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Amino-truncated amyloid β-peptide (Aβ5-40/42) produced from caspase-cleaved amyloid precursor protein is deposited in Alzheimer's disease brain

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ABSTRACT

Caspase activation and apoptosis are implicated in Alzheimer's disease (AD). In view of the finding that the amyloid precursor protein (APP) undergoes caspase-mediated cleavage in the cytoplasmic region, we analyzed amyloid β-peptide (Aβ) production in human neuronal and nonneuronal cells expressing wild-type APP and the caspase-cleaved form of APP (APPΔC). Biochemical analyses, including immunoprecipitation/mass spectrometry, revealed that APPΔC-expressing cells secrete increased levels of amino-terminally truncated Aβ5-40/42 and reduced levels of Aβ1-40/42, compared with wild-type APP-expressing cells. We propose that Aβ5-40/42 is derived from alternative β-cleavage of APP by α-secretase-like protease(s), based on data from treatment of cells with inhibitors of BACE and α-secretase. Apoptosis induction resulted in this alternative cleavage of APP in wild-type APP-expressing cells. Moreover, immunohistochemical staining of the AD brain with an end-specific antibody to Aβ5-40/42 revealed peptide deposits in vascular lesions with amyloid angiopathy. The data collectively suggest that caspase cleavage of APP leads to increased production and deposition of Aβ5-40/42 in the AD brain, and highlight the significance of amino-truncated Aβ in the pathogenesis of AD.

Key words: BACE • α-secretase • apoptosis

Alzheimer's disease (AD) is neuropathologically characterized by the presence of senile plaques, neurofibrillary tangles (NFT), and neuronal death. The major constituent of senile plaques is amyloid β-peptide (Aβ), which is produced from sequential endoproteolytic processing of the transmembrane amyloid precursor protein (APP) by β- and γ-secretases. Alternative processing of APP by α- and γ-secretases generates a shorter peptide designated p3 (1). In addition to Aβ1-40/42, amino-terminally truncated forms of Aβ, such as Aβ11-40/42, are deposited in the AD brain (2, 3).

Although the mechanism of AD neurodegeneration is currently unclear, Aβ accumulation appears to play a critical role in AD pathogenesis. A body of evidence suggests that apoptosis is one of the mechanisms underlying neuronal death in AD (4–6). In fact, activation of caspases has been shown to occur in postmortem AD brain tissues (7–10). APP acts as a caspase substrate, and is cleaved in its cytoplasmic domain, generating an APP fragment lacking the C-terminal 31 amino acids (APPΔC) (11–14). APP derivatives from this specific cleavage are present in the AD brain (11, 15, 16). However, it remains to be elucidated whether the caspase-mediated cleavage of APP contributes to the neuropathology of AD. It is particularly important to clarify whether this APP cleavage influences Aβ generation.

In this study, we used human neuronal and nonneuronal cell lines expressing either APP or its caspase-cleaved form, APPΔC, to analyze the generation of secreted and intracellular Aβ. Our data indicate that amino-terminally truncated Aβ5-40/42 is specifically generated from APPΔC, probably through alternative APP processing by α-secretase-like protease(s). We additionally provide evidence that Aβ5-40/42 is deposited in pathological lesions in AD brains.

MATERIALS AND METHODS

Antibodies and chemicals

Three APP antibodies were used: monoclonal antibody 22C11, which is specific for the N-terminal region (aa 66-81) of APP (Roche, Mannheim, Germany); goat polyclonal antibody 207, which recognizes aa 18-686 of human APP770 (provided by Dr. S. G. Younkin, Center for Neuroscience, Mayo Clinic, Jacksonville, FL) (17); and rabbit polyclonal antibody AC24, which was raised against the C-terminal 24 amino acids of APP (18). The following monoclonal Aβ antibodies were used: BAN50 (specific for Aβ1-16; the epitope comprises Aβ1-10), BNT77 (specific for Aβ11-28; the epitope comprises Aβ11-16), BA27 (specific for Aβ40), BC05 (specific for Aβ42) (gifts from Takeda Chemical Ind., Osaka, Japan), 6E10 (against Aβ1-16) and 4G8 (against Aβ17-26) (BioSource International, Nivelles, Belgium), and 1A10 (specific for Aβ40; IBL, Gunma, Japan). The anti-APP cleavage product antibody (AB5942) was obtained from Chemicon (Temecula, CA). OM99-2 and TAPI-1 were purchased from Bachem (Budendorf, Switzerland) and Calbiochem (San Diego, CA), respectively.

Expression vectors and cell lines

Human APP695 cDNA was cloned into the *Xba*I site of pUC18 (yielding pUC18-APP) and introduced into the *Eco*RV site of the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) to produce pcDNA-APP. An APP695 cDNA fragment lacking the C-terminal 31 amino acids (APPΔC) was generated by polymerase chain reaction, using the following oligonucleotides: 5′-GAGGAGATCTCTGAAGTGAA-3′ (sense) and 5′-TCCCGGGTTAGTCAACCTCCACCACACCATG-3′ (antisense). The amplified product was digested with *Bgl*II/*Sma*I and ligated into the corresponding restriction sites of pUC18-APP. The resulting APPΔC cDNA was sequenced and cloned into the *Eco*RV site of pcDNA3.1 (yielding pcDNA-APPΔC).

Human neuroblastoma SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium/F-12 (Invitrogen) supplemented with 10% fetal bovine serum and 1%

penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. For generating cell lines stably expressing APP or APPΔC, SH-SY5Y cells were transfected with pcDNA-APP or pcDNA-APPΔC using the calcium phosphate method (CellPhect Transfection Kit; Amersham Biosciences, Piscataway, NJ). Transfectants were cultured in the presence of 400 μg/ml G418, and isolated clones were screened for the expression of APP by Western blot analysis. Human embryonic kidney 293 (HEK293) cells were transiently transfected with APP or APPΔC using Lipofectamine 2000 (Invitrogen).

Western blot analysis of cellular APP

Cells were washed with ice-cold PBS and lysed in RIPA buffer containing protease inhibitors, as described previously (19). Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA). Membranes were incubated with anti-APP antibodies (22C11 or AC24), and then with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG, and protein expression was detected with chemiluminescence reagents (Perkin Elmer Life Sciences, Boston, MA).

Immunoprecipitation and Western blot analysis of C-terminal fragments of APP

Cell lysates were incubated with 4G8 and protein G agarose (Roche) at 4°C overnight. Immunoprecipitated proteins were eluted in Tris-Tricine SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 2% 2-mercaptoethanol, 5% glycerol) and subjected to Tris-Tricine SDS-PAGE, followed by Western blotting with BAN50 or 4G8 or AB5942 antibodies.

Immunoprecipitation and Western blot analysis of secreted forms of APP (sAPP)

Cells were cultured in 10-cm dishes and incubated with fresh medium for 2 h. The conditioned media were harvested and centrifuged briefly to remove cell debris, and then 1 ml of each sample was made in ×1 RIPA buffer and incubated with antibody 207 and protein G agarose at 4°C overnight. Immunoprecipitates were washed with ice-cold RIPA buffer, treated with ×2 SDS sample buffer, and subjected to SDS-PAGE and Western blot analyses with 22C11 or BAN50 antibodies.

