Truncated Carboxyl-Terminal Fragments of β-Amyloid Precursor Protein Are Processed to Amyloid β-Proteins 40 and 42†

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Received March 29, 2004; Revised Manuscript Received June 15, 2004

ABSTRACT: We previously showed that β-amyloid precursor protein (APP) is cleaved not only in the middle of the membrane (γ-cleavage) but also at novel cleavage sites close to the membrane/cytoplasmic boundary (ε-cleavage), releasing APP intracellular domains (AICDs) 49−99 and 50−99. To learn more about the relationship between γ- and ε-cleavage, C-terminally truncated carboxyl-terminal fragments (CTFs) of APP, especially CTFs1−48 and 1−49 (the postulated products that are generated by ε-cleavage), were transiently expressed in CHO cells. Most importantly, the cells expressing CTF1−49 secreted predominantly amyloid β-protein (Aβ) 40, while those expressing CTF1−48 secreted preferentially Aβ42. This supports our assumption that ε-cleavage precedes Aβ production and that preceding ε-cleavage determines the preference for the final Aβ species. The γ-secretase inhibitors, L-685,458 and DAPT, suppressed Aβ production from CTF1−49. Regarding Aβ production from CTF1−48, L-685,458 suppressed it, but DAPT failed to do so. A dominant negative mutant of presenilin 1 suppressed the production of Aβ40 and 42 from both CTFs1−48 and 1−49. These data should shed significant light into the mechanism of Aβ production.

Alzheimer's disease (AD)[1] is the most common cause of dementia among the elderly. According to the amyloid cascade hypothesis of AD, amyloid β-protein (Aβ) is a trigger molecule. Its aggregated form brings about all of the subsequent events, including the formation of senile plaques, neurofibrillary tangles, neuronal death, and clinical dementia. Aβ is a small protein consisting of 38−43 residues and is derived from β-amyloid precursor protein (APP) through successive cleavage by β- and γ-secretases (*1*). β-Secretase is a membrane-bound aspartyl protease, β-site APP-cleaving enzyme (BACE) (*2*), but the nature of the γ-secretase remains an enigma. Accumulating evidence, however, strongly suggests that γ-secretase is also an aspartyl protease with its catalytic site(s) sitting within the membrane. Mutation of either of two conserved aspartates in the transmembrane domains 6 and 7 abolishes Aβ production (*3*). It has recently been shown that the γ-secretase is a multiprotein complex that contains at least four integral membrane proteins, Aph-1, nicastrin, Pen-2, and presenilin (PS), and that these four components are indispensable for the emergence of γ-secretase activity (*4*−*8*). Heterodimer formed by N- and C-terminal fragments of PS1/2 is thought to function as a catalytic core of the γ-secretase complex (*9*).

In addition to APP, several other transmembrane proteins are known to serve as substrates for γ-secretase, including Notch, delta, E-cadherin, CD44, and ErbB4 (*10*−*14*), all of which are key molecules in many cellular processes. This suggests that simply blocking γ-secretase activity for prevention of Aβ generation would cause severe adverse effects in vivo. Thus, it is very important to elucidate the mechanism of Aβ generation.

We and others previously showed that APP is cleaved not only in the middle of the transmembrane domain (γ-cleavage) but also at novel sites close to the membrane/cytoplasmic boundary (ε-cleavage) and that APP intracellular domains (AICDs) of ∼6 kDa (mainly AICD50−99) are released (*15*−*17*). This ε-cleavage as well as γ-cleavage is suppressed by γ-secretase inhibitors and by the expression of a dominant negative mutant of presenilins (*15*−*18*). Subsequently, we found that, whereas the cells expressing wild-type (wt)PS1/2 or wtAPP release mainly AICD50−99, those expressing mutant (mt)PS1/2 or mtAPP increase the proportion of AICD49−99 (*18*).

Here we report that C-terminally truncated CTFs, the products that should be generated by ε-cleavage, indeed become substrates for γ-secretase and generate Aβ40 and 42.

† This work was supported in part by a grant-in-aid for Scientific Research on Priority Areas, Advanced Brain Science Project (to Y.I.), and by a grant-in-aid for Scientific Research on Encouragement of Young Scientists (B) (to S.F.) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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[1] Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-protein; APP, β-amyloid precursor protein; BACE, β-site APP-cleaving enzyme; CTF, C-terminal fragment; PS, presenilin; AICD, APP intracellular domain; CHO, Chinese hamster ovary; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *tert*-butyl ester; wt, wild type; mt, mutant; PCR, polymerase chain reaction.

10.1021/bi049399k CCC: $27.50 © 2004 American Chemical Society
Published on Web 09/29/2004

