$\beta_{40/42}$, we first determined whether Aβ_{46} is processed into A$\beta_{40/42}$. To address this issue, a system that contains pre-existing Aβ_{46} 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 Aβ_{46} (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

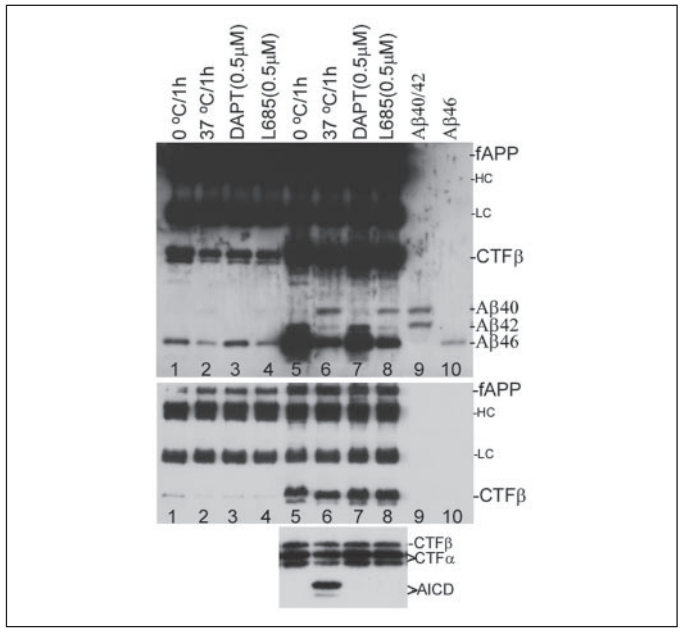

FIGURE 2. **L-685,458 does not inhibit the turnover of Aβ_{46} into A$\beta_{40/42}$ in a cell-free system.** Membrane preparation and the cell-free assay were performed as described under "Materials and Methods." After incubation in the absence or presence of inhibitors, the reaction mixtures were subjected to the centrifugation, and the resulting supernatants (*lanes 1–4*) and pellets (*lanes 5–8*) were then subjected to immunoprecipitation using 6E10 in IP buffer. The immunoprecipitates were analyzed by 10% urea-SDS-PAGE followed by Western blotting using 6E10. *Lanes 1* and *5* are immunoprecipitates from the supernatant and pellet, respectively, obtained from membranes incubated at 0 °C for 1 h. *Lanes 2* and *6* are immunoprecipitates from the supernatant and pellet, respectively, obtained from membranes incubated at 37 °C for 1 h. *Lanes 3* and *7* are immunoprecipitates from the supernatant and pellet, respectively, obtained from membranes incubated at 37 °C for 1 h in the presence of DAPT. *Lanes 4* and *8* are immunoprecipitates from the supernatant and pellet, respectively, obtained from membranes incubated at 37 °C for 1 h in the presence of L-685,458 (*L685*). *Lane 9* is the mixture of synthetic A$\beta_{40/42}$, and *lane 10* is the synthetic Aβ_{46} standard. Since 6E10 was used for both immunoprecipitation and Western blotting, the bands between the fAPP and CTFβ bands are the heavy chain (*HC*) and light chain (*LC*) of mouse IgG. The *middle panel* is the light exposure of the Western blot for observing the changes in the amount of CTFβ. *Bottom panel,* prior to centrifugation, aliquots of the reaction mixture were separated by 10–18% regular SDS-PAGE and probed with C15, an APP C-terminal specific antibody (9), to detect AICD. As discussed in Fig. 3, the two CTFα bands correspond to the Myc-tagged recombinant CTFα and the endogenous CTFα. Similarly, the two AICD bands correspond to the Myc-tagged recombinant AICD and the endogenous AICD.

pellets were subjected to immunoprecipitation by using 6E10. As shown in Fig. 2, *top panel*, A$\beta_{40/42}$ was not immunoprecipitated from the pellet of membranes incubated at 0 °C (*lane 5*). However, A$\beta_{40/42}$ was indeed immunoprecipitated from the pellet of membranes incubated at 37 °C with a concomitant decrease in Aβ_{46} (Fig. 2, compare *lane 6* with *lane*

