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Alzheimer's disease amyloid ß-clipping enzyme (APP secretase): Identification, purification, and characterization of the enzyme

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SUMMARY: Alzheimer's disease (AD) is the most frequent cause of dementia, although no genetic abnormality has been identified. Recent studies have elucidated the molecular defect in AD, including the abnormal deposition of amyloid ß peptide (ß/A4) in senile plaques of affected individuals. Normal brain contains the enzyme, APP secretase, which cleaves inside the ß/A4 portion of the precursor protein (APP); abnormal processing of APP occurs in AD brain. Until now, no evidence has been provided that APP secretase is an intracellular proteinase. We have now prepared two synthetic substrates of APP secretase, both of which contain the cleavage point and are much more sensitive than substrates previously available to identify APP secretase. Using these substrates, we found an intracellular proteinase that has APP secretase activity. This proteinase has been identified as cathepsin B.

Alzheimer's disease (AD) is characterized by senile plaques, neurofibrillary tangles, and cerebrovascular amyloid in the human brain (1,2). The major component of AD amyloid is a 4.2 kDa

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polypeptide, referred to as the amyloid ß peptide (ß/A4) (3-5). ß/A4 is derived from a large transmembrane glycoprotein precursor (amyloid precursor protein:APP) whose amino acid sequence was deduced from the cDNA sequence (6-9). A single APP gene produces at least 4 mRNAs species, APP695, 714 (10), 751 and 770, the last two containing a sequence highly homologous to that of a Kunitz type proteinase inhibitor. The four mRNAs encode the ß/A4 sequence, and the ß/A4 at positions 597 - 638 of the initially identified APP695 is a hydrophobic peptide and C-terminal half of this ß/A4 is buried in the membrane. Normal processing of APP is thought to result in the secretion of a large (>100 kDa on SDS-PAGE), soluble N-terminal APP fragment (11,12) with the generation of an small (9 kDa) membrane-associated C-terminal fragment (13). The C-terminus of the large fragment occurs at Gln^{15} of ß/A4 while the N-terminal sequence of the small membrane-bound C-terminal fragment begins with Leu^{17} of ß/A4 (14). On the other hand, abnormal processing events, in which cleavage by peptide-cutting enzymes occurs both sides of ß/A4 leaving ß/A4 to the deposition, may occur in the AD brain. To date, no evidence of the so-called "APP secretase" that cuts within ß/A4 has been provided. One question concerning the normal processing of APP, a process that leads to dissolution of ß/A4, is how APP molecule anchored to the membrane is cleaved within ß/A4 just outside the cell. We are interested in whether this normal processing is carried out by a single proteolytic enzyme, i.e., a cytosolic proteinase that is able to cleave at Gln^{15}-Lys^{16} or Lys^{16}-Leu^{17} bond of ß/A4 (14). We synthesized two types of substrates that include the tripeptides covalently bound to a fluorogenic aminomethylcoumarin. The degradation of Succinyl-His-His-Gln-methylcoumarinamide(MCA) (SHHQ-MCA) mimics the cleavage of Gln^{15}-Lys^{16} of ß/A4 (His^{13}-His^{14}-Gln^{15}-Lys^{16}), while that of Succinyl-His-Gln-Lys-MCA (SHQK-MCA) mimics the cleavage of Lys^{16}-Leu^{17} of ß/A4 (His^{14}-Gln^{15}-Lys^{16}-Leu^{17}). Using these substrates, it is possible to screen for the "APP secretase" that cleaves ß/A4 from APP. We identified the SHHQ-MCA and SHQK-MCA degrading enzyme in rat liver, ox brain and human brain, and purified to homogeneity. The purified enzyme cleaved Amy-26, a fragment of Asp^{1}-Ser^{26} of ß/A4, and AC-100, a C-terminal fragment of APP that begins at Met^{596} of APP695 and contains the entire ß/A4 peptide (15).

MATERIALS AND METHODS

Materials. Peptidyl methylcoumarinamides and Amy-26, a synthetic peptide fragment of ß/A4, were synthesized by Peptide Institute Inc. (Osaka, Japan). HPLC G3000SW (7.5 mm X 30 cm) and TSK CM2SW (4.6 mm X 25 cm) columns were purchased from Toyo Soda. Hydroxyapatite was purchased from Bio-Rad.

