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Thimet Oligopeptidase Cleaves the Full-Length Alzheimer Amyloid Precursor Protein at a β-Secretase Cleavage Site in COS Cells[1]

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We developed an assay method using a novel quenched fluorescent substrate (QFS) flanking the β-cleavage site of amyloid precursor protein (APP), and purified a candidate β-secretase from bovine brain. N-terminal amino acid analysis showed the candidate to be thimet oligopeptidase (TOP). The cDNA for human TOP was cloned from a human brain cDNA library and expressed in COS cells. The enzyme was further purified on a Ni^{2+}-agarose column. TOP cleaved the Swedish Alzheimer's substrate (SEVNLDAEFR) as well as the normal substrate (SEVKMDAEFR). We then coexpressed TOP with APP695 in COS cells, collected transfected cells and conditioned media, and analyzed them by immunoblotting. The antibody against the specific secreted APP cleaved by β-secretase (sAPPβ) detected the secretion of sAPPβ only from APP/hTOP-overexpressing cells, and not from cells overexpressing of antisense hTOP cDNA. Finally, we analyzed the immunolocalization of overexpressed hTOP in COS cells. Most hTOP was localized in the nuclei, but a small amount was localized in the Golgi or other organelles around the nuclei. These results suggest that TOP has a β-secretase-like activity responsible for the processing of APP.

Key words: Alzheimer's disease, β-secretase, thimet oligopeptidase, APP metabolism, metalloprotease.

Alzheimer's disease is characterized by the formation of amyloid plaques containing amyloid β-protein (Aβ) in brain regions important for intellectual function. The accumulation of Aβ brings about neuronal death and a progressive loss of cognitive function and memory. Aβ, a 39-43 amino acid peptide, is generated by the proteolytic processing of amyloid precursor protein (APP), which has three major alternative spliced isoforms, APP695, APP-751, and APP770 (*1-5*). In normal brain, APP is degraded within the Aβ domain (16 amino acid residues from the N-terminus of Aβ) by α-secretase, and a 90-100 kDa, soluble, non-amyloidogenic N-terminal fragment of APP (sAPPα) is secreted from the cells (*6-10*). In Alzheimer's brain, however, Aβ is produced by the abnormal processing of APP, *i.e.*, β- and γ-secretases specifically cleave the N- and C-termini of Aβ, respectively. Since the site of cleavage by γ-secretase is within the membrane-spanning domain of APP, β-secretase-catalyzed cleavage is a critical step in Aβ production.

Several candidates for the β-secretase have been reported based on *in vitro* experiments using substrates flanking the β-cleavage site, but no *in vivo* cleavage of APP has been confirmed up to now (*11, 12*). In this paper, we describe the purification of a β-secretase candidate from bovine brain, and show that it is thimet oligopeptidase (TOP, EC 3.4.24.15). In addition, we co-expressed human TOP and APP695 in COS cells and found that the amount of secreted sAPPβ is increased in conditioned media.

MATERIALS AND METHODS

Assay for β-Secretase—The QFS for β-secretase, normal KM-QFS [7-methoxycoumarin-4-acetyl-SEVKMDAEFR-K(RR-NH_2)-(2,4-dinitrophenyl)], and Alzheimer NL-QFS [7-methoxycoumarin-4-acetyl-SEVNLDAEFR-K(RR-NH_2)-(2,4-dinitrophenyl)] were synthesized by Peptide Institute (Minoh). A KRR sequence was added for easy solubilization. Recombinant hTOP (0.2 μg) was assayed in 200 μl of EDTA-buffer [25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.1 mM DTT with 10 μg/ml E-64, leupeptin, pepstatin, and 100 μM PMSF (phenylmethylsulfonyl fluoride)] or $MnCl_2$-buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MnCl_2$, and 0.1 mM DTT with 10 μg/ml

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; sAPP, secreted form of APP; Aβ, amyloid β protein; TOP, thimet oligopeptidase; QFS, quenched fluorescent substrates; DTT, dithiothreitol.

E-64, leupeptin, pepstatin, and 100 μM PMSF). Substrate (1 mM in DMSO) was added to a final concentration of 5 μM, and the assay mixtures were incubated at 37°C for 30 min. The addition of 100 μl of 10% SDS and 700 μl of 100 mM sodium acetate (pH 4.0) stopped the reactions. Fluorescence was measured on a JASCO FP-777 fluorescence spectrometer (excitation, 328 nm; emission, 393 nm). Various peptide-MCA substrates (Peptide Institute) were used to determine substrate specificity.