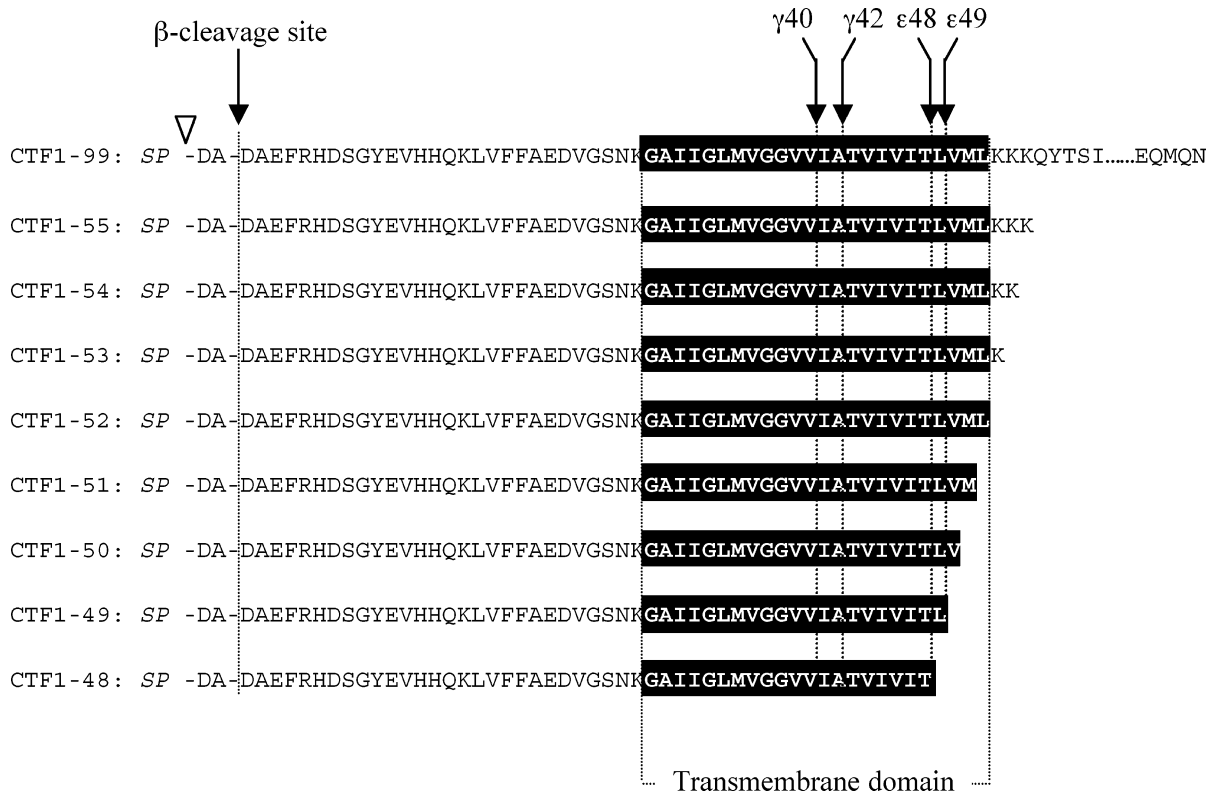

FIGURE 1: C-Terminally truncated CTFs expressed in CHO cells. Various carboxyl-terminally truncated CTFs were fused to the signal peptide sequence (MLPGLALLLLAAWTARA) of APP followed by an Asp-Ala dipeptide intervening in the junction. This insertion allowed precise cleavage by signal peptidase at the β-cleavage site, producing Aβ that starts from Asp-1. Key: γ40, γ-cleavage at the carboxyl terminus of Val-40; γ42, γ-cleavage at the carboxyl terminus of Ala-42; ϵ48, ϵ-cleavage at the carboxyl terminus of Thr-48; ϵ49, ϵ-cleavage at the carboxyl terminus of Leu-49; *SP*, the signal peptide sequence of APP; open arrowhead, an additional cleavage predicted (see the legend to Figure 4).

CTF1−49 produced predominantly Aβ40, while CTF1−48 produced preferentially Aβ42. This suggests that the preceding ϵ-cleavage determines the preference for the final product, Aβ40 or Aβ42.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatments. Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin/streptomycin (Invitrogen). For serum-free culture, cells were maintained in a chemically defined, animal component-free medium, CD-AF (Sigma). For stable transfectants, G418 (Wako, Osaka, Japan) was added to the culture medium at a concentration of 200 μg/mL. For inhibition of γ-secretase activity, 5 h after transfection, cells were treated with either *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *tert*-butyl ester (DAPT) (Calbiochem, San Diego, CA) or L-685,458 (Calbiochem) (*19*, *20*). Following 24 h incubation, cells were harvested, and conditioned media were collected for further analysis.

Constructs and Transfection. Mammalian expression vector pcDNA3.1 containing APP751 was amplified by polymerase chain reaction (PCR) using the primers 5′-GAT GCA GAT GCA GAA TTC CGA CAT GAC TCA-3′ and 5′-CGC CCG AGC CGT CCA GGC GGC CAG-3′ and subjected to self-ligation to fuse the βCTF (C99) coding sequence with the signal peptide sequence of APP. The resultant construct contained additional Asp and Ala residues on the amino-terminal side of the β-cleavage site and is referred to here as CTF1−99. This modification allows the product to be cleaved precisely at the β-cleavage site, generating Aβ that starts from Asp-1 (*21*). For truncation of the C-terminus of CTF1−99, the oligonucleotide primer 5′-TAG ACC CCC GCC ACA GCA GCC TCT-3′ was used for PCR combined with 5′-GGT GAT GAC GAT CAC TGT CGC TAT-3′, 5′-CAA GGT GAT GAC GAT CAC TGT CGC-3′, 5′-CAC CAA GGT GAT GAC GAT CAC TGT-3′, 5′-CAT CAC CAA GGT GAT GAC GAT CAC-3′, 5′-CAG CAT CAC CAA GGT GAT GAC GAT-3′, 5′-CTT CAG CAT CAC CAA GGT GAT GAC-3′, 5′-CTT CTT CAG CAT CAC CAA GGT GAT-3′, or 5′-TTT CTT CTT CAG CAT CAC CAA GGT-3′ (CTFs1−48, 1−49, 1−50, 1−51, 1−52, 1−53, 1−54, and 1−55, respectively). C-Terminal truncation was confirmed by DNA sequencing, and successfully engineered constructs were introduced into CHO cells by Lipofectamine 2000 (Invitrogen), according to the manufacturer's instruction.

Immunoprecipitation and Western Blotting. Conditioned media of transfectants were centrifuged at 20000*g* for 10 min to remove cell debris. The resulting supernatants were incubated overnight with 6E10 (Signet Laboratories, Dedham, MA), a monoclonal antibody raised against amino acid residues 1−17 of human Aβ, and protein G−Sepharose beads (Amersham Biosciences, Piscataway, NJ). After sufficient washing of the beads, bound proteins were separated on a 16.5% Tris−Tricine gel and transferred onto a nitrocellulose membrane (Schleicher & Schnell, Keene, NH).