5), clearly indicating that Aβ_{46} was processed into A$\beta_{40/42}$. Most interestingly, A$\beta_{40/42}$ was also immunoprecipitated from the pellet of membranes incubated at 37 °C, in the presence of L-685,458 (Fig. 2, *lane 8*) but not from the pellet of membranes incubated in the presence of DAPT (*lane 7*). Note that in Fig. 2, *lane 6*, both pre-accumulated Aβ_{46} (*top panel*) and CTFβ (*middle panel*) decreased in comparison with those of the control in *lane 5*, suggesting that the A$\beta_{40/42}$ detected in the absence of inhibitor (*lane 6*) is the sum of the A$\beta_{40/42}$ produced from both accumulated Aβ_{46} and CTFβ. To confirm further that in the absence of inhibitor, CTFβ was processed by γ-secretase, aliquots of the reaction mixtures corresponding to samples in Fig. 2, *lanes 5–8* in the *middle panel*, were separated by 10–18% regular SDS-PAGE and probed with antibody C15, an APP C-terminal specific antibody (9). As shown in Fig. 2, *bottom panel*, in contrast to the basal level of AICD detected in samples in the presence of DAPT and L-685,458 (*lanes 7* and *8*), a significant amount of AICD was detected in the absence of inhibitor with a concomitant decrease in CTFβ and CTFα (*lane 6*). This result provides further support for the conclusion that γ-secretase-mediated processing of CTFβ contributes to the A$\beta_{40/42}$ detected in Fig. 2, *lane 6* (*middle panel*). The fact that CTFβ remains unchanged, in the presence of L-685,458 (compare Fig. 2, *lane 8* with *lane 5, middle* and *bottom panels*), but that the pre-accumulated Aβ_{46} decreased with the concomitant production of A$\beta_{40/42}$ indicates that the A$\beta_{40/42}$ detected in Fig. 2, *lane 8*, is produced solely from the pre-accumulated Aβ_{46}. This also explains why the amount of A$\beta_{40/42}$ detected in Fig. 2, *lane 8*, is slightly less than that detected in *lane 6*. The fact that in the presence of L-685,458, the reduction of Aβ_{46} is similar to that of a membrane incubated in the absence of any inhibitors (compare *lane 8* with *lane 6, top panel*) indicates that L-685,458 has no effect on the turnover of Aβ_{46}. It was noted that only a trace amount of detectable A$\beta_{40/42}$ was immunoprecipitated from the supernatants (Fig. 2, *lanes 2* and *4, upper panel*). One possibility is that the secretion of A$\beta_{40/42}$ from the living cells might be an energy-dependent procedure. Therefore, the secretion of A$\beta_{40/42}$ from the membrane of a cell-free system is not as efficient as in the living cells. Notably, small amounts of Aβ_{46}, CTFβ, and full-length APP were also detected in these supernatants. It is possible that the A$\beta_{40/42}$, as well as other APP derivatives detected in the supernatants, may be associated with the low density membranes contained in the supernatant fraction. The other possibility is that once the A$\beta_{40/42}$ is released from the membrane, or more precisely from the γ-secretase complex, it might be rapidly degraded by the proteases released, during resuspension and incubation, from the membrane, which contains many kinds of protease-containing vesicles. In contrast, the A$\beta_{40/42}$, which still remains in the membrane or, more precisely, before being released from the γ-secretase complex, was protected from the degradation. In this regard, it has been reported that the association of CTFβ with PS1 protects CTFβ from random degradation (17). This degradation of A$\beta_{40/42}$ released from the membrane may also account for the fact that the amount of A$\beta_{40/42}$ detected is smaller than expected, compared with the decrease in precursor Aβ_{46}.

Aβ_{46} Is Processed into A$\beta_{40/42}$ in Living Cells in the Presence of L-685,458—As shown in Fig. 2, Aβ46 was indeed processed into A$\beta_{40/42}$ in a cell-free system. Data presented in Fig. 2 also clearly demonstrate that L-685,458, the transition state analog, did not block the turnover of Aβ_{46} into A$\beta_{40/42}$ in a cell-free system, indicating that L-685,458 has no detectable effect on γ-cleavage that produces A$\beta_{40/42}$ from Aβ_{46}. The 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 Aβ_{46} and A$\beta_{40/42}$ and the role of the new ζ-cleavage in the formation of secreted A$\beta_{40/42}$ in a living cell system. As shown in Fig. 3*A*, 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. 3*A*, *lanes 1* and *2*, were the controls incubated with the vehicle dimethyl sulfoxide (Me_2SO) only. As shown in Fig. 1, at the specified concentrations, DAPM completely blocked the formation of secreted A$\beta_{40/42}$ and caused marked accumulation of Aβ_{46} (Fig. 1, *lane 18*), and L-685,458 completely blocked the formation of A$\beta_{40/42}$ and Aβ_{46} (Fig. 1, *lane 4*). After 12 h of incubation, L-685,458 (0.5 μM) was added to the cells in Fig. 3*A*, *lane 2*. L-685,458 was also added to the cells in Fig. 3*A*, *lanes 5* and *7*, in addition to the existing DAPM, and was continuously incubated for 40 min to completely stop the generation of new Aβ_{46} in these cells, because at this concentration, L-685,458 blocked the formation of Aβ_{46} (Fig. 1, *lane 4*). Since L-685,458 has no effect on the turnover of Aβ_{46} (Fig. 2), this treatment also allows the complete turnover of the Aβ_{46} possibly existing in the cells of *lane 2* of Fig. 3*A*. As a control, cells in Fig. 3*A*, *lane 1*, were cultured in the presence of Me_2SO throughout