Production of hTOP Antibodies—A truncated human TOP (amino acids Met^{1}-Ile^{372}) was produced in *Escherichia coli* with the prokaryotic expression vector pET16b (Novagen, Madison, WI, USA). The expressed hTOP was separated in 10% polyacrylamide gels by the method of Laemmli (*13*), stained with KCl, cut out, crushed, and homogenized in Milli Q. One-third of the homogenate was mixed with an equal volume of Freund's complete adjuvant (Difco Lab., Detroit, MI, USA) and injected into an adult male New Zealand White rabbit. Two booster injections were administered at 10-day intervals. Blood was drawn 10 days after the final booster injection. The antibody obtained was designated the anti-hTOP antibody.

Distribution of β-Secretase Activity in Various Rabbit Tissues, and the Subcellular Fractionation of Bovine Brain—All subsequent steps were carried out at 0-4°C. Various rabbit tissues were homogenized in a Teflon homogenizer in buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.1 mM DTT) and centrifuged at 1,000×g for 10 min. The protein concentrations of all fractions were determined with a DC protein assay kit (Bio-Rad, Richmond, CA, USA) using bovine serum albumin as the standard. The β-secretase activity was determined (add 20 μl fraction) as described above.

Bovine brain was homogenized in a Teflon homogenizer in buffer A (10 volumes) and centrifuged at 200×g for 5 min (precipitate=debris). The supernatant was centrifuged, first at 600×g for 10 min (precipitate=nuclei), then at 8,000×g for 10 min (precipitate=mitochondria), and finally at 70,000×g for 30 min (precipitate=microsomes). The resultant supernatant was designated as cytosol. The protein concentrations of all supernatant and precipitate fractions were determined with the DC protein assay kit.

Purification of β-Secretase from Bovine Brain—Bovine brain was purchased from the Shibaura abattoir sanitation inspection station (Tokyo). All subsequent steps were carried out at 0-4°C. The bovine brain (360 g) was homogenized in 2 liters of buffer A with the mixer set at maximum speed for one min and centrifuged (8,000×g, 30 min). The supernatant, 1.2 liters, was filtered through four layers of gauze, and adsorbed onto a DEAE-cellulose (DE52; Whattman, Maidstone, UK) column (3.2×25 cm) previously equilibrated with buffer D (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 5 mM β-mercaptoethanol). The column was washed with 600 ml of buffer D and then eluted with a linear 0-0.6 M NaCl gradient (total 1,000 ml) in buffer D (flow rate 1 ml/min); 16 ml fractions were collected. Active fractions as detected by β-secretase assay (fraction Nos. 15-26) were adsorbed onto a hydroxyapatite (Bio-Gel HTP; Bio-Rad) column (3.2×15 cm) previously equilibrated with buffer H (10 mM potassium phosphate, pH 7.5, and 5 mM β-mercaptoethanol). The column was washed with 240 ml of buffer H and then eluted with a linear 10-300 mM potassium phosphate gradient (total 360 ml) in buffer H (flow rate 1 ml/min); 4 ml fractions were collected. Solid ammonium sulfate was added to the β-secretase-active fractions (fraction Nos. 32-51) to 40% saturation and the mixture was gently stirred for 30 min. The precipitate was removed by centrifugation at 10,000×g for 10 min, dissolved in 1 ml of buffer D, and dialyzed overnight against buffer A. The dialyzed sample was applied to a Superdex 200 HiLoad 16/60 FPLC column (Pharmacia, Uppsala, Sweden) previously equilibrated with buffer A. Proteins were eluted with buffer A (flow rate 1.25 ml/min); 1.25 ml fractions were collected. The active fractions as detected by β-secretase assay (fraction Nos. 19-26) were pooled and concentrated to 2.4 ml by ultrafiltration through a YM-10 membrane (Amicon, Lexington, MA, USA). The concentrated sample was applied to a Mono Q HR 10/10 FPLC column (Pharmacia) equilibrated with buffer D. Proteins were eluted with 87.5 ml of a linear gradient of 0-0.5 M NaCl in buffer A (flow rate at 1.25 ml/min); 2.5 ml fractions were collected. β-secretase activity was detected as a single peak at about 0.2 M NaCl. Fractions containing activity were pooled, dialyzed against buffer A, and stored at 4°C.

Amino Acid Sequence Determination—The N-terminal amino acid sequence of the β-secretase-like protein was determined by precipitating the purified sample (42 μg of protein) with 10% trichloroacetic acid (TCA). The precipitate was washed with ethanol-ether (1:1), dried, and solubilized in SDS-buffer [62.5 mM Tris-HCl, pH 6.8, 15% glycerol, 1% sodium dodecyl sulfate (SDS), 2.5% β-mercaptoethanol, and 0.1% bromophenol blue]. The protein was subjected to electrophoresis in 10% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane (ProBlott™; Perkin Elmer). The membrane was stained with 0.1% amide black in 50% methanol-10% acetic acid. The protein band to be examined was cut out and washed with Milli Q. Amino acid sequences were determined with an Applied Biosystems model 477A-120A amino acid sequencer (Foster City, CA, USA).