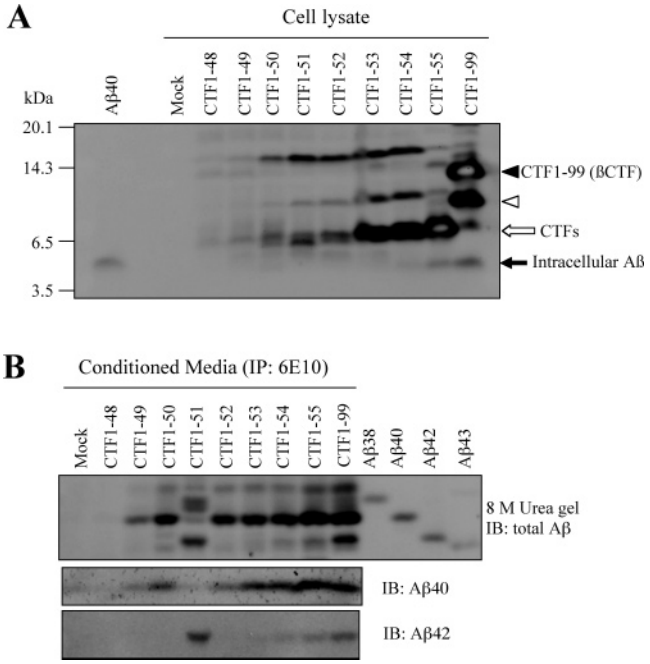

FIGURE 2: Truncated CTFs in the lysate and Aβ in the media. After transfection of the expression vector carrying each CTF, the cells were harvested for the preparation of cell lysates, and Aβ in the conditioned media was subjected to immunoprecipitation with 6E10. Cell lysates were subjected to western blotting with 6E10 (A). In addition to CTFs (open arrow), intracellular Aβ (closed arrow) was detectable in cells expressing CTFs1−99 and 1−55. Closed and open arrowheads in (A) indicate CTF1−99 and a potential caspase-cleaved product, respectively (*25*, *26*). The latter was not observed in 7WD10 cells (see Figure 3). The more CTFs were C-terminally truncated, the less CTFs accumulated in the cell. This was especially the case with CTFs1−48 and 1−49. Detection of Aβ40 and Aβ42 using specific antibodies, BA27 and BC05, respectively, and of total Aβ using 6E10 following separation of Aβ species by urea gel (B). The immunoblot shows the presence of Aβ in the conditioned media of cells expressing truncated CTFs. The amount of Aβ produced from CTF1−48 was under the detection limit. These data demonstrate that the truncated CTFs can be immediate substrates for γ-secretase. Most interestingly, Aβ42 was a predominant species secreted from cells expressing CTF1−51.

Alternatively, the samples were separated on a 10% Tris−Tricine gel containing 8 M urea. The blots were visualized by an ECL system (Amersham Biosciences) using monoclonal antibodies, 6E10, BA27 (highly specific for Aβ40), and BC05 (raised against Aβ35−43, specific for Aβ42, but cross-reactive with CTFs and full-length APP), for assessing total Aβ, Aβ40, and Aβ42, respectively (*22*). For quantification of Aβ, chemiluminescence was captured using a LAS-1000plus luminescent image analyzer (Fuji Film, Tokyo, Japan) and quantified by Image Gauge software version 3.4 (Fuji Film) with defined amounts of synthetic Aβ40 or 42 as an authentic control (*18*, *23*). To visualize CTFs in the cell lysate, lipids were extracted from harvested cells with chloroform/methanol (2:1), and protein residues were subjected to western blotting, after being extracted with 70% formic acid. CTFs in the microsomal fractions were detected as follows. Cells were homogenized in buffer A (20 mM PIPES, pH 7.0, 140 mM KCl, 0.25 M sucrose, 5 mM EGTA) with a glass/Teflon homogenizer. The homogenates were centrifuged at 800*g* for 10 min to remove nuclei and cell debris. The postnuclear supernatants were recentrifuged at 100000*g* for 1 h. The resulting pellets were defined as the microsomal fractions and extracted lipids before western blotting.

RESULTS

Expression of Carboxyl-Terminally Truncated CTFs. ϵ-Cleavage sites of APP have been identified to be at the carboxyl termini of Thr-48 and Leu-49 (according to Aβ numbering), which are ~10 residues downstream of γ-cleavage sites (*15*−*18*). Our failure to identify longer AICDs including AICDs41−99 and 43−99 (*15*−*18*) and the previous report showing the presence of Aβ1−46 in the extract of skeletal muscles (*24*) led us to assume that ϵ-cleavage is the primary cleavage mediated by γ-secretase. We have asked whether there is any correlation between γ- and ϵ-cleavage of APP and found a significant relationship between the two kinds of cleavage; ϵ-cleavage at the carboxyl side of Leu-49, releasing AICD50−99, is linked to Aβ40 production, while that at the carboxyl side of Thr-48, releasing AICD49−99, is linked to Aβ42 production (*15*, *18*). On the basis of these observations, we speculated that ϵ-cleavage precedes γ-cleavage and that ϵ-cleavage sites determine the preference for the final product, Aβ40 or Aβ42. To test this hypothesis, we first examined whether carboxyl-terminally truncated CTFs, postulated intermediates generated by ϵ-cleavage close to the membrane/cytoplasmic boundary, produce Aβ. Figure 1 shows a schematic depiction of the carboxyl-terminally truncated CTFs examined here. These CTFs were fused to the signal peptide sequence of APP followed by an Asp-Ala dipeptide intervening at the junction site. This insertion warranted specific cleavage at the β-cleavage site by signal peptidase, producing an Aβ that starts at Asp (*21*).

After transfection of the expression vector carrying each CTF, CHO cells were harvested for the preparation of cell lysates, and conditioned media were subjected to immunoprecipitation and western blotting using 6E10. In the cells expressing CTF1−99, the band at 11.1 kDa represents CTF1−99, and that at 7.5 kDa is likely a caspase-cleaved fragment, as reported previously (shown by closed and open arrowheads, respectively, in Figure 2A) (*25*, *26*). In cells expressing CTF1−55, the robust CTF band and intracellular Aβ were detected, as seen in the CTF1−99 cells. When the stop transfer signal (triple lysines) at the membrane/cytoplasmic boundary is shortened (CTFs1−54 and 1−53), the levels of intracellular CTFs and production of Aβ were substantially reduced (Figure 2). Complete deletion of the stop transfer signal and further truncation of the transmembrane domain dramatically reduced the levels of intracellular CTFs (CTFs1−48 to 1−52), presumably because of unstable insertion into the membrane (Figure 2A, white arrow).