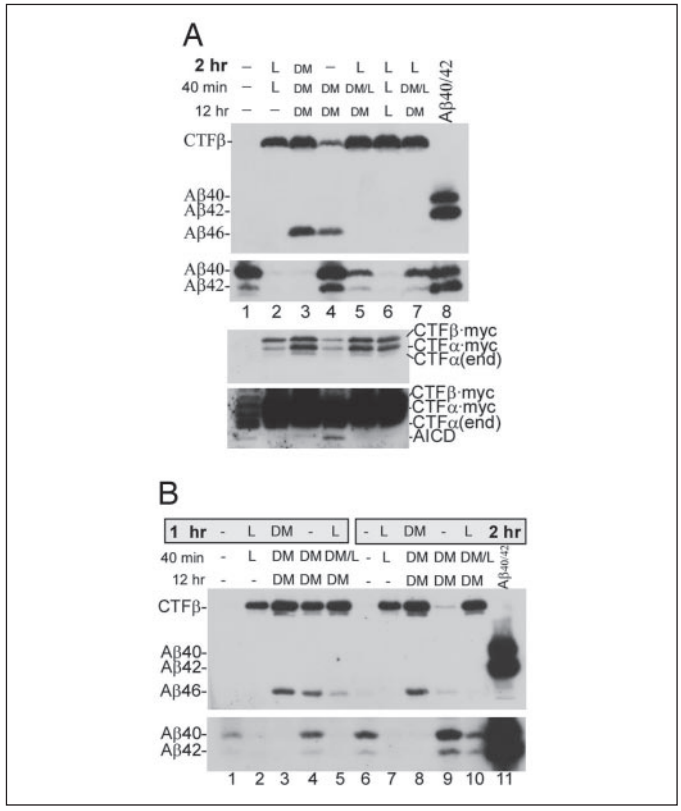

FIGURE 3. **Generation of secreted A$\beta_{40/42}$ from Aβ_{46} in the presence of L-685,458 in living cells.** *L* indicates the cells cultured in the presence of L-685,458 (0.5 μM) alone; *DM* indicates the cells cultured in the presence of DAPM (100 nM) alone; *DM/L* indicates the cells cultured 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 Aβ_{40} and Aβ_{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, CTFβ and CTFα generated from exogenous APP, which is expressed with a Myc tag fused to its C terminus, were designated as CTFβ·myc and CTFα·myc, respectively; CTFα generated from endogenous APP were designated as CTFα(end), as described in a previous study (27). Considering the low level of the endogenous CTFα (*3rd panel*), the detected AICD (*bottom panel*) is possibly the one derived from exogenous APP by ϵ-cleavage. *A,* generation of A$\beta_{40/42}$ from Aβ_{46} in the presence of L-685,458 in living cells. *B,* time course of the generation of A$\beta_{40/42}$ from Aβ_{46} in the presence of L-685,458 in living cells.

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. $A\beta_{40/42}$ was immunoprecipitated using 6E10 from CM of the last 2-h cultures. Both cell lysates and $A\beta_{40/42}$ immunoprecipitated from CM were analyzed by 10% Bicine/urea-SDS-PAGE followed by Western blotting using 6E10.

As shown in Fig. 3*A*, *2nd panel,* secreted $A\beta_{40/42}$ was detected in cells cultured in the absence of any inhibitors throughout the course of the experiment (*lane 1, 2nd panel*). Secreted $A\beta_{40/42}$ 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 $A\beta_{46}$ (Fig. 3*A*, *lane 4*). As expected, secreted $A\beta_{40/42}$ was not detected in cells cultured in the presence of L-685,458 either throughout the course of the experiment (Fig. 3*A*, *lane 6*) or during the last two incubation periods (40 min and 2 h) (*lane 2*). Also, secreted $A\beta_{40/42}$ was not detected in cells cultured in the presence of DAPM throughout the course of the experiment (Fig. 3*A*, *lane 3*). However, when the DAPM was replaced by L-685,458 during the last 2-h incubation period, secreted $A\beta_{40/42}$ was detected in the media, and concomitantly, the pre-accumulated $A\beta_{46}$ disappeared (Fig. 3*A*, compare *lanes 5* and *7* with *lane 3, top panel*). Note that the level of CTFβ in Fig. 3*A*, *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. 3*A*, *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. 3*A*, 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. 3*A*, *lane 4*, were cultured in the presence of DAPM, which causes accumulation of both $A\beta_{46}$ 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. 3*A*, *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 $A\beta_{40/42}$ detected in Fig. 3*A*, *lanes 5* and *7,* should have been produced solely from the pre-accumulated $A\beta_{46}$ 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 $A\beta_{46}$ during the first 12 h of culture, $A\beta_{40/42}$ was not detected in cells (Fig. 3*A*, *lane 2*) cultured in the presence of L-685,458 during the last two incubation periods (40 min and 2 h). Since both $A\beta_{46}$ and CTFβ decreased, the secreted $A\beta_{40/42}$ detected in Fig. 3*A*, *lane 4,* is apparently the sum of the $A\beta_{40/42}$ produced from both pre-accumulated $A\beta_{46}$ and CTFβ, and the CTFβ was most likely first converted to $A\beta_{46}$, and the resulting $A\beta_{46}$ was further processed to $A\beta_{40/42}$. A small amount of AICD was also detected in cells cultured in the presence of DAPM throughout the course of the experiment (Fig. 3*A*, *lane 3, bottom panel*). This result further confirmed that the nontransition state inhibitor DAPM has less effect on the turnover of CTFβ by ϵ- and ζ-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 ϵ-cleavage or, alternatively, results from the accumulation of $A\beta_{46}$, 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