Cloning of the Sense and Antisense cDNA for Human Thimetoligopeptidase (hTOP)—A full-length human TOP cDNA was isolated from a human brain cDNA library. Two oligonucleotide primers from human TOP were synthesized on a Beckman Oligo 1000 DNA synthesizer with restriction enzyme sites added (underlined in the following sequences). The 5′-primer sequence was 5′-CGGAATTCAG-ACCACCCGCCATGAAG-3′ and the 3′-primer sequence was 5′-TGCCAGCTCGAGGCAGACCTGCGG-3′. The amplified DNA of 2.1 kb was cloned into pUC119 and then pUC119/hTOP DNA sequences were determined with an LI-COR® DNA sequencer model 4000L (Aloka). In addition, a full-length antisense cDNA for hTOP was constructed.

Expression of hTOP in COS Cells and Purification of Recombinant Human TOP—The pUC119/hTOP was digested with *Eco*RI and *Xho*I, separated by agarose gel electrophoresis, and purified on a GeneElute™ agarose spin column (Sigma Chemical, St. Louis, MO, USA). The 2.1 kb DNA fragment was cloned into pSecTag (Invitrogen, San Diego, CA, USA). The pSecTag/hTOP was transfected into COS-7 cells by the electroporation method (*14*). After incubation for 72 h, transfected cells were harvested and sonicated in buffer A. The homogenate was subjected to

continuous centrifugation, first at 8,000×*g* for 10 min at 4°C, then at 100,000×*g* for 30 min at 4°C. The supernatant was applied to a Ni-NTA column (Qiagen GmbH, Germany) equilibrated with buffer N (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 10 mM imidazole) and washed with 20 ml of 20 mM imidazole in buffer N. Proteins were eluted with 20 mM to 120 mM imidazole in buffer N (flow rate, 1 ml/min); 2 ml fractions were collected. The fractions were dialyzed overnight against buffer A and the protein concentrations of all fractions were determined with the DC protein assay kit. The fractions were diluted in 2×SDS-buffer and samples were subjected to electrophoresis in 10% polyacrylamide gels. Proteins were transferred onto a PVDF membrane (Finetrap NT-32; Nihon Eido, Tokyo) and incubated with anti-*myc* (Invitrogen) and anti-hTOP antibodies (1:1,500 dilution) for 1 h at room temperature. The results were visualized with a VECTASTAIN *Elite* ABC kit (Vector Lab., Burlingame, CA, USA) and POD immunostain set (Wako, Osaka).

Co-Expression of Human TOP and APP695s in COS Cells and Immunoblot Analysis—Various human APP695 cDNA fragments (APP-WT, wild type APP; APP-NL, Swedish APP mutant) were constructed with the p91023 vector. APP-NL contained Asn^{595} and Leu^{596} instead of the Lys^{595} and Met^{596} in APP-WT. An APP plasmid and hTOP plasmid were used to transfect COS-7 cells by the electroporation method (*14*). After 72 h incubation, transfected cells were harvested and sonicated in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100. After centrifugation at 8,000×*g* for 5 min, the protein concentration of the supernatant was determined, and the supernatant was diluted in 2×SDS-buffer. The conditioned medium (8 ml) was concentrated by the addition of TCA (final concentration 10%) and centrifuged at 10,000×*g* for 15 min. The precipitate was washed with ether-ethanol (1:1) and then dissolved in 300 μl of 2×SDS-buffer. The samples were subjected to electrophoresis in 10% polyacrylamide gels and then transferred onto a PVDF membrane (Finetrap NT32). The membrane was incubated with anti-APP antibodies overnight at 4°C, and visualized first with a VECTASTAIN *Elite* ABC kit (Vector Lab.) and finally with a POD immunostain set (Wako). The anti-APP antibodies were the anti-22C11 antibody (Boehringert Mannheim, Germany), anti-Aβ1-16 antibody (*15*), and anti-sAPPβ antibody (T.C. Saido, unpublished data). The detected bands were quantified with an Imagemaster Ver. 2.0 (Pharmacia).

Immunofluorescence Microscopy—For indirect immunofluorescence microscopy, COS cells transfected with hTOP or APP695-WT were grown under the conditions described above and fixed in 5% formaldehyde for 20 min at 37°C. The fixed cells were washed with PBS and then permeabilized with 0.2% Triton X-100, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ in PBS for 5 min at room temperature. The preparations were treated with blocking solution (1% normal donkey serum in PBS) for 30 min, and soaked in the first antibody solution (anti-22C11 or anti-*myc* antibody) for 1 h at 37°C. The cells were then washed three times with PBS and incubated with Oregon Green™-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) for 1 h at 37°C. The cells were viewed and examined with a confocal scanner (Leica).