Measurement of Aβ in conditioned media by sandwich enzyme-linked immunosorbent assay (ELISA)

Cells were cultured in 12-well plates with fresh medium (0.6 ml), and conditioned media were harvested after 24 h. The levels of Aβ in the conditioned media were measured by sandwich ELISA, using Aβ antibodies (BAN50 or BNT77) for capture and HRP-conjugated BA27 or BC05 for detection, as described previously (20, 21). In brief, Aβ standard solutions (100 μl) and samples of conditioned media were applied to coated 96-well plates at 4°C overnight, followed by three washes with 0.05% Tween-20 in PBS. The plates were then incubated overnight with HRP-conjugated BA27 or BC05 at 4°C. Bound enzyme activity was measured using the TMB microwell peroxidase substrate system (Kirkegaard Perry Laboratories, Gaithersburg, MD). Synthetic Aβ1-40, Aβ1-42, and Aβ17-40 peptides were purchased from BioSource International.

Immunoprecipitation and Western blot analysis of secreted Aβ

Conditioned media were filtered using a Centricon 30 (Millipore) to remove APP and made in ×1 RIPA buffer. Secreted Aβ was immunoprecipitated from 1 ml aliquots of the media with BAN50 or 4G8 or BNT77 plus anti-mouse IgA. Immunoprecipitates were washed with ice-cold RIPA buffer, solubilized in Tris-Tricine SDS-PAGE sample buffer containing 7.8 M urea, and subjected to Tris-Tricine SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane (Pore size 0.22 μm, Schleicher and Schuell, Dassel, Germany), which was then boiled for 5 min in PBS to enhance signals (22), and reacted with BA27 or BC05 or 1A10 antibodies.

Immunoprecipitation and mass spectrometric analysis of secreted Aβ

Secreted Aβ in conditioned media was immunoprecipitated with BNT77 or 4G8 and incubated with 8 M guanidine hydrochloride for protein dissolution. Extracts were applied to Ziptip C18 (Millipore), washed, and eluted with 80% acetonitrile containing 10 mg/ml α-cyano-4-hydroxycinnamic acid. Then samples were analyzed using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Protein tip reader PBS-II, Ciphergen Biosystems, Palo Alto, CA; AXIMA-CFR, Shimadzu, Kyoto, Japan).

Western blot analysis of intracellular Aβ

Sensitive Western blot analysis of intracellular Aβ was performed as described previously (23). Briefly, the membrane fraction containing intracellular Aβ was delipidated with chloroform and methanol, and solubilized using 70% formic acid. The extracted protein samples were dissolved in sample buffer containing 8 M urea and subjected to Western blotting as above. Aβ40 and Aβ42 levels in samples were semiquantified by comparing band intensities with those of the synthetic Aβ1-40 and Aβ1-42 peptide standards, respectively, using an LAS-1000 image analyzer (Fuji Film Co., Tokyo, Japan).

Generation of Aβ5 end-specific antibody and immunohistochemistry

Rabbits were immunized with the peptide NH_2-RHDSGYEVC-COOH, which corresponds to the sequence of Aβ5-12 plus a C-terminal cysteine residue, used for coupling to keyhole limpet hemocyanin. Antisera from a single rabbit was purified as follows. The antiserum was passed through a column (Hitrap NHS-activated HP, Amersham Biosciences) coupled with Aβ1-28 to remove immunoglobulins that reacted with Aβ1-28, and the flow-through was then affinity-purified with a column containing the peptide used for immunization. Immunohistochemical staining of human brain sections was conducted according to previously described methods (24, 25). Briefly, sections were incubated with Aβ5 antibody or other Aβ antibodies for 72 h at 4°C. Synthetic Aβ1-40 was then added to the incubation buffer to completely eliminate possible cross-reactions between the Aβ5 antibody and Aβ1-40/42. Following treatment with the appropriate secondary antibody, labeling was detected using the avidin-biotinylated HRP complex (ABC) system (Vector Laboratories, Burlingame, CA) and the diaminobenzidine reaction.

RESULTS

Evidence for altered Aβ secretion from cells expressing APPΔC

We established SH-SY5Y cell lines stably expressing APP or APPΔC (designated SH-APP and SH-APPΔC cells, respectively), and selected two pairs of SH-APP and SH-APPΔC cells that expressed comparable levels of APP. Of these, SH-APP-2 and SH-APPΔC-2 expressed approximately twice as much APP as did SH-APP-1 and SH-APPΔC-1 (Fig. 1*A*).

We used two types of sandwich ELISA to measure levels of Aβ in the conditioned media. BNT77-based ELISA detected both N-terminal-intact and truncated Aβ (Aβ40total and Aβ42total), but not the p3 fragment (21), whereas BAN50-based ELISA detected only N-terminal-intact Aβ (mainly Aβ1-40 and Aβ1-42). BNT77-based ELISA revealed that the levels of Aβ40total and Aβ42total in SH-APP-1, SH-APPΔC-1, SH-APP-2, and SH-APPΔC-2 cells were ~250/~50, ~240/~40, ~500/~110, and ~450/~120 fmol/ml, respectively. Thus, both SH-APP and SH-APPΔC cells produced comparable amounts of secreted Aβ40total and Aβ42total (Fig. 1*B*). In contrast, BAN50-based ELISA showed that the levels of Aβ1-40 and Aβ1-42 in SH-APP-2 cells were ~300 and ~40 fmol/ml, which were ~60% of Aβ40total and ~40% of Aβ42total respectively. In contrast, Aβ1-40 and Aβ1-42 levels in SH-APPΔC-2 cells were ~90 and ~20 fmol/ml, which were only ~20% of Aβ40total and ~15% of Aβ42total, respectively. Therefore, the N-terminal-intact forms of Aβ40 and Aβ42 in SH-APPΔC-2 cells were decreased to ~30% of the levels found in SH-APP-2 cells (Fig. 1*C*). A similar decrease in N-terminal-intact Aβ40 and Aβ42 was observed in SH-APPΔC-1 cells, compared with SH-APP-1 cells. Our data show that the ratios of Aβ1-40 to Aβ40total and Aβ1-42 to Aβ42total were significantly reduced in cells that expressed APPΔC, suggesting that N-terminally truncated Aβ was increased relative to total Aβ in SH-APPΔC cells.

Analyses of APP C-terminal fragments and sAPP in cells expressing APPΔC

We next analyzed the C-terminal fragments of APP (CTF) by immunoprecipitation/Western blotting with 4G8 antibody. Two CTF bands (~10 kDa α-CTF and ~12 kDa β-CTF) and a faint band (β'-CTF, ~11 kDa) were detected in cell lysates of SH-APP. Similarly, two bands (~6 kDa α-CTFΔC and ~8 kDa β-CTFΔC) and a faint band (β'-CTFΔC, ~7 kDa) were observed in SH-APPΔC cell samples (Fig. 2*A*). Interestingly, the relative level of β'-CTFΔC was increased in SH-APPΔC cells. Steady-state levels of β-CTFΔC and α-CTFΔC in SH-APPΔC cells were lower than those of β-CTF and α-CTF in SH-APP cells. The data indicate that APPΔC is processed as APP695 to produce APP-CTFΔC. We suggest that the lower levels of the CTF bands (α- and β-CTFΔC) in SH-APPΔC cells are due to instability of APP-CTFΔC, in view of the finding that treatment of cells with a proteasome inhibitor, MG132, augmented band intensities (data not shown).

We additionally compared the generation of secreted APP (sAPP) in SH-APP and SH-APPΔC cells. Western blotting with 22C11 and BAN50 showed that the levels of both total sAPP and sAPP-α (sAPP derived from α-secretase cleavage) were approximately fourfold higher in SH-APPΔC cells, compared with SH-APP cells (Fig. 2*B*). This observation is consistent with

previous reports that sAPP release from APP lacking the internalization signal is enhanced (26, 27).