We were indeed able to detect Aβ in all of the media examined in this study. It should be noted that Aβ in the medium of cells expressing CTF1−48 was under the detection limit, as shown in Figure 2 (see also Figure 4). The major product from CTFs except for CTF1−51 (and CTF1−48; see below) is Aβ40, just as in the case of CTF1−99 and full-length APP. Separation of Aβ species by means of 8 M urea gel revealed that CTFs1−49 to 1−50, CTFs1−52 to 1−55, and CTF1−99, predominantly produced Aβ40, followed by Aβ42 and Aβ37. Most interestingly, CTF1−51 generated predominantly Aβ42 and some Aβ38

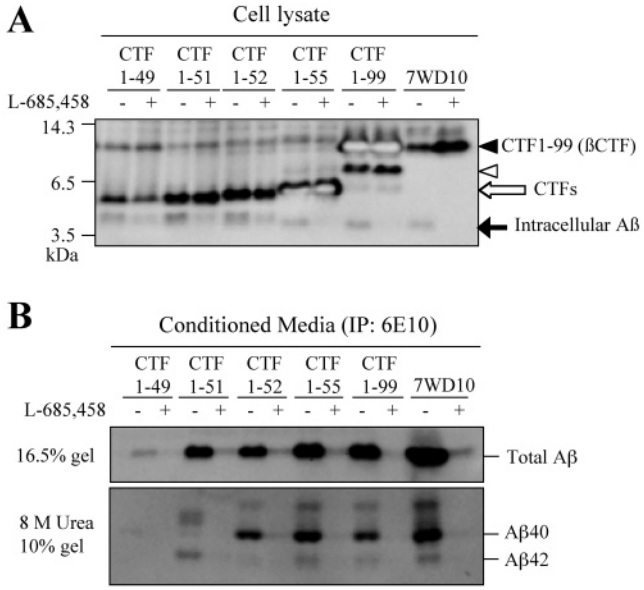

FIGURE 3: Treatment with L-685,458. Cells were incubated for 24 h in the presence of 2 μM L-685,458, a transition state analogue inhibitor of γ-secretase. Cell lysates were subjected to western blotting with 6E10 (A). Intracelluar Aβ (closed arrow) in cells expressing CTF1−55 or 1−99 (closed arrowhead) was suppressed by the treatment. In cells expressing CTF1−49, 1−51, or 1−52 (open arrow), 6E10-reactive fragments migrating slightly slower than Aβ were observed and suppressed by the treatment. The open arrowhead indicates a potential caspase-cleaved product. The origins of bands at ~10 kDa in the lanes for CTFs1−49 to 1−55 are unknown. Aβ secreted into media from cells expressing CTFs1−49 to 1−55 (B). Aβ production from cells expressing the CTFs was significantly suppressed by the inhibitor. The suppression by the inhibitor indicates that Aβ production from CTFs is mediated by γ-secretase.

and Aβ39 (Figure 2B, upper panel). Immunodetection of Aβ in the conditioned media showed that the more CTF was carboxyl-terminally truncated, the smaller were the amounts of Aβ produced (Figure 2B). These data clearly show that those truncated CTFs serve as substrates for γ-secretase, generating Aβ40 and Aβ42.

To further confirm that CTFs are substrates for γ-secretase, but not other distinct protease(s), we treated the cells with L-685,458, a transition state analogue inhibitor of γ- secretase (*9*, *19*). As expected, this treatment with L-685,458 caused a significant accumulation of βCTF in 7WD10 cells, a stable cell line expressing APP751 (Figure 3A) (*27*). In contrast, no such accumulation of those truncated CTFs was noted after the same treatment of the cells expressing CTFs1−49 to 1−99 (Figure 3A, open arrow). However, the amounts of Aβ secreted into the media were significantly suppressed with L-685,458 (Figure 3B). This suggests that those truncated CTFs are indeed substrates of γ-secretase and that truncated CTFs may be rather efficiently degraded through an alternative pathway(s), when γ-secretase is blocked.

Aβ Production from CTFs1−48 and 1−49. We next asked whether ϵ-cleavage at the carboxyl side of Leu-49 leads to predominant production of Aβ40 and whether that at Thr-48 leads to preferential production of Aβ42. Aβ produced from CTFs1−48 and 1−49 cells was quantified by immunoprecipitation/western blotting with defined amounts of synthetic Aβ as a control, and Aβ42/Aβ40 ratios were determined (Figure 4).

Because only a trace amount of Aβ was found in the conditioned medium of the cells expressing CTF1−48 (Figure 2), large amounts (30 mL) of the medium were subjected to immunoprecipitation. This made it possible to detect Aβ secreted from the cells expressing CTF1−48 (Figure 4A,B, lower panels). However, very small amounts of Aβ were detected also in mock transfectants, but its levels were consistently lower than those in CTF1−48 cells (Figure 4A,B, lower panels). Because 6E10 used for immunoprecipitation does not recognize rodent Aβ derived from endogenous APP in CHO cells (data not shown), the detected Aβ in mock transfectants should be derived from bovine serum. To confirm that the Aβ detected in the medium of CTF1−48 cells was originated from these cells, the cells were maintained in serum-free medium after transfection. Western blotting of the immunoprecipitate from the serum-free medium revealed a bona fide Aβ, which indicates that Aβ was indeed produced by CTF1−48 cells.