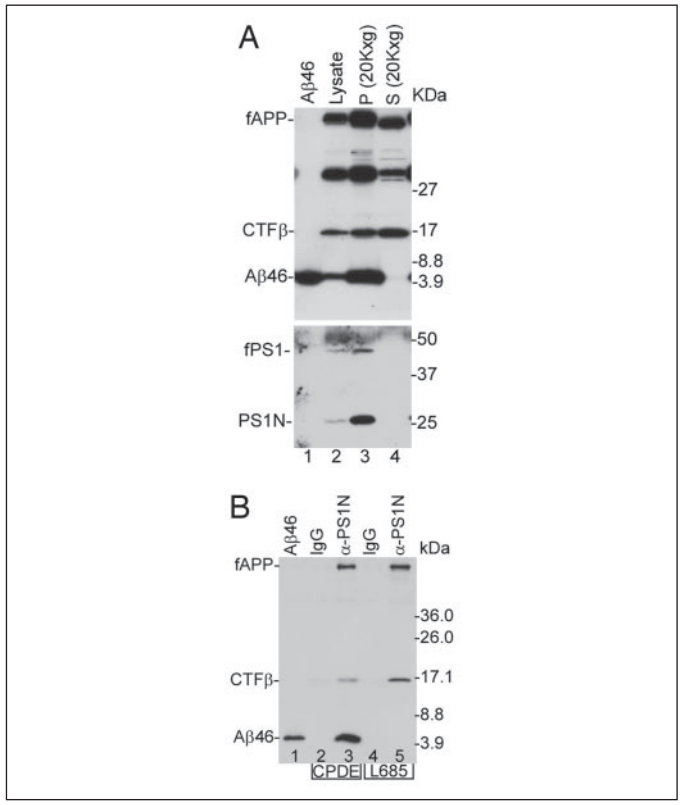

FIGURE 4. **$A\beta_{46}$ is associated with PS1.** Cell lysate fractionation and co-immunoprecipitation were performed as described under "Materials and Methods." The resulting fractions and immunoprecipitates were analyzed by 10–18% SDS-PAGE for $A\beta_{46}$ and CTF_β (*A, upper panel*, and *B*) and 10% SDS-PAGE for PS1 (*A, lower panel*) followed by Western blotting. $A\beta_{46}$ and CTFβ were detected with 6E10. PS1 was detected with anti-PS1N, which recognizes the N terminus of PS1 (9). *A*, *lane 1* is the synthetic $A\beta_{46}$; *lane 2* is the cell lysate; and *lanes 3* and *4* are the subcellular fractions, pellet (*P* (*20Kxg*)) and supernatant (*S* (*20Kxg*)) of 20,000 × *g* centrifugation, respectively. *B*, *lane 1* is the synthetic $A\beta_{46}$; *lane 2* and *lane 3* are samples from compound E(CPDE)-treated cells immunoprecipitated with preimmune rabbit IgG (*lane 2*) and anti-PS1N (*lane 3*), respectively; *lanes 4* and *5* are samples from L-685,458 (*L685*)-treated cells immunoprecipitated with preimmune rabbit IgG (*lane 4*) and anti-PS1N (*lane 5*), respectively. *A,* $A\beta_{46}$ is co-localized with PS1 in the TGN-containing subcellular fraction. *B,* $A\beta_{46}$ is co-immunoprecipitated with PS1.

AICD in DAPM-treated cells clearly indicates that at the tested concentration DAPM has less effect on ϵ-cleavage, even though at the same concentration DAPM completely blocked the formation of secreted $A\beta_{40/42}$. In contrast, L-685,458 completely inhibits the formation of AICD and this is in agreement with a previous report (5).

To confirm further the finding that $A\beta_{40/42}$ was produced solely from pre-accumulated $A\beta_{46}$ in the presence of L-685,458, a time course experiment was performed. As shown in Fig. 3*B*, and as described in Fig. 3*A*, cells were treated with either 100 nM DAPM (*lanes 3–5* and *8–10*) or with the vehicle Me_2SO (*lanes 1, 2, 6,* and *7*) for 12 h. Then L-685,458 (0.5 μM) was added to the cells in Fig. 3*B*, *lanes 2* and *7*. In addition to the existing DAPM, L-685,458 was also added to the cells in Fig. 3*B*, *lanes 5* and *10,* and was continuously incubated for 40 min to stop completely the generation of new $A\beta_{46}$ in these cells. As controls, cells in Fig. 3*B*, *lanes 1* and *6,* were cultured in the presence of Me_2SO 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 were cultured for an additional 1 (Fig. 3*B*, *lanes 1–5*) or 2 h (*lanes 6–10*), either in the presence or absence of inhibitors as indicated. By using 6E10, $A\beta_{40/42}$ was immunoprecipitated from CM of the last 1- and 2-h cultures. Both cell lysates and $A\beta_{40/42}$ immunoprecipitated from CM were analyzed by 10% Bicine/urea-SDS-PAGE followed by Western blotting using 6E10.

