Processing of β-Amyloid Precursor Protein by Cathepsin D*

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Jeffrey Higaki‡, Rosanne Catalano‡, Andrew W. Guzzetta‡, Diana Quon‡, Jean-François Navé¶, Celine Tarnus†, Hugues D’Orchymont, and Barbara Cordell‡

From ‡Scios, Inc., Mountain View, California 94043 and ¶Marion Merrell Research Center, Strasbourg 67084, France

The events leading to the formation of β-amyloid (βA4) from its precursor (βAPP) involve proteolytic cleavages that produce the amino and carboxyl termini of βA4. The enzyme activities responsible for these cleavages have been termed β- and γ-secretase, respectively, although these protease(s) have not been identified. Since βA4 is known to possess heterogeneity at both the amino and carboxyl termini, β- and γ-secretases may actually be a collection of proteolytic activities or perhaps a single proteolytic enzyme with broad amino acid specificity.

We investigated the role of cathepsin D in the processing of βAPP since this enzyme has been widely proposed as a γ-secretase candidate. Treatment of a synthetic peptide that spans the γ-secretase site of βAPP with human cathepsin D resulted in the cleavage of this substrate at Ala42–Thr43. A sensitive liquid chromatography/mass spectrometry technique was also developed to further investigate the ability of cathepsin D to process longer recombinant βAPP substrates (156 and 100 amino acids of βAPP carboxyl terminus) in vitro. The precise cathepsin D cleavage sites within these recombinant βAPP substrates were identified using this technique. Both recombinant substrates were cleaved at the following sites: Leu69–Val80, Asp84–Ala89, Phe89–Phe94. No cleavages were observed at putative γ-secretase sites: Val40–Ile44 or Ala42–Thr43, suggesting that cathepsin D is not γ-secretase as defined by these βA4 termini. Under conditions where the βAPP156 substrate was first denatured prior to cathepsin D digestion, two additional cleavage sites near the amino terminus of βA4, Glu3–Val2 and Glu2–Phe4, were observed, indicating that cathepsin D cleavage of βAPP is influenced by the structural integrity of the substrate. Taken together, these results indicate that in vitro, cathepsin D is unlikely to function as γ-secretase; however, the ability of this enzyme to efficiently cleave βAPP substrates at nonamyloidogenic sites within the molecule may reflect a role in βAPP catabolism.

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‡ To whom correspondence should be addressed: Scios, Inc., 2450 Bayshore Parkway, Mountain View, CA 94043. Tel.: 408-523-7253; Fax: 408-523-7272.

¶ The abbreviations used are: AD, Alzheimer’s disease; βA4, β-amyloid protein; βAPP, β-amyloid precursor protein; bFGF, basic fibroblast growth factor; LC, liquid chromatography; MS, mass spectrometry; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; RP, reverse phase; HPLC, high performance liquid chromatography; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl. The amino acid sequence numbering system used in this report is relative to the βA4 sequence beginning with Asp1.
tions and the expression of aberrant levels and/or isoforms of βAPP (18). This amyloidogenetic pathway was initially thought to involve the lysosomal-endosomal system based on the observation that lysosomal inhibitors stabilized amyloidogenetic carboxy-terminal fragments (15, 16, 19–22). The observation that full-length surface βAPP can be reinternalized (15) via a coated-pit mechanism (23, 24) added further support for the role of the lysosomal-endosomal system. However, the observation that lysosomal inhibitors have little or no effect on βA4 levels suggests that the subcellular site of βA4 production may alternatively involve nonlysosomal compartments such as the trans-Golgi or early endosome (17, 21, 25, 26).

Using a variety of peptide or protein substrates that span the α-secretase cleavage site, a number of protease activities have been implicated as α-secretase (27), but the true enzyme(s) has yet to be definitively identified. The search for α-secretase is confounded by the possibility that multiple proteases may be involved in cleaving this site (28).