BC05-reactive fragments of 5.1 and 5.3 kDa were detected in the media of CTFs1−48 and 1−49 transfected cells, respectively (Figure 4B, closed arrowheads). Western blotting with authentic synthetic peptides Aβ1−48 and Aβ1−49 indicated that those 5.1 and 5.3 kDa fragments represented CTFs1−48 and 1−49, respectively, and that those truncated CTFs were also released into the media (Figure 4B). Thus, the amounts of Aβ40 and Aβ42 produced from CTFs1−48, 1−49, and 1−99 were quantified. As shown in Figure 4C, Aβ production from the truncated CTFs was remarkably reduced, especially for CTF1−48. Table 1 shows the relative amounts of Aβ in the media and those of CTFs located in the microsomal fractions from CTFs1−48, 1−49, and 1−99 cells, in which those values from CTF1−55 cells are assumed to be 100%. The amounts of Aβ40 in the media of CTFs1−48 and 1−49 cells relative to that in the medium of CTF1−55 cells were 0.04% and 34.7%, respectively. For Aβ42, the corresponding figures in the media of CTFs1−48 and 1−49 were 0.31% and 9.99%, respectively, of the amount of Aβ42 secreted from CTF1−55 cells. A likely explanation for this profoundly decreased production of Aβ from CTFs1−48 and 1−49, compared with that from CTF1−55 (and 1−99), is greatly reduced levels of the substrates in the membrane. The amounts of CTFs1−48 and 1−49 in the cells were 3.03% and 4.47%, respectively, relative to those of CTF1−55. Thus, reduced levels of substrates are not sufficient to explain the lower production of Aβ from CTF1−48.

Overall, the cells expressing CTF1−49 preferentially produced Aβ40, while those expressing CTF1−48 preferentially produced Aβ42 (Figure 4D). This is consistent with the assumption that the ϵ-cleavage at the carboxyl terminus of Leu-49 is linked to Aβ40 production and that ϵ-cleavage at the carboxyl terminus of Thr-48 is linked to preferential production of Aβ42. Thus, the above results support our hypothesis that ϵ-cleavage largely determines the production of particular Aβ species.

Aβ42 Production from CTF1−48 Is Mediated by Presenilin. We next examined the effects of two γ-secretase inhibitors, L-685,458 and DAPT. L-685,458 is known as a transition state analogue inhibitor of γ-secretase, while DAPT, a nontransition analogue inhibitor, is also a very potent inhibitor (*19*, *20*). Treatment with L-685,458 or DAPT significantly suppressed Aβ production from CTFs1−49 and