γ-Cleavage Depends on ζ-Cleavage

As shown in Fig. 3*B*, *lanes 1* and *6* (*lower panel*), in the absence of inhibitors, A$\beta_{40/42}$ 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. 3*B*, *lanes 2* and *7*, CTFβ accumulated in a time-dependent manner, but neither secreted A$\beta_{40/42}$ nor intracellular Aβ_{46} was detected in the cells treated with L-685,458 during the last two incubation periods (40 min and 1 or 2 h). As shown in Fig. 3*B*, *lanes 3* and *8*, no A$\beta_{40/42}$ was detected in the CM of cells treated with DAPM throughout the course of the experiment (*lower panel*). Instead, an accumulation of intracellular Aβ_{46} and CTFβ was observed in these cells (Fig. 3*B*, *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 A$\beta_{40/42}$, were decreased in a time-dependent manner (Fig. 3*B*, 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. 3*B*, compare *lane 10* with *lane 5, upper panel*) and the concomitant increase in secreted A$\beta_{40/42}$ (compare *lane 10* with *lane 5, lower panel*) was also observed. It is notable that the accumulated CTFβ remained unchanged (Fig. 3*B*, compare *lane 10* with *lane 5, upper panel*) during this time course. These results clearly indicate that the secreted A$\beta_{40/42}$ 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 A$\beta_{40/42}$ was detected in CM of cells cultured in the presence of L-685,458 during the last 1 and 2 h (Fig. 3*B*, *lanes 2* and *7*). The secreted A$\beta_{40/42}$ detected in Fig. 3*B*, *lane 9*, is the sum of the A$\beta_{40/42}$ produced from both accumulated Aβ_{46} and CTFβ.

The fact that the secreted A$\beta_{40/42}$ 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 A$\beta_{40/42}$ in cells treated with L-685,458, which blocks the formation of Aβ_{46} from CTFβ by ζ-cleavage, indicates that A$\beta_{40/42}$ cannot be generated directly from CTFβ by γ-cleavage. In other words, formation of Aβ_{46} by ζ-cleavage is an indispensable step during the course of γ-secretase-mediated processing of CTFβ to produce A$\beta_{40/42}$.

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 A$\beta_{40/42}$, 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 800 × *g* centrifugation 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. 4*A*, 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. 4*A*, 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. 4*B*, 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. 4*B*, *lane 5, lower panel*). As controls, neither Aβ_{46} nor CTFβ was immunoprecipitated by pre-immune rabbit IgG (Fig. 4*B*, *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).