Proteolytic assays. The standard assay mixture contained 0.1 mM Suc-His-His-Gln-MCA or Suc-His-Gln-Lys-MCA, 50 mM citric-phosphate buffer (pH 6.5), 10 mM ß-mercaptoethanol (ß-ME) and the enzyme in a total volume of 0.1 ml. Incubation was carried out for 30 min at 37 °C, and the reaction was terminated by the addition of SDS to a final concentration of 5 %. Fluorescence of liberated aminomethylcoumarin was measured with a Hitachi F-3000 Fluorescence Spectrophotometer (ex., 380 nm; em., 460 nm). One fluorescent unit (FU ; 0.208 nM) is defined as the amount of the enzyme that catalyzes a fluorescence increase of 1.0 in 30 min (for benzyloxycarbonyl-Phe-Arg-MCA (ZFR-MCA) only, in 5 min) under our standard assay conditions.

Preparation of APP secretase. All operations were performed at 0 - 4 °C. Rat liver tissue was suspended in 10 vol. of 50 mM acetate buffer (pH 5.0) containing 10 mM ß-ME and 1 mM sodium azide, and homogenized with a Waring blender. The homogenate was then filtrated through gauze. Ammonium sulfate was added to 45 % (w/v) saturation to the crude filtrate, and the mixture was stirred for 2 h, and centrifuged at 8,000 x g for 30 min. The supernatant was adjust to 75 % saturation with ammonium sulfate, stirred for 2 h, and was centrifuged at 8,000 x g for 30 min. The precipitate was dialyzed overnight against 20 mM acetate buffer (pH 5.0) containing 10 mM ß-ME and 1 mM sodium azide.

A rat liver extract was applied onto a hydroxyapatite column equilibrated with 5 mM phosphate buffer (pH 6.0) containing 10 mM ß-ME and 1 mM sodium azide and eluted with a linear gradient of phosphate from 5 to 300 mM. The SHQK-MCA and SHHQ-MCA hydrolyzing fractions were collected, dialyzed against acetate buffer (pH 5.0) and applied to a HPLC CM2SW column equilibrated with 20 mM acetate buffer (pH 5.0) containing 10 mM ß-ME and 1 mM sodium azide. Further purification was achieved by chromatography on gel filtration HPLC (TSK-G3000SW).

SDS-PAGE. SDS-PAGE was performed by the method of Laemmli (16).

Western blotting. After SDS-PAGE, the isolated protein bands were transferred from the gel to a nitrocellulose membrane and detected with antisera (1/200 dilution), followed by 4-Chloro-1-naphthol (Vector Lab.) according to the avidin-biotin-peroxidase method (17).

Peptide digestion. Overexpressed AC-100 in E.coli (15) was sonicated and centrifuged to remove unsolved materials. The clear supernatant, AC-100 (10 µg), was used digestion experiments.

Amy-26 was digested with the purified enzyme in 50 mM citric-phosphate buffer (pH 6.5) and 10 mM ß-ME at 37 °C for 10 h. The reaction was terminated by the addition of TFA and acetonitrile to a final concentration of 0.1 % and 10 %, respectively. The mixture was then separated by HPLC on an ODS (C_{18}) column (10-60 % acetonitrile gradient) and the peptides were detected by absorbance 220 nm. Amino acid analysis and sequence were analyzed with an Applied Biosystems automated analyzer.

RESULTS

Purification of APP secretase.

Suc-His-Gln-Lys-MCA (SHQK-MCA) and Suc-His-His-Gln-MCA (SHHQ-MCA) and were used to detect the APP secretase activity that cleaves at the Gln^{15}-Lys^{16} or Lys^{16}-Leu^{17} of ß/A4.

An enzyme hydrolyzing SHQK-MCA eluted at 0.25 M NaCl as a single symmetrical peak. This enzyme also hydrolyzed SHHQ-MCA as well as ZFR-MCA (Fig.1). This peak was collected, dialyzed against 20 mM acetate buffer (pH 5.0) containing 0.1 M NaCl, 10 mM ß-ME, and 1 mM sodium azide and applied to a gel filtration (G3000) HPLC column. Since little SHQK-MCA or SHHQ-MCA

