Metabolism of amyloid precursor protein in COS cells transfected with a β -secretase candidate

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Abstract

Thimet oligopeptidase (TOP) is a thiol- and metallo-dependent peptidase and has been shown to be one of the β -secretase candidates. TOP expressed in COS cells cleaved amyloid precursor protein (APP) at the β -secretase site, and we found a proteolytic product of APP called secreted form of APP by β -secretase (sAPP β) in the conditioned media. Here we demonstrate that sAPP β was increased in conditioned media when TOP was coexpressed in COS cells with APP and treated with an ADAM inhibitor SI-27. In addition, although TOP expressed in COS cell was localized at nuclei or Golgi apparatus, it exclusively colocalized at Golgi apparatus when APP was coexpressed with TOP.

Abbreviations: A β , amyloid β ; ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; FITC, fluorescein isothiocyanate; QFS, quenched fluorescent substrates; sAPP, secreted form of APP; TOP, thimet oligopeptidase; PMSF, phenylmethylsulfonyl fluoride

Introduction

Alzheimer's disease is characterized by the formation of amyloid plaques containing amyloid β -protein (A β) in brain regions important for intellectual function and deposition of A β brings about neuronal death and a progressive loss of cognitive function and memory. A β , a 39–43 amino acid peptide, is generated by the proteolytic processing of amyloid precursor protein (APP) by β - and γ -secretases (Goldgaber et

al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). APP metabolism is normally processing at the A β domain (16 amino acid residues from N-terminus of A β) by α -secretase and non-amyloidogenic N-terminal fragment of APP (sAPP α) is secreted from the cells (Schubert et al., 1989; Weidemann et al., 1989; Esch et al., 1990; Sisodia et al., 1990; Wang et al., 1991). Therefore, APP processing at the α -secretase site dominantly occurs in the normal brain, but in the Alzheimer's brain, β - and γ -

Figure 1. The cleavage activity for KM-QFS and NL-QFS of purified hTOP is specifically inhibited by metalloprotease inhibitors. The β -secretase activities for KM- and NL-QFS were determined as described in the Materials and Methods section. Columns 1 and 2 are negative control containing EDTA and positive control containing $MnCl_2$ in assay buffer, respectively. Columns 3–7 are pepstatin, E-64, TLCK, phosphoramidon, and BE16627B (final concentrations; 10 μ g/ml), respectively. The white and black columns are the activities for KM-QFS and NL-QFS, respectively.

secretases are activated and $A\beta$ is produced. However, a small amount of $A\beta$ is secreted in the normal cultural medium, and $A\beta$ deposition is also detected in the normal brain (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992). Consequently, APP metabolism is fluctuating to some extent and balance of α - and β -secretase activity could regulate the onset of Alzheimer's disease.

Previously, we reported that the purification of a β -secretase candidate, thimet oligopeptidase (TOP, EC 3.4.24.15), from bovine brain. In addition, we coexpressed human TOP and APP695 in COS cells and found that the amount of secreted sAPP β is increased in conditioned media (Koike et al., 1999a). Independently, we reported that the membrane anchored metalloprotease MDC9 has an α -secretase activity and

activation of α -secretase suppresses the secretion of sAPP β (Koike et al., 1999b). In this article, we describe that the expression of TOP decreases sAPP α secretion into conditioned media and addition of a metalloprotease inhibitor enhanced the secretion of sAPP β . We also show that TOP and APP are colocalized in the same compartment in COS cells.

Materials and methods

Assay for β -secretase

The quenched fluorescent substrates (QFS) for β -secretase, normal KM-QFS (7-methoxycoumarin-4-acetyl-SEVKMDAEFR-K(RR-NH₂)-(2,4-dinitrophenyl)),

Figure 2. Inhibit of α -secretase enhanced sAPP β secretion from COS cells. (A) Conditioned media that APP-WT and/or hTOP were cotransfected with/without SI-27 (20 μ g/ml), were electrophoresed (15 μ g protein of each fraction) and immunostained with anti-sAPP α (upper) and anti-sAPP β (lower) after transfer to PVDF. The black triangle and open triangle indicate sAPP α and sAPP β , respectively. (B) Quantitation results of sAPP α and sAPP β : values are the ratio of densitometric scores for sAPP α and sAPP β in lane 2 (Figure 2A). Lanes and columns 1 and 3 are expressed APP-WT, APP-WT/hTOP without SI-27 (final concentrations; 20 μ g/ml), respectively. Lanes 2 and 4 are expressed APP-WT, APP-WT/hTOP with SI-27 (final concentrations; 20 μ g/ml), respectively.