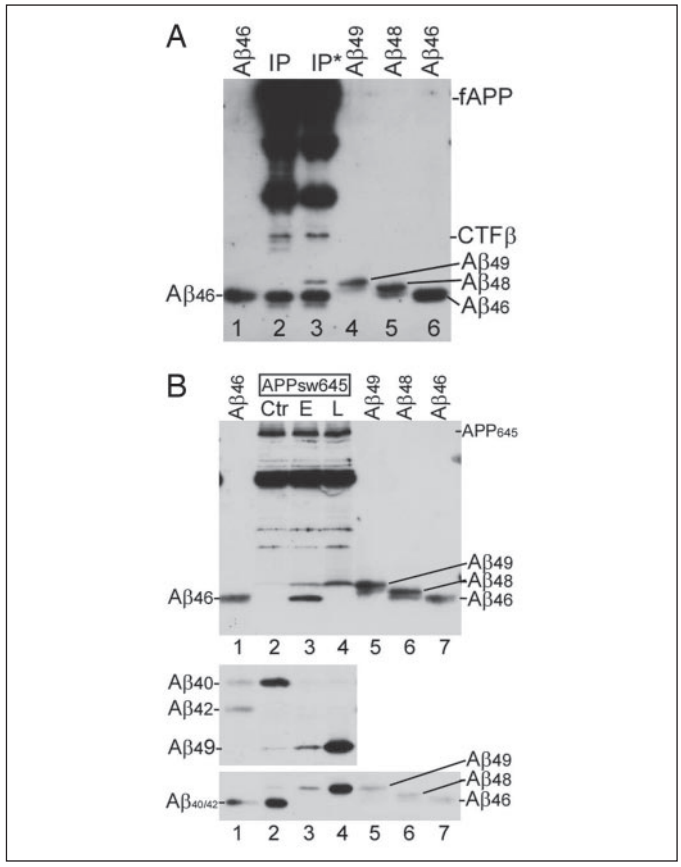

FIGURE 5. *A,* detection of Aβ_{49}. N2a cells stably expressing both PS1 and APPsw were cultured in the absence of inhibitor. Cell lysis, and immunoprecipitation were performed as described under "Materials and Methods." The immunoprecipitates (*IP*) were analyzed by 10–18% regular SDS-PAGE followed by Western blotting using 6E10 (*lanes 2* and *3*). Note, *IP** indicates that immunoprecipitation was carried out in the presence of DAPT. *Lanes 1* and *6* are synthetic Aβ_{46}. *Lane 4* is the synthetic Aβ_{49}. *Lane 5* is the synthetic Aβ_{48}. *B,* generation of secreted A$\beta_{40/42}$ from Aβ_{49} is mediated by the formation of Aβ_{46}. N2a cells stably expressing PS1wt were further transfected with APPsw645. Stable clones were selected with both G418 (for APPsw) and hygromycin (for presenilin). Cells were cultured in the absence (*Ctr*, *lane 2*) or presence of compound E (*E*, *lane 3*) or L-685,458 (*L*, *lane 4*). Cell lysates were directly analyzed by 10–18% regular SDS-PAGE followed by Western blotting using 6E10 (*top panel*). Secreted Aβ in CM was immunoprecipitated with 6E10 and analyzed by 10% urea-SDS-PAGE (*middle panel*). The *bottom panel* is the reanalysis of the same samples in the *middle panel* (urea gel), using 10–18% regular SDS-PAGE to determine the secreted Aβ_{49}. *Lanes 1* and *7* in the *top panel* are the synthetic Aβ_{46}. *Lanes 5* and *6* in the *top panel* are synthetic Aβ_{49} and Aβ_{48}, respectively. *Lane 1* in the *middle* and *bottom panels* is the mixture of synthetic A$\beta_{40/42}$. *Lanes 5–7* in the *bottom panel* are synthetic Aβ_{49}, Aβ_{48}, and Aβ_{46}, respectively. *A,* detection of Aβ_{49} in intact cells. *B,* generation of A$\beta_{40/42}$ from Aβ_{49} is mediated by the formation of Aβ_{46}.

Detection of the Possible Aβ_{49}—The data presented above clearly demonstrate that Aβ_{46} is an intermediate precursor of secreted A$\beta_{40/42}$. We next attempted to determine the possible presence of Aβ_{49} generated by ε-cleavage. Lysates of cells cultured in the absence of inhibitor were subjected to immunoprecipitation followed by Western blotting

using 6E10. As shown in Fig. 5*A*, Aβ_{46} 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 Aβ_{46}, a band with a slower migration rate was detected (Fig. 5*A*, *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, Aβ_{46}, Aβ_{48}, and Aβ_{49}. As the new Aβ species migrates at the same rate as that of the synthetic Aβ_{49} (Fig. 5*A*, *lane 4*), it is most likely the long sought after intermediate, Aβ_{49}, generated by ε-cleavage. This conclusion is also supported by the fact that the majority of AICD starts from Aβ_{50} as reported by previous studies (5–8). It should be noted that under normal conditions, Aβ_{49} can only be detected after enrichment by immunoprecipitation carried out in the presence of DAPT, indicating its rapid turnover.

Aβ$_{49}$ Is the Precursor of Aβ$_{46}$—Data presented in Fig. 3*A* clearly indicate that L-685,458 inhibits ε-cleavage that produces AICD. To determine further the effect of L-685,458 on the ζ-cleavage, which produces Aβ_{46}, and the relationship between Aβ_{49} and Aβ_{46}, we created a construct, APPsw645, that expresses a C-terminal truncated APPsw ending at the ε-cleavage site Aβ_{49}. N2a cells, which stably express wild type PS1, were stably transfected with APPsw645. As shown in Fig. 5*B*, secreted A$\beta_{40/42}$ 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 A$\beta_{40/42}$ was detected in the medium with a concomitant accumulation of intracellular Aβ_{46} (Fig. 5*B*, *lane 3, top panel*). This result clearly indicates that formation of secreted A$\beta_{40/42}$ from Aβ_{49} is also mediated by the formation of the intermediate Aβ_{46}. Most interestingly, when cells were treated with L-685,458, neither A$\beta_{40/42}$ nor Aβ_{46} was detected (Fig. 5*B*, *lane 4, top* and *middle panels*), with concomitant accumulation of intracellular Aβ_{49} (*lane 4, top panel*). Given the fact that L-685,458 has no effect on the γ-cleavage that produces A$\beta_{40/42}$ (Figs. 2 and 3), this result indicates that the blockage of the formation of A$\beta_{40/42}$ from Aβ_{49}, by L-685,458, is not due to inhibition of γ-cleavage but rather due to inhibition of ζ-cleavage that produces Aβ_{46}, which can be further processed into A$\beta_{40/42}$ even in the presence of L-685,458.