Immunoprecipitation/Western blot and mass spectrometric analyses of secreted Aβ

We then analyzed altered Aβ secretion in SH-APPΔC cells using immunoprecipitation/Western blotting. BAN50 antibody immunoprecipitated both Aβ1-40 and Aβ1-42 (band 1, comigrating with synthetic Aβ1-40/42). These immunoreactivities were decreased in media from SH-APPΔC cells, compared with SH-APP cell media. N-terminal-intact Aβ (Aβ1-40 and Aβ1-42) and the smaller fragment (band 3, most likely Aβ11-40 and Aβ11-42) were detected in media from SH-APP cells by BNT77 immunoprecipitation. In contrast, the intensities of bands 1 and 3 were reduced, and the intensity of band 2 (located between these bands) was increased in SH-APPΔC media (Fig. 3*A*). We did not observe any band comigrating with Aβ17-40 (p3 fragment) in the BNT77 immunoprecipitates.

To identify the secreted Aβ species, we analyzed BNT77 immunoprecipitates using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer. Two major peaks (Aβ1-40 and Aβ11-40) and a small peak (Aβ1-38) were detected in conditioned media from SH-APP cells. Conversely, in SH-APPΔC media, the relative peak intensity of Aβ5-40 was markedly increased, whereas the peak intensities of Aβ1-40 and Aβ11-40 were decreased. In addition, we observed an Aβ5-38 peak in SH-APPΔC media (Fig. 3*B*). These data show that N-terminally truncated Aβ (starting at Arg5) is markedly increased in media from SH-APPΔC cells. Aβ11-40/42 appears to be generated through processing of APP by BACE1 and γ-secretase, as BACE1 alternatively cleaves between Tyr10-Glu11 in the Aβ sequence (28, 29).

Sensitive Western blot analysis of intracellular Aβ

We further analyzed the levels and species of intracellular Aβ in SH-APP and SH-APPΔC cells by sensitive Western blotting. Aβ antibodies BA27 and BC05 detected bands comigrating with synthetic Aβ1-40/42 peptides in both cell lines. However, we did not detect N-terminally truncated forms of Aβ or any other smaller peptides. The levels of Aβ1-40 and Aβ1-42 in samples from SH-APPΔC cells were similar to those in samples from SH-APP cells (Fig. 4*A*, 4*B*). The intracellular Aβ1-42/Aβ1-40 ratio was ~0.4 in both cell lines. These results suggest that the generation of intracellular Aβ is unaffected by the C-terminal truncation of APP.

Aβ5-40/42 generation involves altered β-cleavage of APP

To evaluate the mechanism by which Aβ5-40/42 is produced from APPΔC, we treated SH-APPΔC and SH-APP cells with a specific inhibitor of BACE, OM99-2 (30), and analyzed the amount of secreted Aβ using immunoprecipitation/Western blotting and mass spectrometry (MS). In OM99-2-treated SH-APPΔC cells, the Aβ1-40 concentration was significantly decreased, but Aβ5-40 secretion was not altered, compared with untreated cells (Fig. 5*A*, 5*B*). Interestingly, SH-APP cells treated with the inhibitor secreted significantly reduced amounts of Aβ1-40 and increased amounts of Aβ5-40 (Fig. 5*A*, 5*B*). These data suggest that cleavage between Phe4 and Arg5 is not mediated by BACE1. Moreover, BACE1 inhibition promotes Aβ5-40 secretion. To

determine whether α-secretase-like proteases are responsible for Aβ5-40/42 generation, we incubated SH-APPΔC cells with a TACE (tumor necrosis factor α converting enzyme) inhibitor, TAPI-1, which blocks α-secretase (31). Incubation with 20 μM TAPI-1 resulted in increased secretion of Aβ1-40 and decreased secretion of Aβ5-40 (Fig. 5*A*, 5*B*), suggesting that α-secretase-like proteases are involved in Aβ5-40/42 generation.

The effects of caspase cleavage of APP on Aβ production were additionally investigated in HEK293 cells. We performed immunoprecipitation/mass spectrometric analysis of Aβ in conditioned media from HEK293 cells transiently transfected with wild-type APP or APPΔC. The data demonstrate that APPΔC-transfected cells secrete more Aβ5-40 and less Aβ1-40 and Aβ11-40 than wild-type APP-transfected cells (Fig. 5*C*), providing evidence of altered Aβ production from APPΔC in both neuronal and nonneuronal cells.

Immunohistochemical analysis of Aβ5-40/42 in AD brain

To determine whether Aβ5-40/42 is present in human brain tissues, we generated a specific antibody to the N-terminal end region of this Aβ species (designated the Aβ5 antibody). In Western blots, the Aβ5 antibody reacted with Aβ5-40, but not with Aβ1-40, whereas the BAN50 antibody recognized only Aβ1-40 (Fig. 6*A*). The Aβ5 antibody immunostained a number of vessels in the AD brain, indicating the deposition of Aβ5-40/42, particularly in vascular lesions with amyloid angiopathy (Fig. 6*B*). It was noted that in nearby sections, another Aβ antibody (6E10) labeled more vessels than did the Aβ5 antibody (Fig. 6*C*). Amyloid angiopathy in the smaller-sized vessels tended to be negative or weakly positive for Aβ5. In addition, the Aβ5 antibody stained numerous neurofibrillary tangles (NFT), suggesting that Aβ5-40/42 may be deposited in the NFT (Fig. 6*D*). Although a small number of senile plaques were positive for Aβ5 in some cases, the immunostaining was not so consistently seen as those of vessels and NFT.

Cleavage at the Aβ5 site occurs during apoptosis of wild-type APP-expressing cells

Finally, we investigated whether APP processing to generate Aβ5-40/42 occurs when wild-type APP-expressing cells undergo apoptosis. SH-APP cells were treated with 1 μM MG132 or MG132 plus 30 nM staurosporine, which induce apoptosis in various cell lines. Exposure to these agents induced caspase-mediated cleavage at the cytoplasmic region of APP, as revealed by Western blots using the AB5942 antibody specific for the caspase-generated neo epitope of APP (Fig. 7). We examined CTF generation from caspase-processed APP by immunoprecipitation/Western blot analysis with 4G8 and AB5942 antibodies. Cells treated with MG132 plus staurosporine contained significant amounts of β-CTFΔC, β'-CTFΔC, and α-CTFΔC, consistent with data obtained with SH-APPΔC cells, suggesting that cleavage at the Aβ5 site occurs during apoptosis in SH-APP cells (Fig. 7). However, Aβ5-40/42 was not detectable in conditioned media from these apoptotic cells, possibly due to extremely low concentrations.

DISCUSSION

A body of evidence suggests that apoptosis possibly plays a role in AD neurodegeneration (4–6). It is particularly significant that active forms of caspases (7–10) and the caspase-cleaved APP (11, 15, 16) have been detected in AD brain tissues. We conducted the present study to clarify whether

the caspase cleavage of APP affects Aβ generation. Using human neuronal and nonneuronal cell lines, we clearly demonstrate that this APP cleavage promotes the secretion of a distinct amino-truncated Aβ species (Aβ5-40/42). We further provide immunohistochemical evidence that this Aβ species is deposited in AD brain. Thus, our data indicate for the first time the connection between caspase activation and the formation of amino-truncated Aβ.