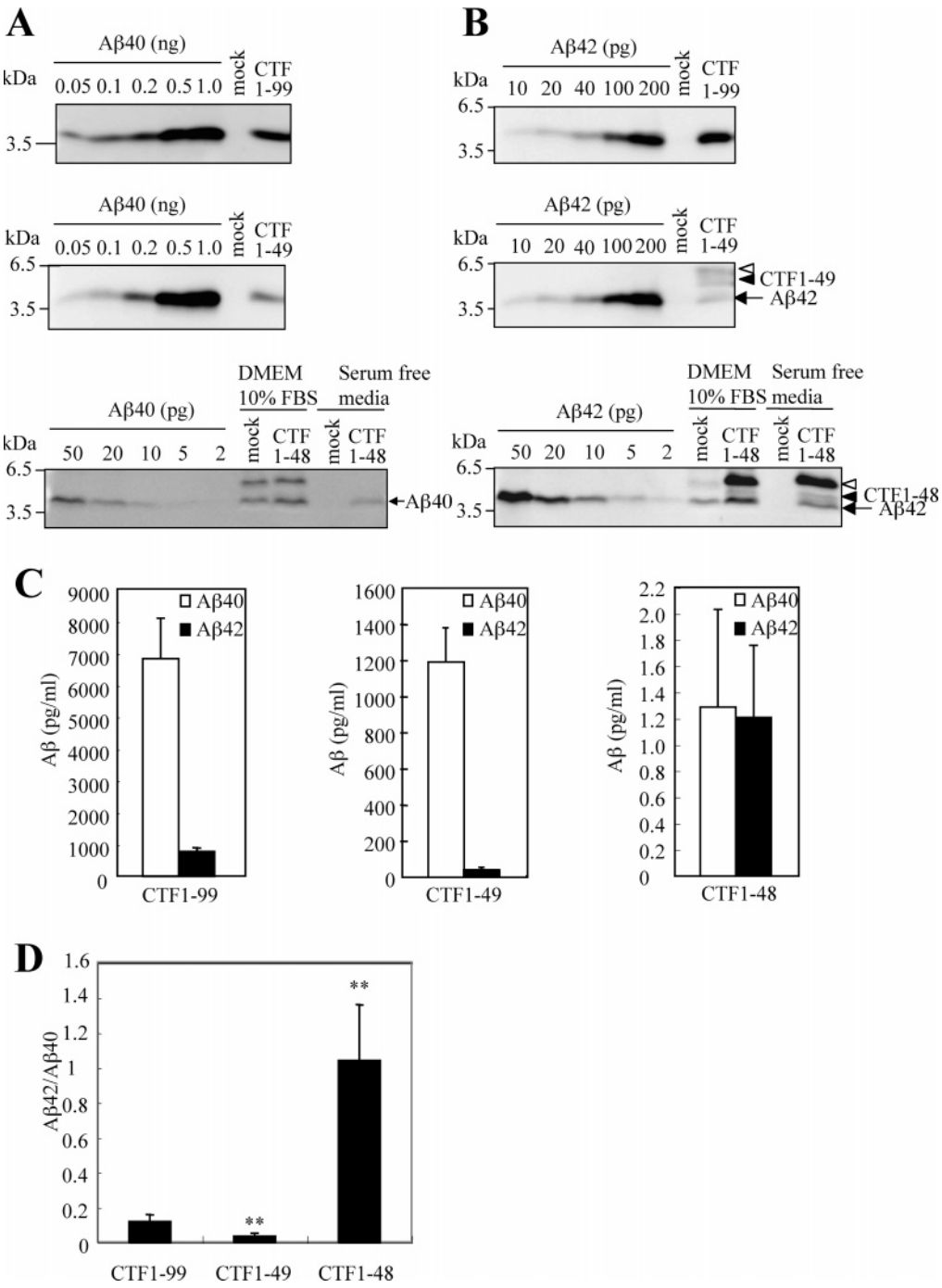

FIGURE 4: Production of Aβ40 and Aβ42 from CTF1−99 (βCTF), CTF1−49, and CTF1−48. Conditioned media of cells were collected for immunoprecipitation 24 h after transfection of various CTF constructs. Immunoprecipitates from the conditioned media with 6E10 were subjected to western blotting, together with defined amounts of synthetic Aβ for quantification. Aβ40 was visualized by BA27 (A). 5.5 kDa fragments reactive with BA27 were detected in media of mock and CTF1−48 cells, but their origins are unknown. In serum-free media, these fragments were undetectable. Aβ42 was detected by BC05 (B). Two BC05-positive fragments at 5.1 and 5.3 kDa in the media of CTFs1−48 and 1−49 cells comigrated with authentic synthetic peptides Aβ1−48 and Aβ1−49, respectively (data not shown), strongly suggesting that those CTFs1−48 and 1−49 were released into the media [closed arrowheads in (B)]. In addition, 5.3 and 5.4 kDa fragments were detected in the media of CTF1−48 and 1−49 cells, respectively [open arrowheads in (B)]. Signal peptide cleavage prediction by SignalP V2.0 (http://www.cbs.dtu.dk/services/SignalP-2.0/) indicates the possibility for an additional signal peptide cleavage before DA insert (Figure 1, open arrowhead). Presumably, those 5.3 and 5.4 kDa fragments were CTFs−2−48 and −2−49, respectively. Quantification of Aβ produced from the CTFs1−99, 1−49, and 1−48 (C). The amounts of Aβ in mock cells as a background were subtracted from those of Aβ in CTF-expressing cells. The cells expressing CTFs1−48 and 1−49 released much smaller amounts of Aβ than did those expressing CTF1−99. Values represent means ± SD of six independent experiments. Panel D shows ratios of Aβ42/Aβ40 in CTF1−99, CTF1−49, and CTF1−48 cells. Values represent means ± SD of six independent experiments. The Aβ42/Aβ40 ratio for CTF1−49 was significantly decreased, and that for CTF1−48 was greatly increased, as compared with that of CTF1−99. ** = $P < 0.01$ vs the ratio for CTF1−99.

1−99 (Figure 5A,B,D,E). L-685,458 also suppressed Aβ production from CTF1−48. Unexpectedly, however, DAPT failed to suppress Aβ production from CTF1−48 (Figure 5C,F). Even when the concentrations of DAPT were increased to 10 μM, its inhibitory effect was undetectable (data not shown).

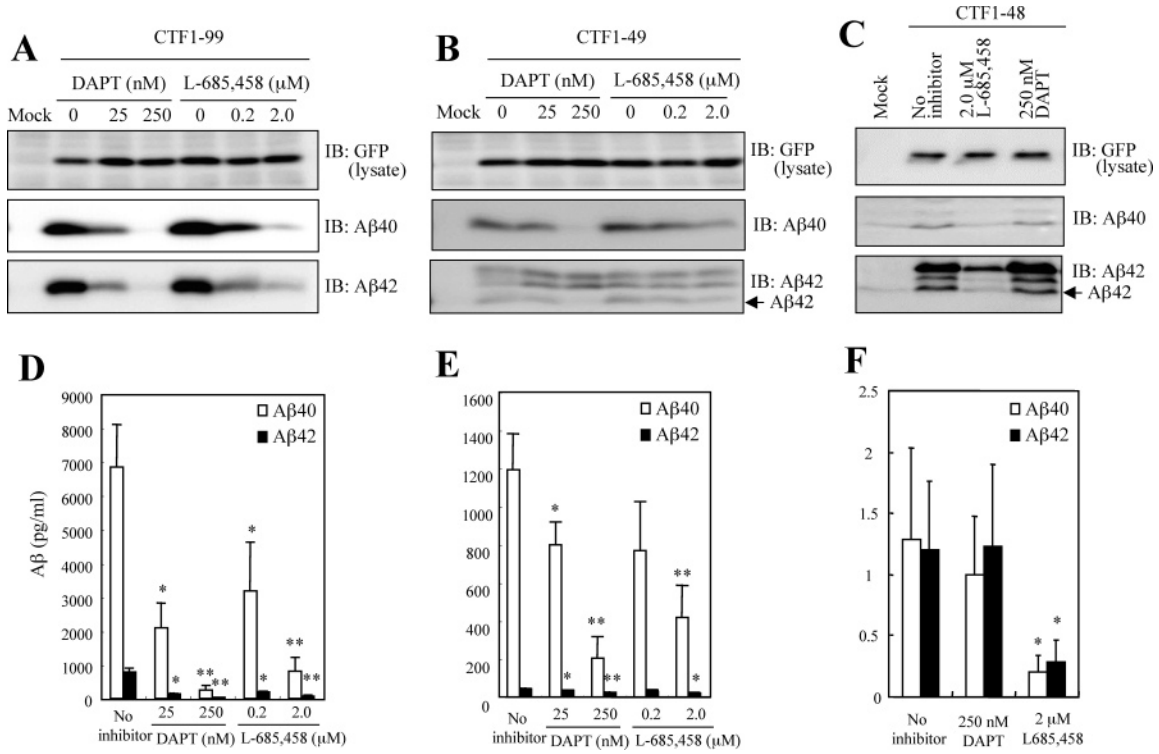

FIGURE 5: Treatment with L-685,458 and DAPT of the cells expressing CTFs1−99, 1−49, and 1−48. After transfection of the CTF constructs, the cells were incubated for 24 h in the presence of L-685,458 and DAPT at the concentrations indicated. Aβ40 and Aβ42 in the conditioned media of cells expressing CTFs1−99, 1−49, and 1−48 were quantified by BA27 and BC05 (A, B, and C, respectively). The amounts of Aβ in mock cells were subtracted from those of Aβ in CTF-expressing cells. Green fluorescent protein (GFP) was coexpressed with those CTFs, as an internal standard. Quantification of Aβ produced from CTFs1−99 and 1−49 in the presence of γ-secretase inhibitors (D and E, respectively). Values represent means ± SD of three to four independent experiments. Panel F represents quantification of Aβ produced from CTF1−48 in the presence of γ-secretase inhibitors. DAPT failed to suppress Aβ production from CTF1−48. Values represent means ± SD of four independent experiments. * = P < 0.05 vs value for no treatment; ** = P < 0.005 vs value for no treatment.