RESULTS

Distribution of β-Secretase Activity in Rabbit Tissues—First, we determined the tissue distribution of the candidate β-secretase in rabbit tissues, employing a normal β-secretase substrate flanking β-cleavage site. According to previous reports (*11, 12*), the APP-WT substrate flanking β-cleavage site is cleaved between Met and Asp by thimet oligopeptidase. Figure 1 shows that the β-secretase activities for KM-QFS are highest in brain (four times higher than in other tissues). Since Aβ production is observed exclusively in the brain, this result indicates that the β-secretase activity can be measured using synthetic peptide substrates. We also employed a Swedish Alzheimer substrate (NL-QFS) with KM replaced by NL, and found that brain extracts cleaved NL-QFS as well as KM-QFS (data not shown). This result corresponds with that of a rabbit brain extract in a previous report (*11*).

Purification of β-Secretase from Bovine Brain—Next, we determined the subcellular localization of the candidate β-secretase in bovine brain. Figure 2 shows that high β-secretase activities for both KM-QFS and NL-QFS are detected in the cytosol fraction (fluorescence intensity is the mean value of three independent experiments). Next, we tried to purify this activity from the cytosolic fraction of bovine brain, because larger amounts of material can be obtained than from rabbit. After several purification steps, we purified the candidate β-secretase.

Figure 3 shows the chromatographic pattern of proteins eluted from the final Mono Q chromatography column with the candidate β-secretase eluting at fraction 14 (0.2 M NaCl). The overall purification results are summarized in Table I. The N-terminal amino acid sequence of the β-secretase candidate was determined to be TGDALDVVAP. This is almost identical to the predicated amino acid sequence of residues 8-17 of porcine thimet oligopeptidase (AGDALDVAAP). Therefore, we conclude that the candi-

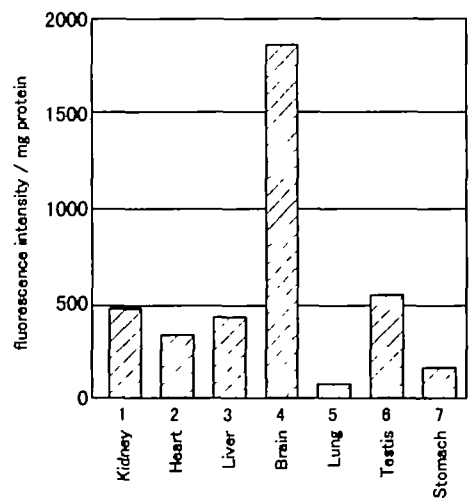

Fig. 1. The candidate β-secretase activity in rabbit tissues. The β-secretase activities for KM-QFS were determined as described in "MATERIALS AND METHODS." Columns 1-7 are kidney, heart, liver, brain, lung, testis, and stomach, respectively. The hatched columns are the activities for KM-QFS. The β-secretase activity is shown as fluorescence intensity per mg protein.

date β-secretase purified from bovine brain is bovine thimet oligopeptidase. Slight contamination was observed in the SDS gel with amino acid sequencing showing the impurity to be a lactate dehydrogenase (data not shown).

Recombinant Human TOP Activity—Figure 4 shows the SDS gel electrophoretic patterns of proteins eluted from the Ni-NTA column. hTOP was eluted in fractions 8 and 9 with 70 mM imidazole. hTOP was identified by immunoblot analysis using anti-*myc* and anti-TOP antibodies.

To determine the substrate specificity of the recombinant hTOP, we used KM-QFS and NL-QFS as well as 6 peptide substrates with different specificities towards various proteases (Fig. 5). hTOP cleaved none of these 6 peptide substrates, including Suc-LM-MCA (substrate for Met-endopeptidase), Boc-QAR-MCA [substrate for trypsin (*16*)], Boc-QGR-MCA [substrate for factor XIIa (*16*)], Suc-GPLGP-MCA [substrate for prolyl oligopeptidase (*17*)], Z-VKM-MCA [substrate for proteasome (*18*)], and Suc-HQK-MCA (substrate for putative α-secretase). hTOP specifically cleaved KM-QFS and NL-QFS from the β-secretase site in APP. The Alzheimer substrate (NL-QFS) should be quickly cleaved by the β-secretase, because Aβ production is enhanced in Alzheimer's brain. To determine whether hTOP is a β-secretase or not, we coexpressed hTOP and APP in COS cells.

Effect of Human TOP Overexpression on APP695 in COS Cells—To investigate hTOP as a potential β-secretase, hTOP and human APP695-WT or APP695-NL were coexpressed in COS cells. hTOP expression was evaluated by immunoblot analysis using anti-*myc* antibody (Fig. 6A, upper). Anti-sAPPβ recognizes the C-terminus of secreted