The β- and γ-secretases responsible for processing βAPP through the amyloidogenetic pathway also remain a mystery despite numerous attempts to identify them. Using a variety of synthetic peptide and recombinant βAPP substrates, a number of proteases have been implicated as candidates for β-secretase based on their ability to cleave at or near the Met0–Asp1 bond of βA4. These proteases include multicatalytic proteases, serine proteases, metalloproteases, and aspartic acid proteases (as reviewed in Refs. 27 and 29). Proteases implicated as γ-secretases include multicatalytic protease, prolyl endopeptidase, and cathepsin D (27, 29). Of these enzymes, cathepsin D is of particular interest, since many of the properties associated with this protease favor its role in the amyloidogenetic processing of βAPP.

The features of cathepsin D relevant to βA4 generation include the following observations. Cathepsin D is an abundant aspartic protease in brain tissue (30) and is located in and is active within acidic lysosomal and endosomal compartments (31). This protease is also associated with amyloid deposits in AD tissue (32, 33), is up-regulated in AD neurons (34), and shows an age-related change in activity in human and rodent brain tissue (35, 36). Furthermore, cathepsin D exists in both a soluble and a membrane-associated form (37, 38), the latter of which favors its role in processing membrane-associated proteins such as βAPP. Cathepsin D also displays an amino acid specificity consistent with the amino acid sequences in the vicinity of putative β- and/or γ-secretase sites (39). Evidence against a role of cathepsin D in the formation of βA4 includes the fact that the levels of cathepsin D activity in AD tissue are not significantly different from those of controls (40) and that the overexpression of this enzyme in cell culture does not enhance the level of βA4 secretion (41).

To determine the ability of cathepsin D to function as either a β- or γ-secretase, in vitro assays were developed to study the proteolytic activity of cathepsin D on synthetic peptide substrates that span either the β- or γ-secretase sites (27, 29, 42, 43). Although these studies showed that cathepsin D is able to cleave these substrates at sites consistent with either a β- or γ-secretase, better evidence implicating cathepsin D as a secretase enzyme was obtained by studying the in vitro activity of cathepsin D on βAPP itself. The production of amyloidogenetic carboxy-terminal fragments of βAPP starting from a βAPP695 substrate was a demonstration of cathepsin D's ability to cleave βAPP near the β-secretase site and, hence, an illustration of a possible β-secretase activity for this enzyme (44). Likewise, cathepsin D was able to further process an amyloidogenetic carboxy-terminal βAPP substrate (corresponding to a β-secretase product), suggesting a γ-secretase activity for the enzyme (42).

However, in these studies, since the precise cathepsin D cleavage sites were not characterized at the amino acid sequence level and since no βA4 was detected, the ability of cathepsin D to cleave βAPP substrates at relevant sites was not fully established. In this study, we further investigated the potential role of cathepsin D in βAPP processing by developing sensitive in vitro assays for evaluating the proteolytic activity of this enzyme on various βAPP substrates. Improvements in the methods used to detect and to characterize hydrophobic cleavage products has allowed us to identify precise βAPP cleavage sites within the vicinity of the βA4 domain. These cleavage sites provide important clues regarding the function of cathepsin D in βAPP processing.

MATERIALS AND METHODS

Enzyme, Synthetic Substrate, and Antibodies—Bovine cathepsin D (EC 3.4.23.5), 15 units/mg of protein, was from Sigma. Human liver cathepsin D (8 units/mg of protein) was either from Calbiochem or purified according to published procedures (45). Enzyme purity was assessed by standard SDS-polyacrylamide gel electrophoresis and reversed-phase methods. In addition, control assays performed in the presence of the aspartic protease inhibitor, pepstatin, showed complete inhibition of all protease activity, confirming the lack of contaminating protease activities. One cathepsin D unit produces an increase in A500 of 1.0/min at pH 3.0, 37 °C measured as trichloroacetic acid-soluble products using hemoglobin as a substrate. The synthetic peptide Ac-Orn-GGVIAVTI-Orn-NH2 was synthesized by standard solid phase methods on an Applied Biosystems Biosynthesizer (Applied Biosystems, Foster City, CA) and purified to single peak homogeneity by RP-HPLC. This peptide spans the βA4 γ-secretase site (GLT90-Ile100) but is very hydrophobic since it comprises a portion of the transmembrane domain of APP. To enhance the solubility of this peptide at the low pH range, ornithine residues were included at the amino- and carboxyl-terminal ends. The polyclonal antisera, BC-1, directed to the cytoplasmic domain of βAPP (705–730 of βAPP751 sequence) was described previously (17). Anti-FLAG M2 affinity column was from Eastman Kodak Co. Brij35 and Brij30 were purchased from Sigma.