and Alzheimer NL-QFS (7-methoxycoumarin-4-acetyl-SEVNLDAEFR-K(RR-NH₂)-(2,4-dinitrophenyl)) were synthesized by Peptide Institute Inc. (Minoh, Japan). A KRR sequence was added for easy solubilization. The human TOP (hTOP) expressed COS cells was purified by Ni-NTA column as described (Koike et al., 1999a). hTOP (2 μ g) was assayed in 200 μ l of buffer A (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM DTT, 10 μ g/ml leupeptin and 100 μ M PMSF) containing 5 mM EDTA or MnCl₂. Substrate (1 mM in DMSO) was added to a final concentration of 5 μ M, and the assay mixtures were incubated at 37 °C for 30 min. The addition of 100 μ l of 10% sodium dodecyl sulfate (SDS) and 700 μ l of 100 mM sodium acetate (pH 4.0) stopped the reactions. Fluorescence value was measured on a JASCO FP-777 fluorescence spectrometer (excitation, 328 nm; emission, 393 nm). Various inhibitors (E-64, TLCK: *N* $^{\alpha}$ -tosyl-L-lysyl chloromethyl ketone, pepstatin, phosphoramidon and BE16627B: hydroxamate-based metalloprotease inhibitor (A. Okuyama, unpublished data)) were used to determine protease activity for KM- and NL-QFS.

Co-expression of human TOP and APP695 in COS cells and immunoblot analysis

Human APP695 cDNA fragment (APP-WT, wild type APP) was constructed with the p91023 vector. hTOP cDNA fragment was constructed with pSecTag vector (Invitrogen, San Diego, CA, U.S.A.). An APP plasmid and hTOP plasmid were used to transfect COS-7 cells by the electroporation method (Chu et al., 1987). After 12 h incubation, transfected cells were transferred into serum-free medium with or without a hydroxamate-based cell-permeable metalloprotease inhibitor: SI-27 (final concentration 20 μ g/ml). 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 8000/ $\times$ /g for 5 min, the protein concentration of the supernatant was determined. The supernatant was diluted in 2/ $\times$ /SDS-buffer (125 mM Tris-HCl, pH 6.8, 30% glycerol, 2% SDS, 5% β -mercaptoethanol, and 0.2% bromophenol blue). The conditioned medium (8 ml) was concentrated by the addition of trichloroacetic acid (TCA; final concentration 10%) and centrifuged at 10 000/ $\times$ /g for 15 min. The precipitate was washed with ether-ethanol (1:1) and then dissolved in 300 μ l of 2/ $\times$ /SDS-buffer. The samples were subjected to electrophoresis in 10% polyacrylamide gels (Laemmli, 1970) and then transferred onto

a polyvinylidene difluoride (PVDF) membrane (Nihon Eido, Tokyo, Japan). The transferred membrane was incubated with anti-APP antibodies overnight at 4 °C, visualized with a VECTASTAIN *Elite* ABC kit (Vector Lab., Burlingame, CA, U.S.A.) and finally with a POD immunostain set (Wako, Osaka, Japan). The anti-APP antibodies used were anti-22C11 antibody (Boehringer Mannheim, Germany), anti-A β 1-16 antibody (Kinouchi et al., 1995) and anti-sAPP β antibody (Koike et al., 1999). The anti-TOP antibodies were raised according to Koike et al. (1999a). The detected bands were quantified with an Imagemaster Ver. 2.0 (Pharmacia, Uppsala, Sweden).

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. After washing with PBS, the fixed cells were permeabilized with 0.2% Triton X-100, 1 mM MgCl₂, and 0.1 mM CaCl₂ 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 anti-C100 antibody (Kouchi et al., 1998) and anti-myc antibody (Invitrogen) for 1 h at 37 °C. The cells were then washed three times with PBS and incubated with the second Oregon Green^{TM}-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG antibodies (Molecular Probes Inc., Eugene, OR, U.S.A.) for 1 h at 37 °C. The cells were viewed and examined with a confocal scanner (Leica).