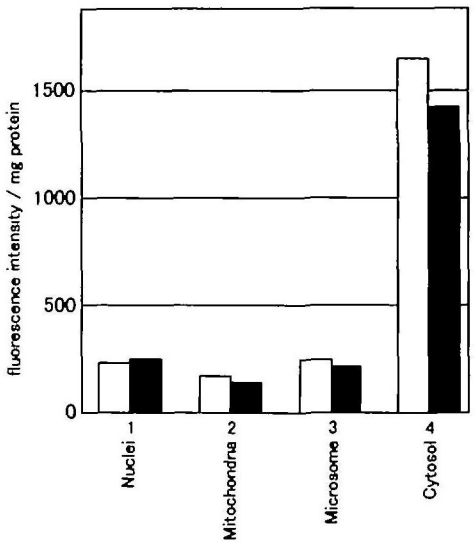

Fig. 2. **Subcellular fractionation of the candidate β-secretase in bovine brain.** The fractionation of a bovine brain extract is described in "MATERIALS AND METHODS." Columns 1-4 are nuclei (600×g precipitate), mitochondria (8,000×g precipitate), microsomes (70,000×g precipitate), and cytosol (70,000×g supernatant), respectively. The white and black columns are KM-QFS and NL-QFS, respectively. The β-secretase activity was analyzed as shown in Fig. 1.

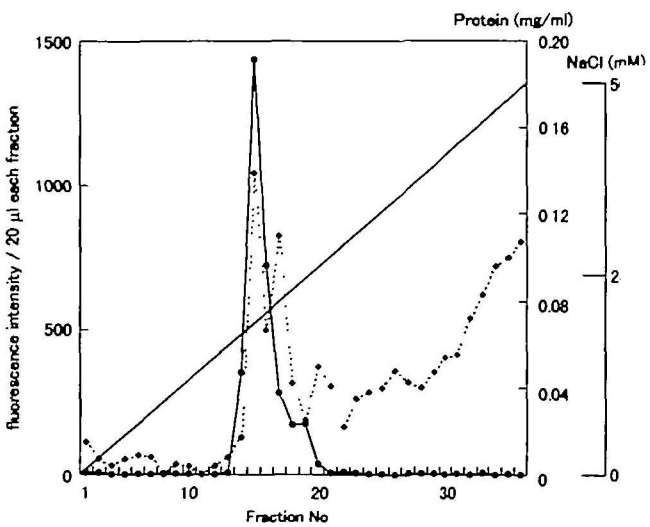

Fig. 3. **Final Mono Q chromatography of bovine β-secret** The cytosolic fraction of bovine brain was fractionated as describe... ... "MATERIALS AND METHODS" and the active gel filtration chromatographic fractions were applied to a Mono Q column (1.0 cm×10 cm). Protein concentration was monitored as absorbance at 280 nm (dotted line and diamonds). Elution was accomplished using a 0-0.5 M NaCl gradient (solid line). The β-secretase activity for KM-QFS was determined (solid line and circles).

TABLE I. **The purification of TOP from bovine brain.** The results are those for typical preparations from 2 liters of bovine brian extract. Activities were determined as described in "MATERIALS AND METHODS," protein was determined using the DC protein assay kit (Bio-Rad). F.I. = fluorescence intensity.

Purification step	Total protein (mg)	Total activity (F.I.)	Specific activity (F.I./mg)	Fold increase	Yield (%)
Crude extract	48,000	7.5×10^7	1.6×10^3	1.0	100
DE52	1,000	1.5×10^7	1.5×10^4	9.6	20
Hydroxylapatite	84	1.5×10^6	1.8×10^4	11	2
Superdex 200	17	6.8×10^5	4.0×10^4	25	0.9
Mono Q	0.35	3.6×10^5	1.0×10^6	660	0.5

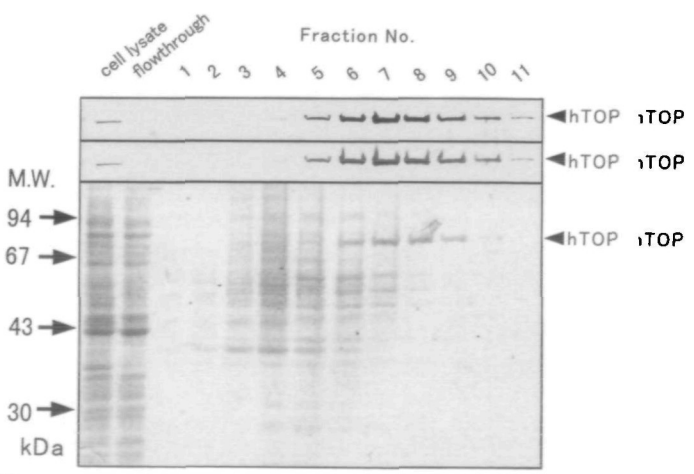

Fig. 4. **Purification of recombinant hTOP from COS cells.** Expressed hTOP was purified by Ni-NTA column chromatography, electrophoresed (10 μl of each fraction), and stained with Coomassie Brilliant Blue (Lower). hTOP was immunostained with anti-hTOP (Upper) or anti-*myc* (Middle) antibodies after transfer to PVDF. The arrowheads indicate hTOP.