It was noted that a low amount of Aβ_{49} was also detected in cells cultured in the absence of inhibitors (Fig. 5*B*, *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 Aβ_{49} with γ-secretase complex may also account for the detection of the unprocessed Aβ_{49} secreted into the medium (Fig. 5*B*, *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 Aβ_{49} was detected in the media (Fig. 5*B*, *lanes 3* and *4, middle* and *bottom panels*). One possibility is that in the presence of these inhibitors, the γ-secretase-bound Aβ_{49} or the intermediate Aβ_{46} occupies the binding site of γ-secretase complex and prevents other unprocessed Aβ_{49} from binding to γ-secretase complex, resulting in the secretion of these unprocessed Aβ_{49} into the media. Fig. 5*B*, *bottom panel*, is the result of reanalysis of the same samples in the *middle panel* (urea gel), using 10–18% regular SDS-PAGE to determine the Aβ_{49}.

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 Aβ_{46} in cells treated with L-685,458 is not due to its failure to block the turnover of Aβ_{46}, but instead is due exclusively to its inhibition of the formation of Aβ_{46}. 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 Aβ_{46} 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 ε-cleavage and the formation of Aβ_{46} by ζ-cleavage, but have no effect on γ-cleavage which produces secreted A$\beta_{40/42}$ from Aβ_{46}. The observation that L-685,458 has no effect on the turnover of Aβ_{46} is important because this made it possible to determine the following: 1) the precursor and product relationship between Aβ_{46} and A$\beta_{40/42}$; 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 ε-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, Aβ_{46} undergoes further γ-cleavage to produce secreted A$\beta_{40/42}$, 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 Aβ_{46} by L-685,458 also blocks the formation of A$\beta_{40/42}$ from CTFβ (Fig. 3) and Aβ_{49} (Fig. 5*B*, *lane 4*) indicates that without the formation of the intermediate Aβ_{46} by ζ-cleavage, A$\beta_{40/42}$ cannot be directly generated from CTFβ or Aβ_{49} by γ-secretase, *i.e.* Aβ_{46} is the intermediate precursor of A$\beta_{40/42}$. However, it cannot be totally ruled out that A$\beta_{40/42}$ can be generated directly from CTFβ or Aβ_{49} by a distinct γ-cleavage, which is inhibited by L-685,458.

To confirm further the notion that Aβ_{46} is the intermediate precursor of A$\beta_{40/42}$ or, in other words, Aβ_{46} 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, Aβ_{46} is indeed tightly associated with PS1. In agreement with the previous study (19), CTFβ was also co-immunoprecipitated with PS1 (Fig. 4*B*, *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. 4*B*, *lane 3*). One possibility is that, in the presence of compound E, which prevents the turnover of Aβ_{46} into A$\beta_{40/42}$, the accumulated intermediate Aβ_{46} 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. 4*A*, *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 Aβ_{46} was co-immunoprecipitating with PS1 in compound E-treated cells indicates that the complex formed between PS1 and Aβ_{46} is more stable than that formed between PS1 and CTFβ. The tight association of Aβ_{46} 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 Aβ_{46} is an intermediate product formed during the γ-secretase processing and that further turnover of Aβ_{46} 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, Aβ_{46}, are closely associated with presenilin until the release of the final product of secreted A$\beta_{40/42}$. Thus, all the data presented support our hypotheses that Aβ_{46} is the precursor of the

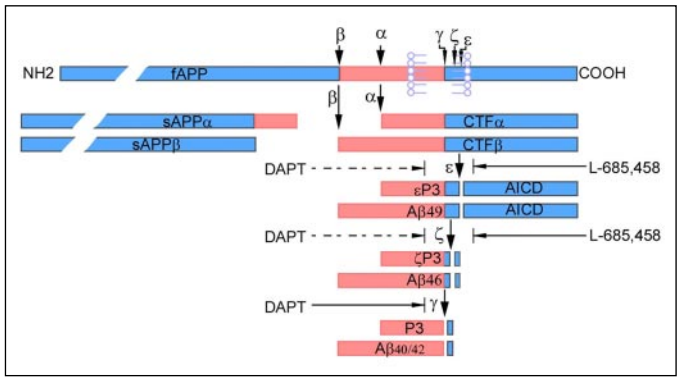

FIGURE 6. **Schematic illustration of APP processing.** L-685,458 specifically inhibits ϵ- and ζ-cleavages (—); DAPT strongly and preferentially inhibits γ-cleavage (—) and also inhibits ϵ- and ζ-cleavages at higher concentration (- - - -). *ϵP3* and *ζP3* are the putative P3-related fragments produced from CTFα by ϵ- and ζ-cleavages, respectively.

secreted A$\beta_{40/42}$ and that the ζ-cleavage, which produces Aβ_{46}, plays a key role in Aβ formation.