Results and discussion

Inhibition of β -secretase activity by metalloprotease inhibitors

Previously, we reported that hTOP has a β -secretase activity toward APP as a substrate as well as synthetic KM- or NL-QFS. To know the specificity of TOP, we determined the inhibitory profile of TOP (Figure 1). hTOP was strongly inhibited by metalloprotease inhibitors (phosphoramidon, BE16627B) and chelating agents (EDTA, 1,10-phenanthroline), but other protease inhibitors (E-64, cysteine protease inhibitor; TLCK, serine protease inhibitor; pepstatin, aspartate protease inhibitor) had no effect. Both KM-QFS and NL-QFS were directly cleaved by hTOP in the presence of metal ions and reducing agent. Interestingly,

Figure 3. Immunofluorescence microscopy images of APP-WT and/or hTOP-transfected COS cells. APP-WT and hTOP were transfected and visualized as described in the Materials and methods Section. 3A-C images are vector, APP-WT, hTOP single-transfected cells, respectively. 3D-F images are APP-WT and hTOP cotransfected cells. 3D and 3E are visualized APP-WT or hTOP in cotransfected cells, respectively. 3F image is merged with 3D and 3E. The green shows APP-WT, and the red shows hTOP.

fluorescence intensities were higher at NL-QFS than KM-QFS under all conditions. NL-QFS was synthesized according to β -secretase flanking site of Swedish Alzheimer APP mutant, in which $A\beta$ was drastically increased. These results indicate that hTOP directly cleaved the β -secretase substrates, and hTOP has a higher activity toward Swedish APP mutant.

Increase in sAPP β secretion by α -secretase inhibitor

In the previous study, we reported that sAPP α secretion in APP-WT overexpressing cells increased with TPA, and it decreased by the addition of α -secretase inhibitor SI-27. However, sAPP β was reciprocally increased by SI-27 treatment (Koike et al., 1999b). It could be possible that the APP metabolism including β -secretase and α -secretase pathways are equally balanced.

To confirm this, we tried to inhibit endogenous α -secretase activity. We coexpressed APP-WT and/or hTOP in COS cells with/without SI-27. Figure 2 shows sAPP α (Figure 2A, upper) or sAPP β (Figure 2A, lower) release in condition media in APP-WT/vector (Figure 2A, lanes 1 and 2) or APP-

WT/hTOP (Figure 2A, lanes 3 and 4) transfected cells. The densitometric analysis was shown in Figure 2B. In COS cells, APP-WT was normally secreted mainly by α -secretase, and only slightly by β -secretase (lane 1). Inhibition of α -secretase by SI-27 (lane 2) increased secretion of APP by β -secretase in a compensatory manner. Expression of hTOP enhanced the cleavage of APP at β -secretase site (lane 3), and the effect of hTOP was promoted by the addition of SI-27. These results indicate that inhibition of endogenous α -secretase by SI-27 increased β -secretase activity of hTOP.

Change in the cellular localization of hTOP

Finally, we immunostained APP-WT and/or hTOP-transfected COS cells with anti-C100 and/or anti-myc antibody. Figure 3A showed no fluorescence was observed in vector-transfected cells. APP-WT- or hTOP-transfected cell was stained mainly at Golgi apparatus or nucleus plus Golgi apparatus, respectively (Figures 3B and 3C). On the other hand, APP-WT/hTOP-cotransfected cell was stained mainly Golgi-like apparatus (Figures 3D-F). These results suggest that

single-expressed hTOP is mainly localized at nuclei, but a small amount of TOP is attached to Golgi apparatus, while hTOP coexpressed with APP-WT colocalized with APP in Golgi apparatus. It was generally said that TOP was a cytoplasmic protease (Orlowski et al., 1983; Silva et al., 1999). However, TOP was also purified from microsomal fraction of the porcine brain (Akopyan et al., 1994). From these results, we predict that the localization of hTOP may change upon unidentified signal(s).

In the present study, TOP expressed in COS cells increased the secretion of sAPP β (Figure 2, lane 3), and enhanced sAPP β secretion is promoted by the SI-27 treatment (Figure 2, lanes 2 and 4). In contrast, sAPP α secretion was decreased in the SI-27 treated condition or TOP coexpressed condition (Figure 2, lanes 2 and 3). Recently a novel β -secretase candidate was cloned. Using an expression cloning strategy to identify β -secretase gene that changes in A β level, Vassar et al. (1999) isolated a protease clone that shared sequence similarity with a lysosomal aspartic protease cathepsin E. Overexpression of this brain-specific membrane-bound protease, termed BACE (beta-site APP cleaving enzyme)/Asp2/p501 (Vassar et al., 1999; Yan et al., 1999; Sinha et al., 1999), increased the peptide cleavage as well as sAPP β production with concomitant decrease in sAPP α . In addition, a substantial proportion of BACE immunostaining appeared in the Golgi apparatus like TOP and endosome. Robust expression of BACE in pancreatic acinar cells (Vassar et al., 1999; Yan et al., 1999) needs further explanation. These and our results suggest that many proteases have a secretase activity for APP.

In conclusion, we have demonstrated that the APP metabolism is regulated by the balance of α -secretase (MDC9, TACE, etc.) and β -secretase (TOP, BACE etc.) activities and that hTOP is one of the β -secretase candidates in living cells.

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