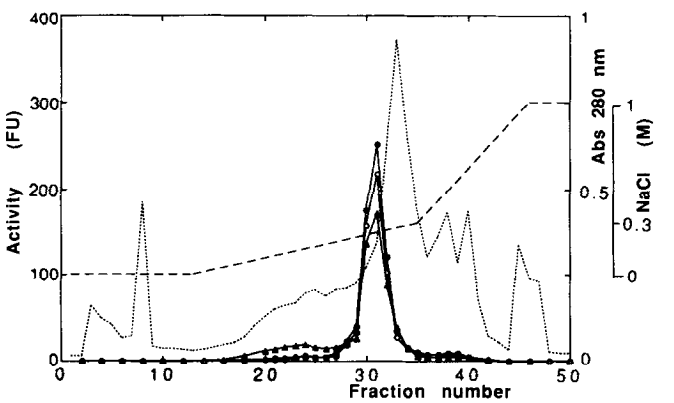

Fig.1. CM-cellulose column chromatography of rat liver APP secretase.
The active enzyme fraction from hydroxyapatite column chromatography (see MATERIALS AND METHODS) was subjected to HPLC CM-cellulose column (Toyo Soda, CM2SW, 4.6 mm X 25 cm) eluted with a linear NaCl gradient (0 to 0.3 M, fractions 13 to 35; 0.3 to 1.0 M, fractions 36 to 46) in 20 mM acetate buffer (pH 5.0) containing 10 mM ß-ME and 1 mM sodium azide. The major enzyme activity was eluted at 0.25 M NaCl.
·····, absorbance 280 nm; ---, NaCl; ○, SHQK-MCA; •, SHHQ-MCA; △, ZFR-MCA.

Table 1. Substrate specificity of APP secretase

Substrate	Activity (%)
Z-Phe-Arg-MCA	(100)
Z-Arg-Arg-MCA	29
Boc-Leu-Arg-Arg-MCA	101
Bz-Arg-MCA	19
Boc-Val-Leu-Lys-MCA	23
Suc-His-Gln-Lys-MCA	2
Suc-His-His-Gln-MCA	2
Ala-Ala-Phe-MCA	5
Z-Val-Ile-Ala-MCA	0
Suc-Ala-Glu-MCA	0

The enzyme and each of the substrates were incubated with 50 mM citric-phosphate buffer (pH 6.5), 10 mM ß-ME and 0.1 mM substrate for 30 min at 37 °C.
The activity is shown as % activity relative to that of Z-Phe-Arg-MCA.

hydrolyzing activity was observed, we conclude that one enzyme processes APP secretase activity in the rat liver extract. Interestingly enough, the enzyme also hydrolyzed the cathepsin substrate, ZFR-MCA (18), especially at low pH. Therefore, we tested the pH optima for two substrates. The enzyme obtained from chromatography on CM-cellulose had a pH optimum of 6.5. Table 1 shows the substrate specificity of the enzyme. It hydrolyzed ZFR-MCA most rapidly, 50 times faster than SHQK-MCA. The enzyme also digested benzyloxycarbonyl-Arg-Arg-MCA (ZRR-MCA), as well as wide range of other substrates. Addition of SH compounds stimulated the peptide hydrolyzing activity by about 10-fold. These results strongly suggest that the purified enzyme has a substrate specificity similar to that of thiol-dependent cathepsins.

Identification of the SHHQ-MCA and SHQK-MCA degrading enzyme as cathepsin B.

The effect of an inhibitor of cathepsins B and L, benzyloxycarbonyl-Phe-Ala-CHN_2 (ZFA-CHN_2), on the purified enzyme was determined (19). The addition of the inhibitor strongly inhibited the enzyme with a calculated Ki value of 10^{-6} M. Our previous experiment showed that cathepsin L is more sensitive to inhibition by ZFA-CHN_2 (Ki=10^{-7}M) than cathepsin B (Ki=10^{-6}M). Therefore we suspected that the purified enzyme might correspond