PS is thought to function as the catalytic core of γ-secretase, and the two conserved aspartates in transmembrane domains 6 and 7 are necessary for its activity (*3*, *9*). We next examined whether PS is involved in Aβ production from CTFs1−48 and 1−49 by expressing the Asp mutant of PS. Stable cell lines expressing human PS1 wild type (PS1 WT) or PS1 D385A were established, and we confirmed that endogenous PS was largely displaced in each cell line (data not shown). PS WT cells (nos. 10, 19, and 28) and parent cells (the cells expressing endogenous PS alone, shown as CHO) generated similar levels of Aβ from CTFs1−49 and 1−99 (Figure 6A,B,D,E). In contrast, the cell lines expressing PS1 D385A (nos. 7, 28, and 39) produced profoundly decreased amounts of Aβ from CTFs1−49 and 1−99. Aβ production from CTF1−48 was also reduced in the PS1 D385A cells, which indicates that Aβ production from CTF1−48 as well as from other CTFs is mediated by PS1-involving γ-secretase (Figure 6C,F).

DISCUSSION

We and others previously identified a novel cleavage site of APP, near the membrane/cytoplasmic boundary, very similar to site 3 cleavage of Notch (*15*−*17*). However, it has remained unclear whether a causal relationship exists between γ- and ϵ-cleavage. Recently, we found that CHO cells expressing wtPS or wtAPP released mainly AICD50−99, while those expressing mtPS or mtAPP increased the proportion of AICD49−99 (*18*). This raises the possibility for a link between Aβ42 production and AICD49−99 production and, thus, the relationship between these two distinct cleavage sites on the APP molecule.

Here we provide the data supporting this causal relationship between γ- and ϵ-cleavages. First, particular expression in CHO cells of the postulated ϵ-cleaved products, CTFs1−48 and 1−49, led to Aβ secretion in the media, indicating that the ϵ-cleaved products become substrates for γ-secretase. Second, whereas CTF1−49, a counterpart of AICD50−99, produced mainly Aβ40, CTF1−48, a counterpart of AICD49−99, produced preferentially Aβ42. This is consistent with our previous observation that the generation of AICDs50−99 and 49−99 by ϵ-cleavage is linked to Aβ40 and Aβ42 production, respectively (*18*). Further, it is of note that CTF1−49 would be a more favorable substrate for γ-secretase, when the amounts of Aβ40 and Aβ42 relative to the amount of substrate are compared with those in CTF1−55 (Table 1).

In this study, we also attempted to perform "reverse experiments" by expressing, in CHO cells, CTF41−99 or 43−99 (the postulated products generated by γ-cleavage) fused with the APP signal peptide and examined whether ϵ-cleavage can occur after γ-cleavage. Although we detected AICDs containing AICDs50−99 and 49−99 by incubation of the membrane prepared from cells expressing signal peptide-fused CTF41−99, the treatment with 1 μM L-685,458 failed to suppress the production of the AICDs, and thus we cannot exclude the possibility of non-γ-secretase-mediated degradation (data not shown). Regarding cells expressing signal peptide-fused CTF43−99, we detected only back-