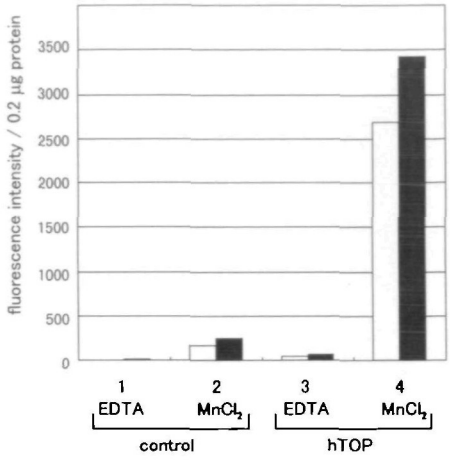

Fig. 5. **The purified hTOP specifically cleaves KM-QFS and NL-QFS.** Columns 1-4 are without hTOP in EDTA-buffer (flow-through), without hTOP in $MnCl_2$-buffer (flowthrough), hTOP in EDTA-buffer (fraction 8 in Fig. 4), and hTOP (fraction 8 in Fig. 4), respectively. The white and black columns are the activities for KM-QFS and NL-QFS, respectively.

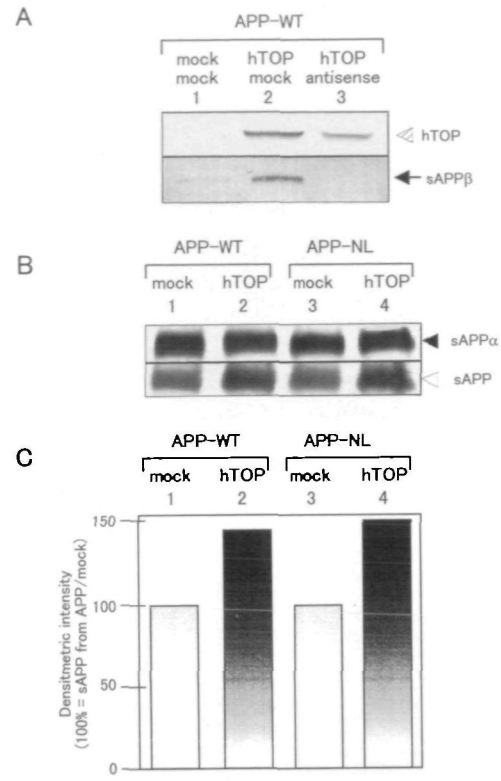

Fig. 6. **hTOP increases the secretion of non-sAPPα from COS cells.** (A) Cell lysates and conditioned media from APP695-WT and/or hTOP sense or antisense DNA transfected cells, were electrophoresed (15 μg protein of each fraction) and immunostained with anti-*myc* (upper; cell lysates) or anti-sAPPβ (lower; conditioned media) after transfer to PVDF. The hatched triangle and arrowhead indicate hTOP and sAPPβ, respectively. Lanes 1-3 are APP695-WT, APP695-WT/hTOP without hTOP antisense DNA, and APP695-WT/hTOP with antisense DNA, respectively. (B) Conditioned media from transfected APPs and/or hTOP cells were electrophoresed (15 μg protein of each fraction) and immunostained with anti-Aβ1-16 (upper) or anti-22C11 (lower) after transfer to PVDF. The filled triangle and open triangle indicate sAPPα and total sAPP, respectively. (C) Quantitation of sAPP: values are the ratios of the densitometric scores for total sAPP and sAPPα. Lanes and Columns 1-4 (B and C) are for expressed APP695-WT, coexpressed APP695-WT/hTOP, expressed APP695-NL, and coexpressed APP695-NL/hTOP, respectively.

APP695-WT, but not that of APP695-NL. Therefore, anti-sAPPβ detects only sAPPβ-WT, and not sAPPβ-NL, in the medium. Anti-sAPPβ clearly detected sAPPβ in APP/hTOP overexpressing cells but only slightly in APP/mock cells (Fig. 6A, lanes 1 and 2, lower). A full-length antisense cDNA for hTOP cotransfected with hTOP cDNA suppressed hTOP expression and sAPPβ secretion (Fig. 6A, lane 3, lower). These results indicate that hTOP is involved in the production of sAPPβ.

Secreted APP695s were detected with anti-22C11 and anti-Aβ1-16 antibodies (Fig. 6B). The anti-22C11, anti-Aβ1-16, and anti-sAPPβ antibodies detected amino acids 66-81, 597-612, and 592-596 of APP695, respectively. Since the anti-22C11 epitope is the N-terminal portion of the APP molecule, immunoblotting the secreted APP with anti-22C11 detects both sAPPα and sAPPβ. However, Aβ1-16 is involved only in sAPPα, not in sAPPβ. Therefore, anti-Aβ1-16 detects only sAPPα in the medium. Densitometric quantification (Fig. 6C) showed an increase in the secretion of anti-22C11 immunoreactive APP-processing products (sAPPα + sAPPβ, Fig. 6B, lower), but no change in the secretion of protein products immunoreactive with the anti-Aβ1-16 antibody (sAPPα, Fig. 6B, upper). These results suggest that sAPPβ (the product of β-secretase) increases in the presence of hTOP, while sAPPα (the product of α-secretase) does not.