Our data are consistent with and expand upon previous studies showing that the levels of Aβ1-40/42 are reduced in conditioned media from N2a, CHO, and B103 cells expressing C-terminal deleted fragments of APP (32, 33). These studies were limited in that they used ELISA systems, which detect only N-terminally intact Aβ, and did not fully analyze amino-truncated Aβ. Immunoprecipitation-MS was highly efficient in Aβ5-40/42 detection. Another study reported that B103 cells expressing APPΔC secrete more total Aβ than cells expressing wild-type APP (11), but they might have detected both Aβ and p3 fragments, since the C-terminal deletion of APP increases the secretion of p3 fragments (33).

Here, we used inhibitors of BACE and α-secretase to investigate the mechanism of Aβ5-40/42 generation. Following treatment of SH-APP cells with a BACE inhibitor, OM99-2, Aβ1-40 levels were decreased, whereas Aβ5-40 levels were increased. Furthermore, treatment of SH-APPΔC cells with TAPI-1 induced a decrease in Aβ5-40 and increase in Aβ1-40 secretion. The data strongly suggest that cleavage at the Aβ5 site is not due to BACE1 activity, but is mediated by α-secretase-like proteases (e.g., ADAM family proteases, including TACE and ADAM10) (34). This result is consistent with the finding that secretion of p3 (Aβ17-40), a product derived from α-secretase cleavage, is significantly increased in cells expressing APPΔC. BACE2 functions as an alternative α-secretase (35, 36). However, BACE2 does not appear to be involved in the generation of Aβ5-40/42, since OM99-2 inhibits both BACE1 and BACE2 (30). Moreover, it is highly unlikely that Aβ5-40/42 is produced by the action of aminopeptidases following the formation of Aβ1-40/42. Thus, our data indicate that Aβ1-40/42 and Aβ5-40/42 are derived from two distinct pathways, possibly reflecting the difference of intracellular trafficking between APP and APPΔC (27).

Since wild-type APP undergoes caspase cleavage during apoptosis, it is reasonable to assume that subsequent cleavage at the Aβ5 site occurs in apoptotic cells. We show evidence that APPΔC is generated in SH-APP cells exposed to MG-132 and staurosporine, and that β'-CTFΔC, which corresponds to a Aβ5-40/42 precursor, is formed in these apoptotic cells. This finding supports our conclusion that Aβ5-40/42 generation occurs following caspase activation.

Immunohistochemical staining with the Aβ5 antibody revealed that Aβ5-40/42 species are deposited in some vessels with amyloid angiopathy in AD brain tissues. This finding possibly reflects the in vivo occurrence of caspase-cleavage of APP. The additional observation that Aβ5 antibody labels NFT is intriguing, considering that activation of caspases is suggested to occur in neurons bearing NFT (9, 10) and to be associated with the extent of NFT formation (37). Significantly, a caspase-truncated form of tau, the major constituent of NFT, is also detectable in NFT (38). Since Aβ induces hyperphosphorylation of of tau (39), intraneuronal Aβ5-40/42 may be relevant to NFT formation. Our data are consistent with previous reports that considerable N-terminal modifications of Aβ are seen in AD cortices and leptomeninges (4, 40). These

amino-truncated Aβ species may have an instrumental role in the amyloidosis process (4). The process by which Aβ5-40/42 is preferentially deposited in vessel walls currently remains unclear. In a recent study, transgenic mice with low neuronal expression of APP harboring Swedish and Dutch/Iowa mutations displayed robust microvascular accumulation of Aβ (41). The authors suggest that the Aβ mutant is retained at the cerebral microvessels due to deficient clearance at the cerebral vasculature. Aβ5-40/42 may be trapped and deposited in blood vessels by a similar mechanism.

Our sensitive Western blot analyses indicate that the majority of intracellular Aβ consists of Aβ1-40/42 in both wild-type APP- and APPΔC-expressing cells. Since two distinct pathways appear to exist for extracellular and intracellular pools of Aβ (42), amino-truncated Aβ peptides, including Aβ5-40/42, are likely to be produced mainly in the extracellular Aβ pathway. Our data also suggest that levels of intracellular Aβ do not necessarily correlate with those of secreted Aβ.

In conclusion, we suggest that caspase activation in the AD brain results in the formation of APPΔC, leading to the increased production and deposition of N-terminally truncated Aβ5-40/42. Further research on the in vivo generation of this Aβ species is required to clarify its pathological role in Aβ deposition and neuronal death in AD.