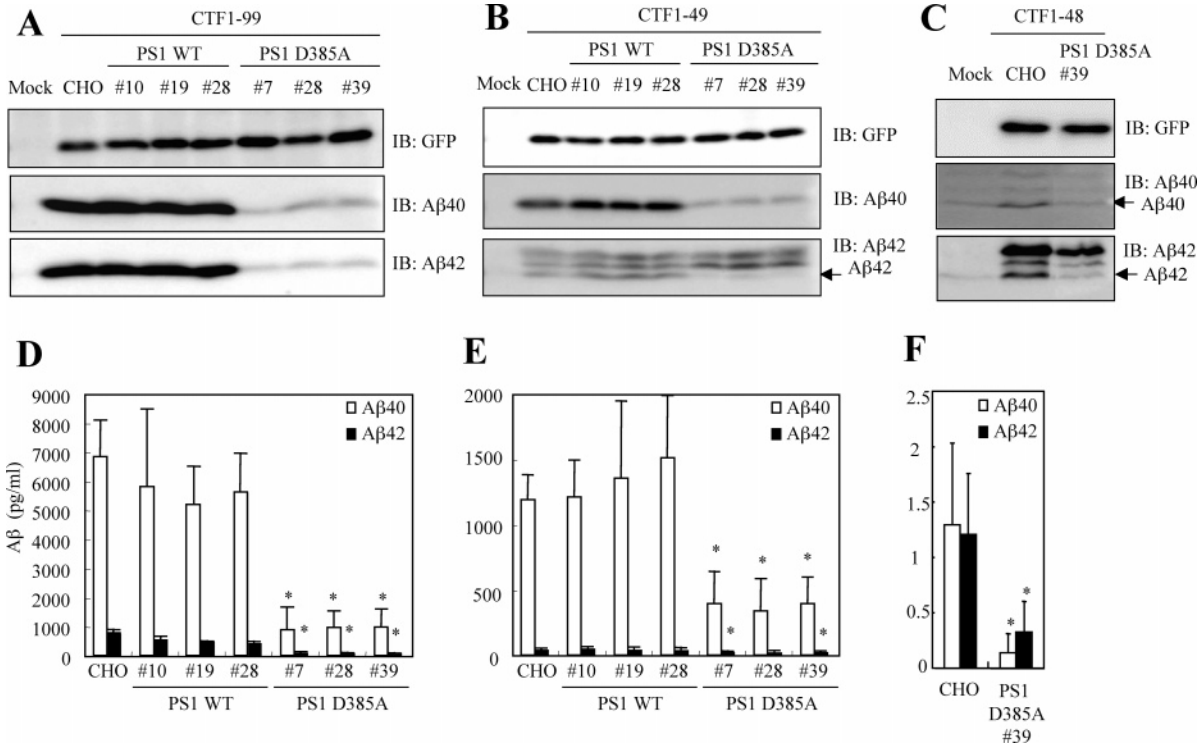

FIGURE 6: Effects of the PS1 D385A mutant on Aβ production. The CHO cells stably expressing wtPS1 or a dominant negative mutant of PS1 (D385A) were transfected with the CTF constructs. Aβ40 and Aβ42 in the conditioned media of cells expressing CTFs1−99, 1−49, and 1−48 were quantified by BA27 and BC05 (A, B, and C, respectively). Cells expressing the PS1 dominant negative mutant produced a trace amount of Aβ from all CTFs tested here, as compared with the cells expressing wtPS1. GFP as an internal standard was coexpressed with those CTFs. Quantification of Aβ produced from PS1 D385A cells transfected with CTFs1−99, 1−49, and 1−48 constructs (D, E, and F, respectively). The amounts of Aβ in mock cells were subtracted from those of Aβ in CTF-expressing cells. Values represent means ± SD of three to four independent experiments. * = $P < 0.05$ vs values for the original CHO cell. PS1 WT and PS1 D385A stand for the cells expressing wtPS1 and the dominant negative mutant of PS1, respectively.

Table 1: Relative Amounts of Aβ40, Aβ42, and CTF Produced[a]

	Aβ40 (%)	Aβ42 (%)	CTF (%)
CTF1−55	100	100	100
CTF1−49[b]	34.7	9.99	4.47
CTF1−48[b]	0.04	0.31	3.03
CTF1−99[b]	198.3	198.4	ND

[a] Aβ40 and Aβ42 in the media of cells expressing CTFs were quantified by western blotting using defined amounts of synthetic peptide as authentic controls (see Experimental Procedures). CTFs in cell lysate were visualized, and relative band intensities were quantified by using Image Gauge software (Fuji Film). The transfer and retained efficiency to the nitrocellulose membrane was assumed to be the same for CTFs1−55, 1−49, and 1−48. The amounts of Aβ and CTF from CTF1−55 cells were defined as 100% for comparison. ND = not determined. [b] $P < 0.05$ vs the value for CTF1−55.

ground level of AICD by western blotting and mass spectrometry (data not shown). Since we have failed to detect longer AICDs, such as CTFs41−99 and 43−99 as counterparts of Aβ40 and 42, respectively (*15*), the present data suggest that ϵ-cleavage precedes γ-cleavage and that ϵ-cleavage sites determine the preference for Aβ species. This may be also supported by the observations that the substitution of Leu-49 with Pro-49 resulted in a significant decrease of AICD and Aβ40 and a slight increase of Aβ42 (*17*; data not shown). It is quite possible that Pro insertion, an α-helix breaker, specifically inhibits ϵ-cleavage at the carboxyl terminus of Pro-49 and subsequently causes a specific reduction in Aβ40 production.

An unexpected observation was that CTF1−51 produces predominantly Aβ42, together with some other minor species, Aβ38 and Aβ39. This could be an important finding which may lead to elucidation of the mechanism of Aβ42 production. This particular CTF fragment may not necessarily be artificial, because AICD prepared from HEK293 cells expressing mtAPP and mtPS has a significant peak representing AICD52−99 (*18*, *28*). This suggests that CTF1−51, a counterpart of AICD52−99, may contribute to the production of Aβ42 at least in HEK cells. CTFs1−48 and 1−51 share similar characteristics in preferentially producing Aβ42 or in being preferentially processed to Aβ42. The distance of three residues between CTFs1−48 and 1−51 leads us to speculate that the α-helical model may explain in part the enigmatic processing characteristics associated with γ-secretase. The last residues of the CTFs1−48 and 1−51, Thr-48 and Met-51, respectively, are aligned on the α-helical surface, because an α-helix needs 3.6 residues for one complete turn. If the same Aβ42-producing γ-secretase is to clip the carboxyl termini of particular residues within the transmembrane domain, it is quite reasonable to assume that this cleavage occurs every three (or sometimes four) residues. This is because its catalytic site(s) of Aβ42-producing γ-secretase should be toward a particular direction within the transmembrane domain, and particular cleavage sites of the APP molecule should face the catalytic site(s) of γ-secretase at every three (or four) residues. According to this α-helical model, the carboxyl termini of Met-51, Thr-48, Ileu-45, and Ala-42 are aligned on the α-helical surface of APP. This may suggest that successive cleavage of CTF1−48 or 1−51 at every three residues may finally

produce Aβ42. However, it should be noted that the majority of CTF1−48 does not come from CTF1−51, because AICD49−99 appears to be a predominant product of ϵ-cleavage, compared with AICD52−99.

DAPT, a nontransition state analogue, has potent γ-secretase inhibitory activity (*20*). As expected, DAPT inhibited Aβ production from CTFs1−49 and 1−99 in dose-dependent manners, but unexpectedly, it failed to inhibit Aβ production from CTF1−48. In fact, even 10 μM DAPT did not suppress the γ-secretase activity that participates in the processing of CTF1−48. One may deduce from this observation the presence of novel enzyme(s) sensitive to L-685,458, but insensitive to DAPT. However, the effects of the dominant negative mutant of PS1 indicate that PS1 itself is involved in the production of Aβ from CTF1−48. Recently, Kornilova et al. reported differential effects of inhibitors on the γ-secretase complex (*29*). In their photoaffinity labeling, DAPT at high concentrations successfully competed with a photoactivatable transition state analogue but could not displace the photoprobe at the concentrations with which a transition state analogue can compete. This suggests that DAPT binds to the active sites of γ-secretase, which are distinct from but overlap with the catalytic sites bound by the transition state analogue. The differential effects of DAPT on Aβ production from CTFs1−48 and 1−49 may be consistent with the view that L-685,458 and DAPT have distinct binding sites on presenilin (*29*).

ACKNOWLEDGMENT

We thank Dr. E. H. Koo, University of California, San Diego, for 7WD10 cells, Dr. T. Iwatsubo, University of Tokyo, for anti-presenilin serum, Dr. A. Asami, Takeda Chemical Industries, for BA27 and BC05, and members of the laboratory for helpful discussion.

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BI049399K