Immunofluorescence Microscopy—Finally, we immunostained hTOP-transfected COS cells with anti-*myc* antibody. Figure 7A and B show no fluorescence in mock transfected cells. On the contrary, an hTOP-transfected cell is stained mainly at the nucleus and slightly at or around the Golgi apparatus (Fig. 7D). This result suggests that expressed TOP is mainly localized at nuclei, but a small amount of TOP is attached to the Golgi apparatus, like APP.

DISCUSSION

We purified a candidate β-secretase and assayed it using quenched fluorescent peptide substrates. Several reports have been published concerning β-secretases, however, these proteases, such as cathepsins (*11, 19-21*), calpain, proteasome (*20*), *etc.*, were all identified using peptidyl-MCA substrates. Our preliminary results suggest that different proteases are identified as β-secretases when Z-VKM-MCA or VKMDAEF are used as substrates (*19, 22*). Peptidyl-MCAs flanking the β-site may not be good substrates for β-secretase. Therefore, we synthesized new quenched peptides containing β-secretase-cleaving sites in addition to fluorescent groups on both sides of the peptide. We also synthesized an Alzheimer substrate that contains the Swedish Alzheimer mutation (KM to NL). Since Alzheimer's disease mutations enhance β-cleavage, it is easy to detect β-secretase activity by the more rapid cleavage of the NL than the KM substrate. Our results and those of Brown *et al.* (*12*) suggest that brain TOPs have higher activity toward the Swedish APP mutant than wild

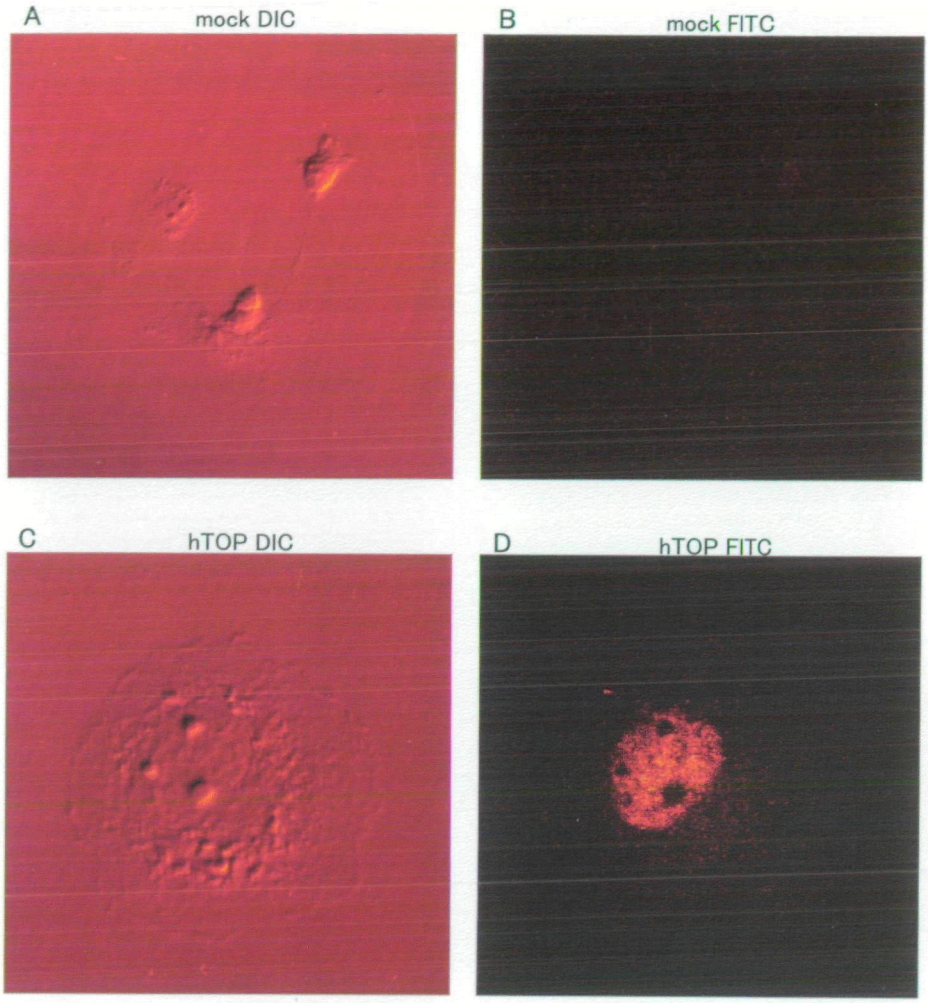

Fig. 7. **Immunofluorescence microscopy images of hTOP-transfected COS cells.** hTOP was transfected and visualized as described in "MATERIALS AND METHODS." Both A and B images are mock transfected cells; C and D are hTOP-transfected cells. A and C are differential interference images; B and D are immunofluorescence images using anti-*myc* antibody.

type APP. However, in mammalian brain extracts, β-secretase has nearly equal activities toward KM-QFS and NL-QFS, while recombinant human TOP cleaves NL-QFS faster than KM-QFS. We measured β-secretase activities under neutral conditions without Ca^{2+}.