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Fig. 1

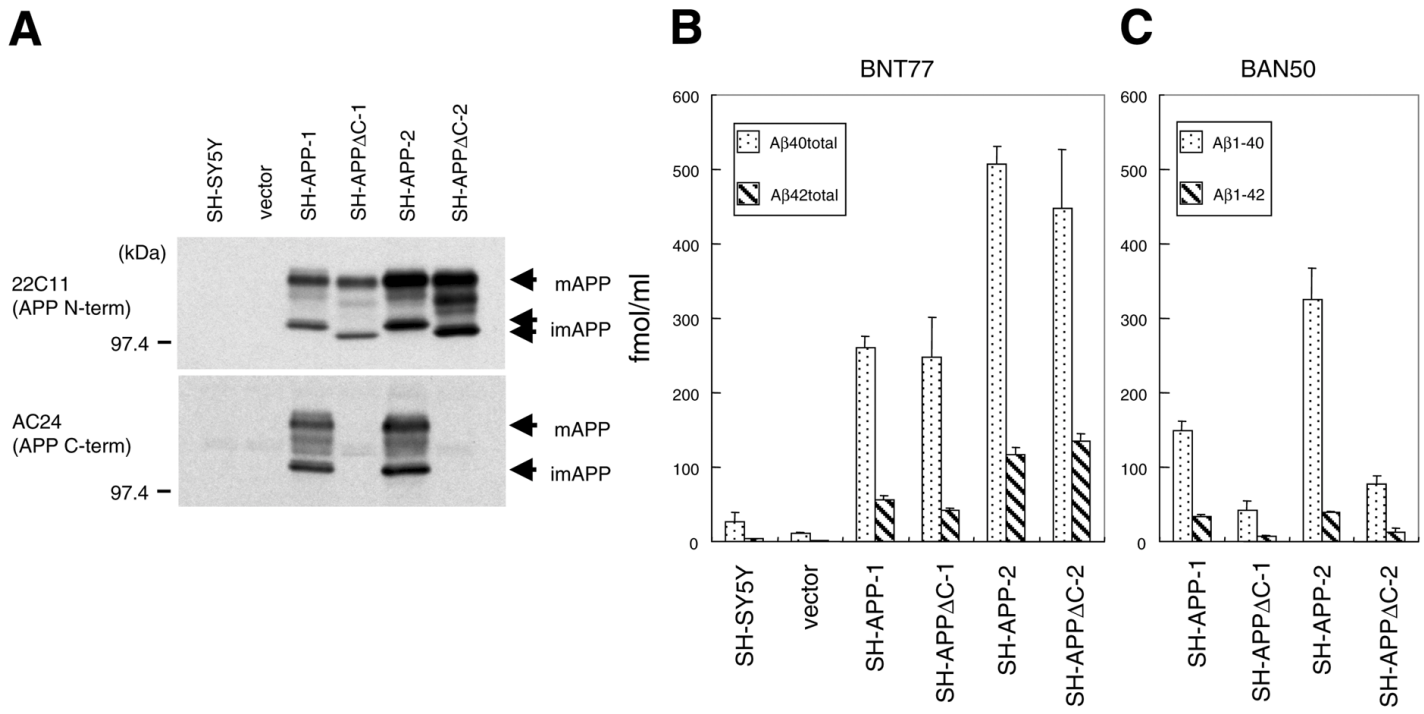

Figure 1. ELISA analysis of secreted Aβ. ***A)*** Western blot analysis of cellular APP. Lysates were prepared from SH-SY5Y cell lines stably transfected with APP (SH-APP-1, -2), APPΔC (SH-APPΔC-1, -2), or vector alone (vector) and were subjected to SDS-PAGE and Western blotting with APP antibodies 22C11 (upper) or AC24 (lower). Immature (im) and mature (m) forms of APP and APPΔC were detected with the 22C11 antibody, whereas neither form of APPΔC was detected with the AC24 antibody. Note that immature APPΔC is slightly smaller than APP. ***B, C)*** The indicated cell lines were cultured in a 12-well plate, and Aβ peptide levels in 24 h-conditioned media were quantified by BNT77-based (*B*) or BAN50-based (***C***) sandwich ELISA. Values represent means ± SD (n=3–4). The levels of Aβ40total and Aβ42total were increased in proportion to cellular APP expression. The Aβ40total and Aβ42total values were similar between SH-APP and SH-APPΔC. The levels of Aβ1-40 and Aβ1-42 were significantly decreased in SH-APPΔC, relative to SH-APP (P<0.05, t test), but the ratio of Aβ1-40 to Aβ1-42 was similar in both cell lines.

Fig. 2

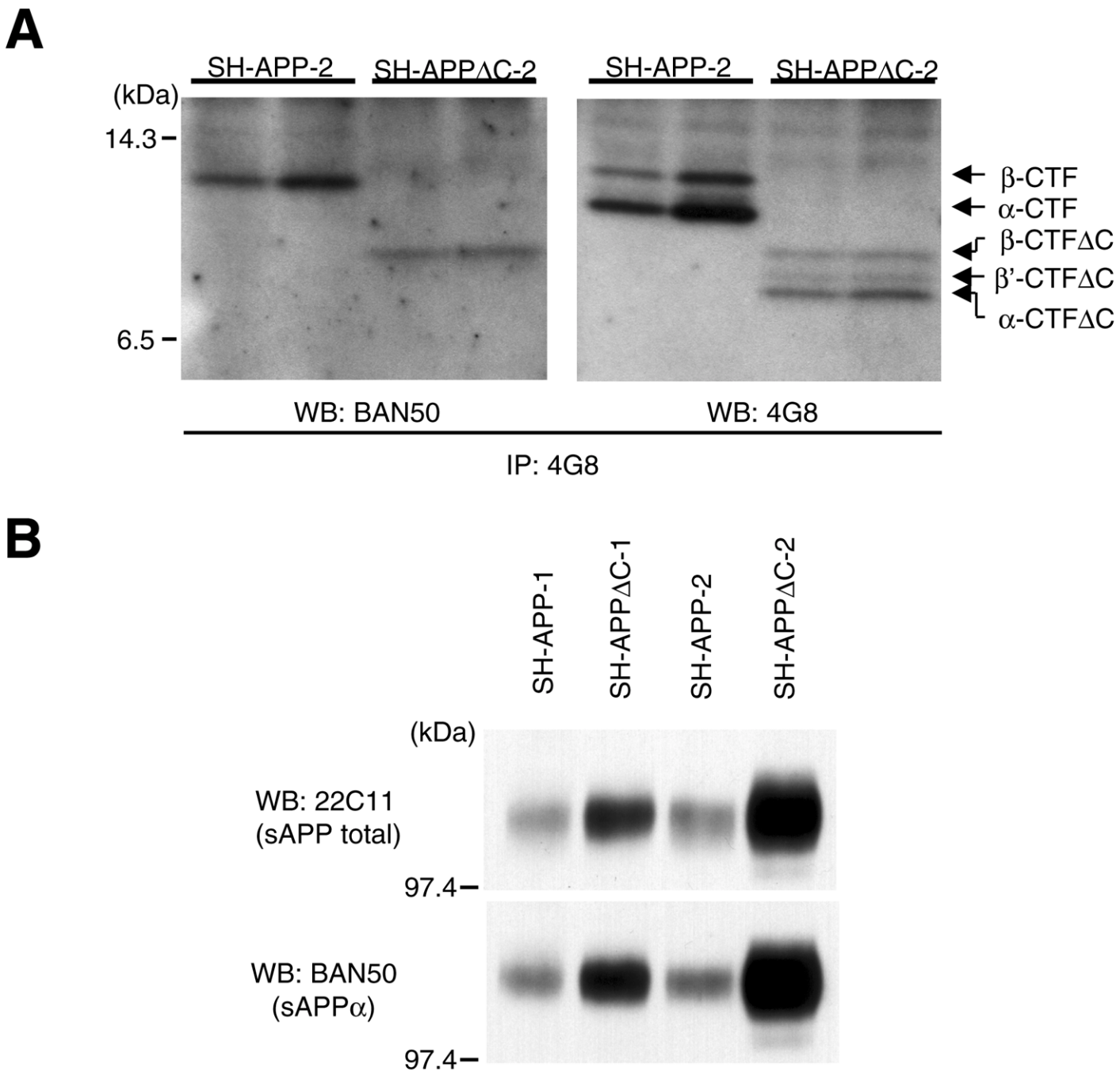

Figure 2. Western blot analysis of APP CTF and secreted APP. ***A)*** Cell lysates were immunoprecipitated with antibody 4G8 and subjected to Western blotting with 4G8 or BAN50. 4G8 detected α-CTF and β-CTF in SH-APP, and α-CTFΔC and β-CTFΔC in SH-APPΔC. In addition, a third CTF (β'-CTFΔC) was observed between the positions of α-CTFΔC and β-CTFΔC in SH-APPΔC. BAN50 recognized only β-CTF and β-CTFΔC. ***B)*** Secreted APP in the media was immunoprecipitated with antibody 207 and subjected to Western blot analysis with antibody 22C11 or BAN50. Antibody 22C11 recognizes all sAPP forms, whereas BAN50 specifically recognizes sAPP containing the N-terminal region of Aβ. The levels of sAPP were markedly increased in SH-APPΔC, compared with SH-APP cells.