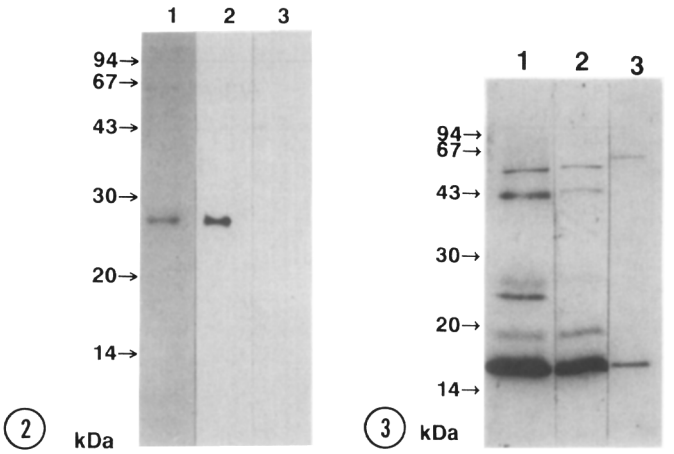

Fig.2. SDS-PAGE (15 %) and immunoblot analysis of purified APP secretase.
Lane 1, silver stain of the SDS-PAGE analysis of rat liver secretase (0.5 μg); lane 2, immunoblot analysis of purified APP secretase (0.5 μg) with anti-cathepsin B antibody; lane 3, same as lane 2 except that anti-cathepsin L antibody was used.

Fig.3. Degradation of AC-100 by APP secretase as determined by 15 % SDS-PAGE followed by immunoblotting.
AC-100 was incubated with purified enzymes (approximately 150 FU/tube) for 12 h at 37 C° and electrophoresed. Antibody to the synthetic C-terminal fragment of APP(672-695) was raised in rabbits.

to cathepsin B. To determine whether this is the case, immunoblotting analysis was performed (Fig.2) and it was found that antiserum to rat liver cathepsin B (20) crossreacted with the purified enzyme. These findings indicate that the enzyme, a candidate for the enzyme that cleaves APP in the interior of the amyloid ß peptide sequence, is identical to cathepsin B.

Digestion of amyloid related peptides.

The overexpressed 17 kDa fragment of APP, AC-100, which contains the whole C-terminal of APP (Met^{596}-Asn^{695}) (15) was digested with the purified enzyme (Fig.3). Immunoblotting showed that the E.coli extract contained several species of AC-100, the major band appearing at 17 kDa, but with several minor aggregation products seen in the gel. These peptides were completely degraded by the purified enzyme after 12 h incubation. The results indicate that cathepsin B is capable of degrading intact APP (see discussion).

In the next step, we determined the proteolytic cleavage site when a shorter, easily solubilized fragment, Amy-26, was digested

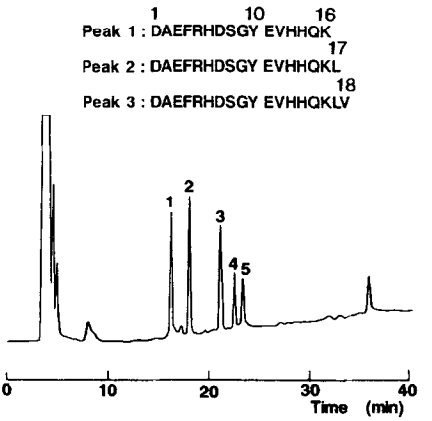

Fig.4. Reverse-phase HPLC on a ODS (C_{18}) column of digested Amy-26.
Peptides were separated on Sumipax PG ODS column (10 μm, 4.6 mm X 25 cm) with a linear gradient of acetonitrile (10-60 %) in 0.1 % TFA. Amino acid sequence of the peptides in peaks 1 to 3 are shown at the top.

by the purified enzyme. The reaction mixture was analyzed by HPLC on reverse phase ODS column. The proteolytic cleavage products appeared as five peaks whose amino acid sequences were analyzed. Fig.4 shows that peaks 4 and 5 were longer than 20 mer (the sequencer is able to detect at most 20 mer). Peak 4 was identified as intact Amy-26, because its retention time was the same as an authentic sample. Peaks 1, 2 and 3 (Fig.4) are the products of proteolytic cleavage of ß/A4 at Lys^{16}-Leu^{17}, Leu^{17}-Val^{18}, and Val^{18}-Phe^{19}, respectively. The peak appearing at 36 min was not due to a peptide.

DISCUSSION

The APP-processing enzyme "APP secretase" is screened for using the artificial substrates, SHHQ-MCA and SHQK-MCA. We found that the enzyme is identical to cathepsin B in term of substrates specificity, inhibitor spectrum, and immunoreactivity. Though the active enzyme hydrolyzing SHQK-MCA and SHHQ-MCA appeared as a single major peak on CM cellulose chromatography, two other minor peaks were also detected. These components at fractions 25 and 39 (Fig.1) crossreacted with rat liver anti-cathepsin B and L, respectively, (data not shown). APP secretase cleaves the Amy-26 peptide, Asp^{1}-Ser^{26} of ß/A4, and also degrades AC-100, Met^{596}-Asn^{695} of APP695. Therefore, we conclude that cathepsin B is a candidate for the APP-processing enzyme "APP secretase".