The fact that L-685,458 has no effect on the turnover of Aβ_{46} (Figs. 2 and 3) and that L-685,458 blocks the formation of Aβ_{46} from Aβ_{49} by ζ-cleavage (Fig. 5*B*), and also blocks the formation of AICD by ϵ-cleavage (Fig. 3), indicates that the transition state analog L-685,458 specifically inhibits both ϵ- and ζ-cleavages but has no effect on γ-cleavage. Moreover, the finding that L-685,458 does not directly inhibit γ-cleavage strongly supports an important notion that this inhibitor inhibits the formation of A$\beta_{40/42}$ in living cells by a mechanism other than directly inhibiting the γ-cleavage, namely by indirectly inhibiting the formation of Aβ_{46} by ζ-cleavage. This idea is supported by the fact that Aβ_{46} is detectable in cells cultured in the absence of any inhibitor, indicating that ζ-cleavage must occur prior to γ-cleavage, *i.e.* ζ-cleavage is upstream of γ-cleavage. Therefore, the finding that inhibition of upstream ζ-cleavage by L-685,458, which does not directly inhibit γ-cleavage, completely prevents the downstream γ-cleavage from taking place strongly suggests that in living cells γ-cleavage not only occurs secondarily but is also dependent on ζ-cleavage occurring first. In this regard, the fact that the putative Aβ_{49}, which contains the ζ-cleavage site at Aβ46, is detectable in living cells (Fig. 5*A*, *lane 3*) strongly suggests a possibility that ϵ-cleavage occurs prior to ζ-cleavage, otherwise the ϵ-cleavage product Aβ_{49} would not have had a chance of being formed. Moreover, our data clearly demonstrate that Aβ_{49} cannot be processed directly into A$\beta_{40/42}$ by γ-cleavage. It has to be first processed into Aβ_{46} by ζ-cleavage and then the Aβ_{46} undergoes further processing by γ-cleavage to produce A$\beta_{40/42}$ (Fig. 5*B*). Taken together, as illustrated in Fig. 6, our data strongly suggest the possibility that under normal conditions, after β- or α-cleavage of APP, the resulting CTFβ and CTFα first undergo ϵ-cleavage, followed by a sequential but rapid ζ-cleavage, and then by a γ-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 γ-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 ϵ-cleavage site is close to the membrane boundary and is easily accessed by water molecules in the cytosol. The initial ϵ-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 ζ-cleavage site and then γ-cleavage site. Accordingly, without removal of the three C-terminal residues from Aβ_{49} by ζ-cleavage, water molecules may not be able to access the γ-cleavage site, resulting in the prevention of γ-cleavage from taking place. Thus, the blockage of water access may account, at least in part, for the fact that γ-cleavage depends on ζ-cleavage occurring first.

Regarding the relationship between ϵ- and ζ-cleavages, as discussed above, one possibility is that ϵ-cleavage occurs before ζ-cleavage. However, since L-685,458 blocks both ϵ- and ζ-cleavages and the inhibitor, which specifically inhibits ϵ-cleavage or ζ-cleavage, has not yet been identified, it cannot be ruled out that ϵ-cleavage and ζ-cleavage may also occur simultaneously. Nevertheless, even though it is currently not clear whether ζ-cleavage is dependent on ϵ-cleavage, the finding that the generation of A$\beta_{40/42}$ from Aβ_{49} has to be mediated by the formation of Aβ_{46} by ζ-cleavage indicates that once ϵ-cleavage occurs it has to be followed by ζ-cleavage to produce Aβ_{46}, which is then further processed into A$\beta_{40/42}$ by γ-cleavage.

Regarding the catalytic mechanism of the γ-secretase, the finding that ϵ-/ζ-cleavages and γ-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 γ-cleavage is dependent on ϵ- and ζ-cleavages occurring first, suggest that γ-, ζ-, and ϵ-cleavages are catalyzed by a single enzyme. The single enzyme model is further strongly supported by the fact that the intermediate Aβ_{46} is tightly associated with PS1, the putative catalytic subunit of the γ-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 ϵ-cleavage, by directly binding to the catalytic site. As a result, the downstream ζ- and γ-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 γ-cleavage, with less effect on the ϵ- and ζ-cleavages, by preventing the γ-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 Aβ_{46} is still processed to A$\beta_{40/42}$ by γ-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 ϵ- and ζ-cleavages and the other engaged in carrying out the γ-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 γ-cleavage, may also inhibit ϵ- and ζ-cleavages by an allosteric mechanism, *i.e.* these compounds may induce conformational changes into the γ-secretase complex, resulting in partial inhibition of ϵ- and ζ-cleavages. The other possibility that may account for the accumulation of CTFβ in cells treated with nontransition state inhibitors is that in the presence of nontransition state inhibitors, which inhibit the turnover of Aβ_{46} into A$\beta_{40/42}$, the accumulated intermediate Aβ_{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.

Acknowledgments—We thank Dr. Sangram S. Sisodia and Dr. Seong-Hun Kim (University of Chicago) for providing mouse N2a neuroblastoma cells stably expressing PS1wt and APPsw. We thank Dr. Gopal Thinakaran (University of Chicago) for providing the APPsw plasmid. We thank Dr. M. Donald McGavin (Department of Pathobiology) and Jada R. Huskey, College of Veterinary Medicine, the University of Tennessee, for their critical reading of the manuscript.

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