Fig. 3

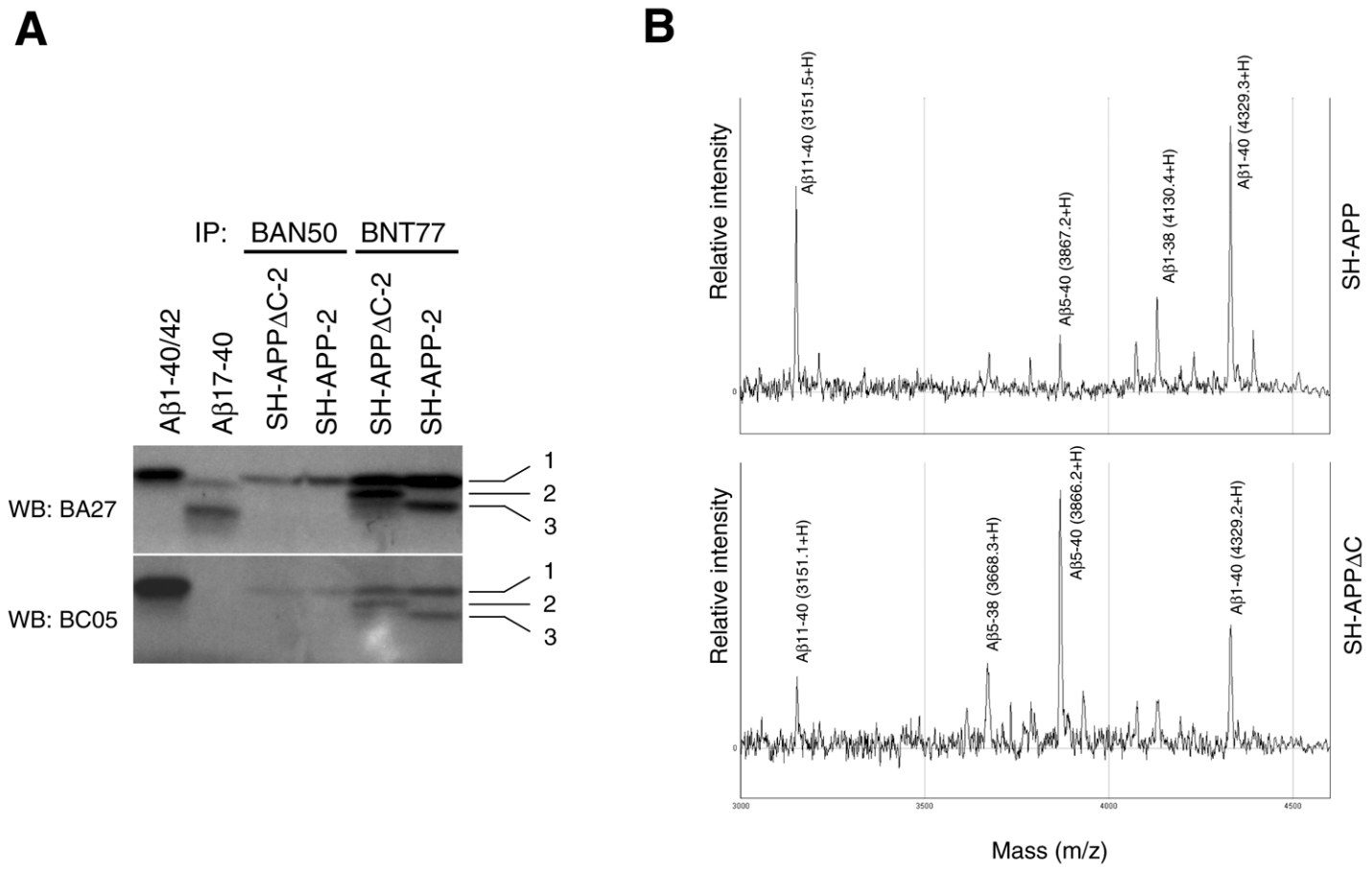

Figure 3. Immunoprecipitation-Western blot and mass spectrometric analyses of secreted Aβ. ***A)*** Secreted Aβ peptides were immunoprecipitated with BAN50 or BNT77 antibodies and subjected to Tris-Tricine SDS-PAGE and Western blot analyses with BA27 or BC05. Synthetic Aβ1-40/42 and Aβ17-40 were simultaneously electrophoresed as markers. In BNT77 immunoprecipitates, three bands were detected. Band 1 corresponded to N-terminally intact Aβ1-40/42, whereas band 3 possibly represented Aβ11-40/42. Bands 1 and 3 were the major Aβ species in SH-APP cells. Conversely, in SH-APPΔC cells, the intensity of band 2 was markedly increased, whereas that of bands 1 and 3 was reduced. In BAN50 immunoprecipitates, only band 1 was detected. ***B)*** Secreted Aβ was immunoprecipitated with BNT77 from conditioned media of SH-APP or SH-APPΔC cells and analyzed by MALDI-TOF-MS. Peaks were identified according to observed molecular and theoretical masses of Aβ and its variants. Aβ1-40, Aβ11-40, and some C-terminally truncated Aβ forms, such as Aβ1-38, were identified in the sample from SH-APP (upper). The peak intensities of Aβ1-40 and Aβ11-40 were reduced, whereas that of Aβ5-40 was markedly increased in the SH-APPΔC sample (lower). Aβ5-38 was also observed in this sample.

Fig. 4

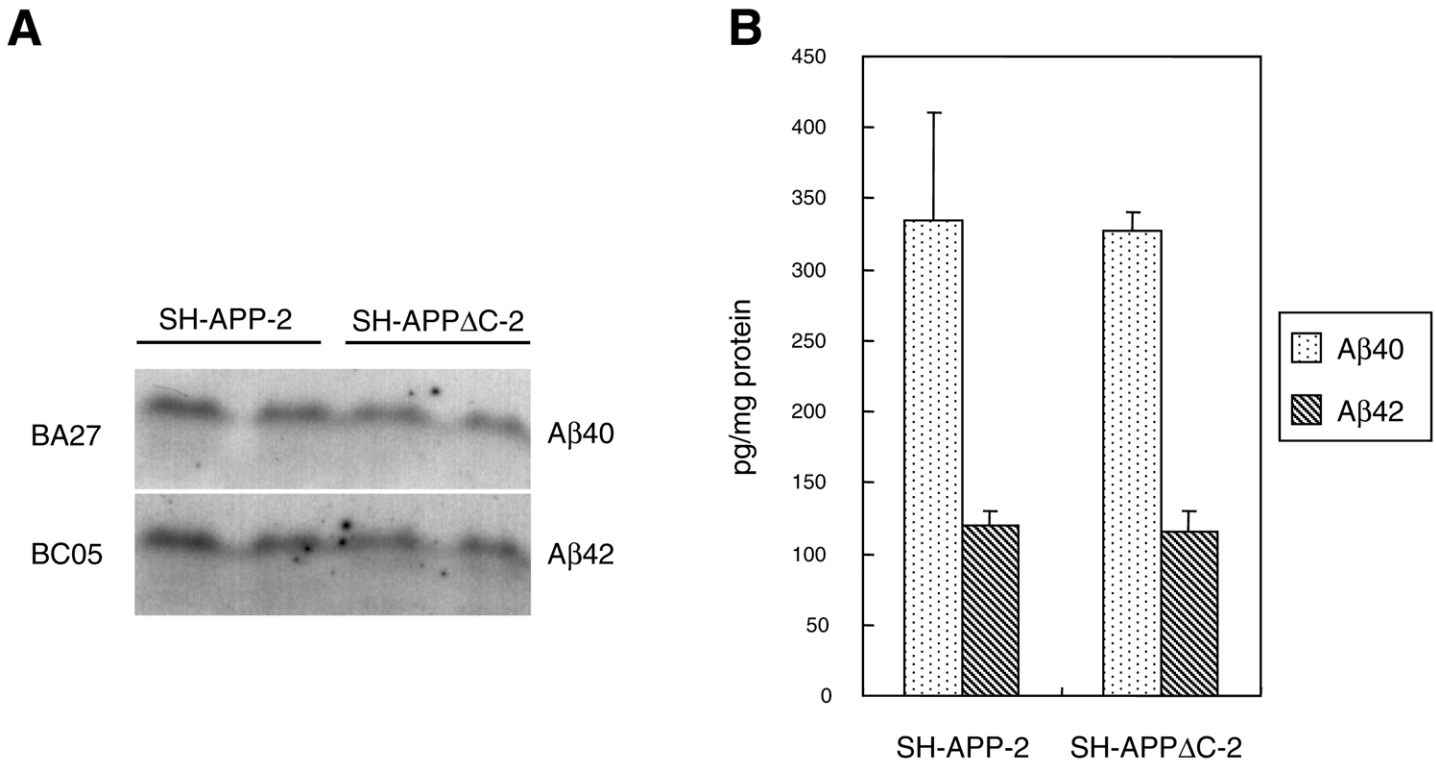

Figure 4. Western blot analysis of intracellular Aβ. ***A)*** Protein extracts were prepared from total membrane fractions with 70% formic acid and were subjected to Western blot analysis with BA27 or BC05 as described in Materials and Methods. Similar levels of intracellular Aβ40 and Aβ42 were detected in SH-APP and SH-APPΔC. These Aβ bands comigrated with synthetic Aβ1-40 and Aβ1-42. ***B)*** The levels of intracellular Aβ40 and Aβ42 were semiquantified as described in Materials and Methods. The graph shows mean values ± SE from three independent experiments.