After several purification steps, we succeeded in purifying the enzyme from bovine brain. Sequencing the protease from the N-terminus revealed it to be thimet oligopeptidase. Thimet oligopeptidase has been cloned from various mammalian sources (*23-25*) and the human TOP gene has been found to localize at 19q13.3 (*26*). The homology between human and porcine TOPs is 86.6% at the DNA level and 91.1% at the amino acid level, while the homologies between human and rat TOPs are 84.2 and 84.3%, respectively. TOP is a metalloprotease that cleaves peptide bonds with hydrophobic residues at P1, P2, and P3′ (*27*), and is present in highest concentrations in the brain (*25*). On the other hand, the peptides adjacent to the β- secretase site on APP are collectively hydrophobic residues.

TOP is a cytosolic protease (*28*) expressed in brain (*25*) considered to be a candidate β-secretase. TOP has been shown to hydrolyze synthetic fluorogenic peptide substrates that harbor the β-secretory cleavage site (*12, 29*). The N-terminal amino acid sequence of bovine TOP is almost identical to that of porcine TOP (amino acid residues 8-17). However, we speculate that the N-terminal residue of TOP is blocked because the amino acid sequence signal was very low; N-terminal blocking might be removed by proteolysis (data not shown). Recombinant hTOP was expressed in COS cells and purified on a Ni-NTA column (Fig. 4). The purified protein shows a molecular mass of 82 kDa, identical to that deduced from the cDNA sequence. This suggests that hTOP is not modified in COS cells such as by phosphorylation, glycosylation, *etc.* We purified hTOP from cultured cells for the first time, since previous purifications of hTOP were from *E. coli* (*23, 29*). Accordingly, our hTOP preparation should be native and suitable for analyzing the biological function *in vitro*. hTOP specifically cleaves NL-QFS better than KM-QFS, which is the β-secretase site of APP (Fig. 5). However, hTOF does not cleave other short peptide substrates. These results suggest that hTOP specifically cleaves peptide substrates containing the β-secretase site of APP.

To determine whether hTOP cleaves APP695 *in vivo*, we transfected both hTOP and APP695-WT or APP695-NL into COS cells. Figure 6A shows the increased secretion of anti-sAPPβ-immunoreactive APP-WT-processing products (sAPPβ-WT). Because the anti-sAPPβ antibody is made from sAPPβ-WT C-terminal fragments, it detects only sAPPβ-WT fragments, and not sAPPβ-NL fragments (data not shown). Moreover, Fig. 6B and 6C show an increased secretion of anti-22C11-immunoreactive APP-

processing products (sAPPα + sAPPβ), but not anti-Aβ1-16-immunoreactive APP-processing products (sAPPα). Since anti-22C11 recognizes APP N-terminal fragments while anti-Aβ1-16 recognizes the fragment comprising amino acids 1-16 of Aβ, these changes in the intensity of the immunoreactive band reflect an alteration in the processing of APP. The specific anti-sAPPβ antibody detected the secretion of sAPPβ only from APP/hTOP-overexpressing cells, with no secretion of sAPPβ observed from antisense expressing cells. Figure 6 clearly demonstrates that the total amount of APP secreted from COS cells increases in the presence of hTOP and that the secreted APP cleaved by hTOP does not contain the N-terminal 16 amino acids of Aβ.

Finally, we tried to determine where the expressed hTOP is localized in COS cells using immunofluorescence microscopy method (Fig. 7). We found most of hTOP localized at the nucleus, however, these was slight but definite, hTOP localized in the Golgi or similar organelles (Fig. 7D). Previous reports indicated that TOP is a soluble protein (*28, 30*), but our results suggest that TOP is not only soluble but also organelle-bound. Since sAPPβ could not be found in APP antisense hTOP-overexpressing cells (Fig. 6A) and hTOP is localized in Golgi-like organelles (Fig. 7D), we concluded that a small portion of expressed TOP colocalizes with APP and cleaves APP at the β-secretase site.

In conclusion, we have demonstrated that hTOP is a potential β-secretase candidate. Previous results suggested that rabbit TOP cleaves the peptide substrate at the β-secretase site of APP-WT (*12*). Our results confirm that TOP is involved in the processing of APP *in vivo*.

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