In our experiments, the purified enzyme degraded AC-100 completely. The molecular weight of the recombinant AC-100 is 17 kDa, which is higher than its theoretical weight based on sequence. The protein expressed in E.coli has a strong tendency to aggregate, i.e., to be potentially amyloidogenic under physiological conditions. After solubilization with 6 M urea and HPLC chromatography on DEAE-cellulose, the dilute solution became soluble in saline. The reason that purified cathepsin B (APP secretase) did not digest the aggregated protein at a specified position in the middle of ß/A4 may be due to difference in conformation in vitro. If cathepsin B only hydrolyzes a nascent soluble precursor peptide in vitro or only cuts at the ß/A4 portion after incorporation into the membrane, further experiments, such as coexpression of cathepsin B with AC-100 in COS-1 cells, will be required to clarify the role of cathepsin B in the processing of the APP molecule. It is also possible that the different susceptibility to AC-100 is due to solubility of the substrates. As indicated, the N-terminal half of ß/A4 (Amy-26) is soluble in 0.1 % TFA. On the other hand, AC-100 accumulates as deposit-like structures in COS-1 cells (15). Wolf et al. (21) who expressed an analogous 100 amino acid C-terminal APP region in CV-1 monkey fibroblasts, also saw deposited materials resembling those of preamyloid (15). These facts indicate that a large C-terminal fragment of APP may self-aggregate, while a small ß/A4-containing fragment does not. Comparable amounts of amyloidgenic enzymes, multicatalytic proteinase (22,23) and prolyl endopeptidase (24), did not degrade AC-100 in vitro.

Purified APP secretase was shown to cleave Amy-26 at Lys^{16}-Leu^{17}, Leu^{17}-Val^{18}, and Val^{18}-Phe^{19} of ß/A4 at approximately the same rates. It has been previously reported that the cleavage site during the normal processing of ß/A4 is at Gln^{15}-Lys^{16} or Lys^{16}-Leu^{17}, which is in agreement with the above results that APP secretase has the heterogeneous specificity toward a synthetic fragment of ß/A4 peptide. The reason we failed to identify any residual fragments after the digestion of Amy-26 is unknown. The remaining fragment of Amy-26 may be present in the flowthrough or small peaks and have gone unnoticed. The minor differences between APP processing in vivo and in our in vitro experiments may be caused by differences in the structure of the substrate.

Does cathepsin B in fact process APP at lysosomes, membranes, or elsewhere? Cathepsin B generally distributes in the lysosomes

of mammalian cells, and acts on proteins mainly as endoproteinase (although it also possesses dipeptidylcarboxypeptidase activity) and cleaves synthetic esters and amides, especially at sites adjacent to arginine [EC.3.4.22.1]. Since anti-APP does not specifically stain plasma membranes and anti-APP staining near nucleus is often observed on immunohistochemistry (25), it is possible that APP is digested in lysosomes. APP has been thought to be localized on the cell membrane because, as deduced from its cDNA, APP has a signal peptide followed by a membrane-associated domain. Since a small membrane-associated C-terminal fragment (9 kDa), a product of APP-processing, is tightly associated with the cell membrane, it is necessary that APP secretase should work either in the extracellular space or at the membrane surface. On the other hand, it is possible to postulate that APP is localized at the Golgi or lysosomal membrane. If APP is incorporated into lysosomal membranes, our results would easily explain the evidence that APP is processed in the middle of the ß/A4 sequence.

The results of immunohistochemical staining by Benowitz et al. (26) also suggests the lysosomal localization of APP in pyramidal cells in hippocampus. It is possible, therefore, that APP may function in neuronal lysosomes or interact with lysosomal membrane proteins. The degradation of APP by a lysosomal enzyme may account for the deposition of ß/A4 amyloid in neuritic plaques, because these plaques contain ubiquitin, a substance known to concentrate in lysosomes, and other lysosomal elements. Since amyloid itself contributes to neurodegeneration, results of these studies are critical for understanding the etiology of ß/A4 deposition in AD.

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