Fig. 5

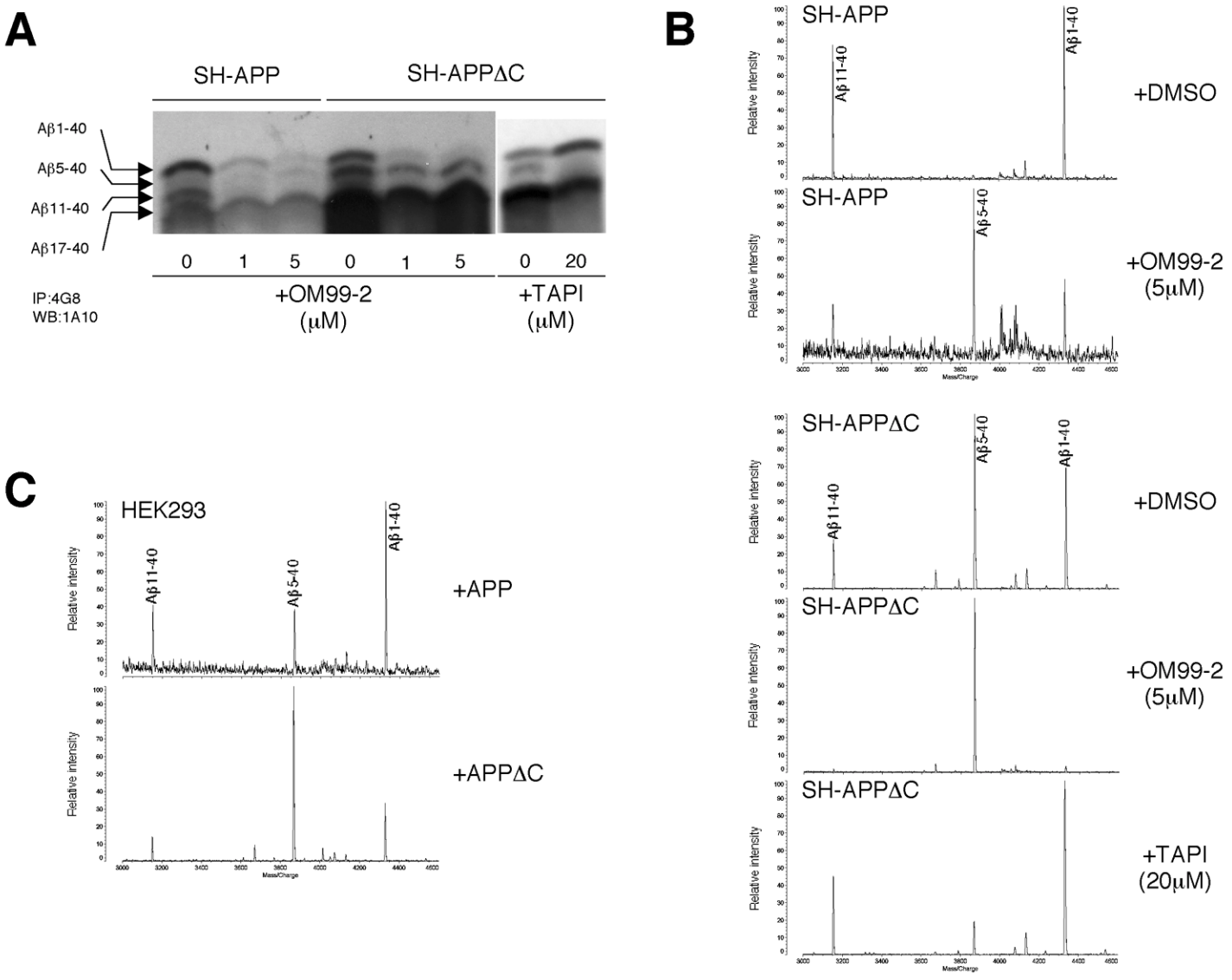

Figure 5. Analysis of secreted Aβ from OM99-2 and TAPI-1-treated cells and transiently transfected HEK293 cells. ***A, B)*** SH-APP and SH-APPΔC cells were treated with the indicated concentrations of OM99-2 for 16 h. Aβ in conditioned media was analyzed by immunoprecipitation-Western blots (***A***) or immunoprecipitation-mass spectrometry (***B***), similar to **Figure 3**, except that 4G8 was used in place of BNT77, and 1A10 was substituted for BA27. SH-APPΔC cells were additionally treated with 20 μM TAPI-1 for 16 h, and secreted Aβ was analyzed as described above. Treatment with OM99-2 significantly reduced Aβ1-40 and Aβ11-40 secretion in both SH-APP and SH-APPΔC cells but increased Aβ5-40 levels in SH-APP cells. Exposure to TAPI-1 decreased Aβ5-40 but increased Aβ1-40 and Aβ11-40 in SH-APPΔC cells. ***C)*** Aβ secreted from HEK293 cells transiently transfected with APP or APPΔC was analyzed by immunoprecipitation-mass spectrometry as in ***B***. Increased Aβ5-40, and reduced Aβ1-40 and Aβ11-40, levels were observed in APPΔC-transfected cells, compared with APP- transfected cells.

Fig. 6

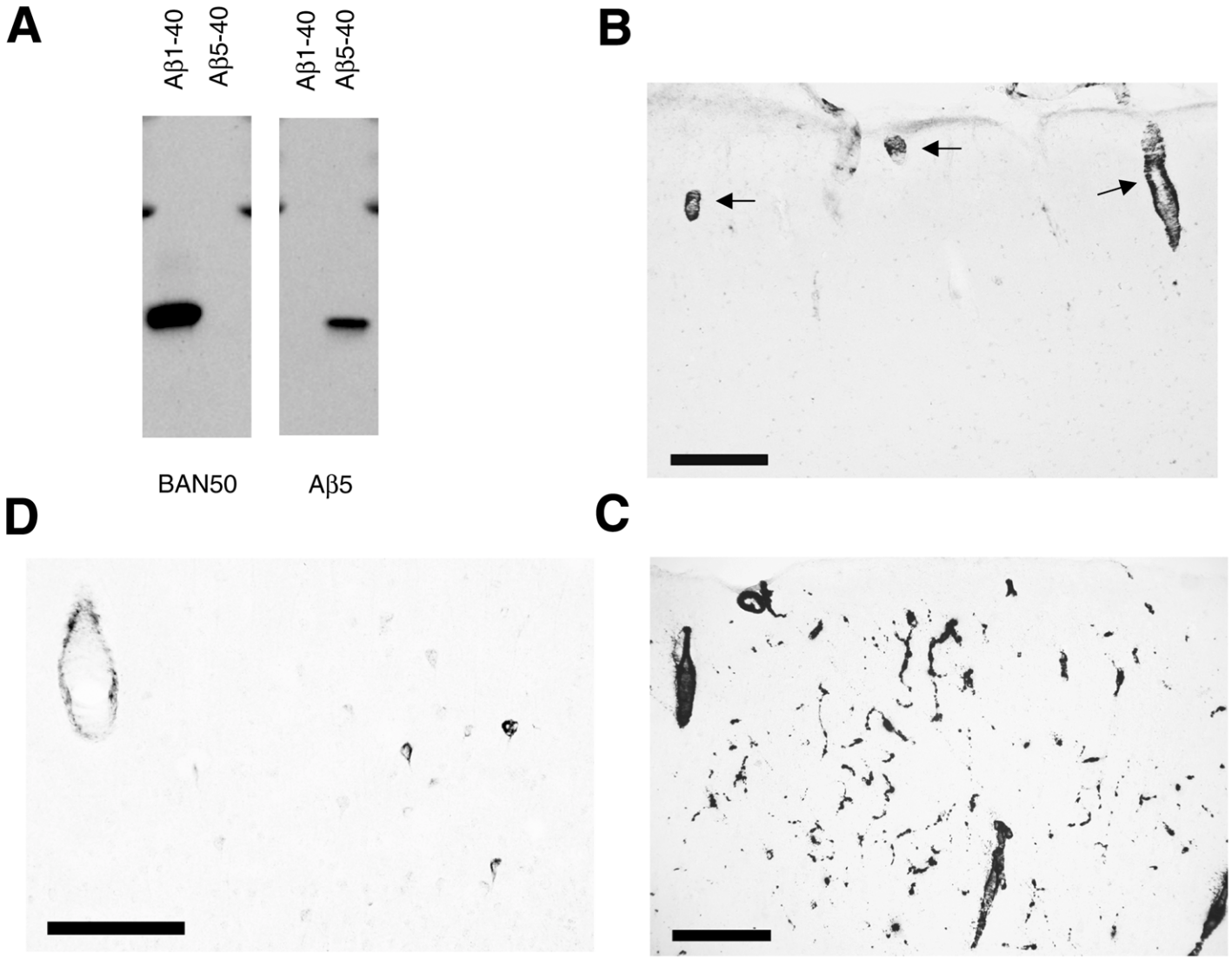

Figure 6. Immunohistochemical analysis of Aβ5-40/42. ***A)*** Specificity of Aβ5 antibody. Synthetic Aβ1-40 and Aβ5-40 (20 ng each) were immunoblotted and detected with BAN50 or Aβ5 antibody. Aβ5 antibody reacted with only Aβ5-40. (***B–D***) Immunostaining of the occipital cortex from an AD case. The sections were stained with Aβ5 antibody (***B***, ***D***) or 6E10 antibody (***C***). Intermediate size vessels were strongly positive for Aβ5 (arrows) (***B***), whereas both small and intermediate size vessels were positive for 6E10 (***C***). Bar, 200 μm. Several NFT were labeled with Aβ5 antibody (***D***). Bar, 100 μm.

Fig. 7

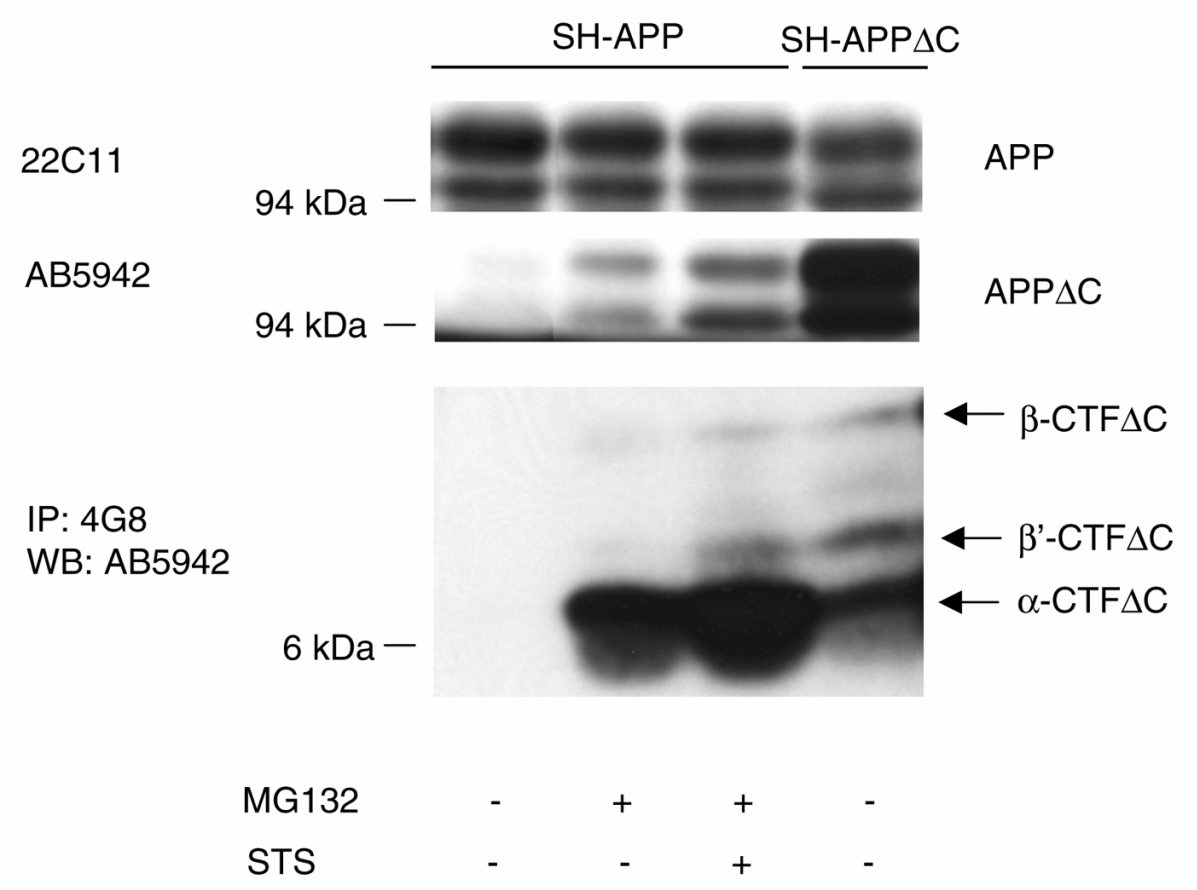

Figure 7. Immunoprecipitation-Western blot analysis of CTF derived from caspase-cleaved APP in wild-type APP-expressing cells after apoptosis induction. SH-APP cells were exposed to 1 μM MG132 or MG132 plus 30 nM staurosporine for 16 h. On Western blots with the BA5942 antibody, caspase-cleaved APP bands were observed in treated cells. In SH-APP cells exposed to MG132 and staurosporine, immunoprecipitation-Western blots with 4G8 and AB5942 disclosed bands of β-CTFΔC, β'-CTFΔC, and α-CTFΔC corresponding to those in SH-APPΔC cells.