Expression and Purification of FLAG-βAPP156-bFGF Substrate—In order to construct the DNA encoding βAPP156 tagged with FLAG at the amino terminus and a bFGF epitope at the carboxyl terminus, a FLAG-βAPP751 was first made by inserting a double-stranded synthetic oligonucleotide encoding the signal sequence of βAPP, a FLAG epitope, and the first three amino acids of βAPP into a Kan-Smal-digested pGEM vector containing βAPP751. This insertion added the FLAG sequence immediately 5′ to the βAPP751 sequence. Next, the sequence encoding a bFGF epitope was added to the 3′ end of the βAPP sequence. This was accomplished by first introducing a MluI restriction site next to the 3′ end of βAPP751 by in vitro mutagenesis. A double-stranded synthetic oligonucleotide encoding the bFGF tag was then ligated into this MluI site. This FLAG-βAPP751-bFGF vector was then used to construct a baculovirus FLAG-βAPP156-bFGF expression vector.

The FLAG-βAPP751-bFGF plasmid DNA was digested with Kan1 and BstNI to remove a 1745-base pair sequence encoding the aminoterminal residues of βAPP751 from Pro4 to Trp602. A 23-base pair double-stranded oligonucleotide was ligated into the Kan1-BstNI sites to create a fusion between βAPP751 Pro4–Leu602. This FLAG-βAPP156-bFGF construct was then digested with Smal-XmnI and the sequence encoding FLAG-βAPP156-bFGF was blunt-end ligated into filled-in NdeI-digested baculovirus expression vector (Invitrogen, San Diego, CA). The chimeric baculovirus expression vector was used to express the βAPP derivative in SF9 insect cells.

A 1-liter culture of infected SF9 cells was harvested and lysed in PBS containing 1% (v/v) Triton X-100 (reduced) and 5 mM EDTA. The cell suspension was adjusted to 0.1% Triton X-100 by dilution with PBS and centrifuged at 27 000 × g, for 20 min. The resulting supernatant was applied to an anti-bFGF affinity column and eluted with 0.1 M sodium citrate, pH 3.0 containing 0.1% Triton X-100. Fractions containing FLAG-βAPP156-bFGF were immediately neutralized with Tris, adjusted to 0.1 M CaCl2, and applied to an anti-FLAG M1 affinity column. FLAG-βAPP156-bFGF was eluted with PBS containing 0.1% Triton X-100 and 5 mM EDTA.

Expression and Purification of βAPP100-FLAG Substrate—The βAPP100-FLAG used in this study was expressed and purified as de-
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Fig. 1. Digestion of Ac-Orn-GGVVIATVI-Orn-NH$_2$ with cathepsin D—A 6-µg sample of Ac-Orn-GGVVIATVI-Orn-NH$_2$ was incubated in 50 mM sodium citrate, pH 3.5, for 2 h at 37 °C in the absence (A) and in the presence (B) of 1 µg of human cathepsin D. Following the incubation period, the sample was analyzed by reversed phase HPLC as described under "Materials and Methods." S, undigested substrate peak; P1 and P2, product peaks. The peaks with retention times of 2, 3.5, and 12.7 min are non-peptide, background components.

Digestion of Ac-Orn-GGVVIATVI-Orn-NH$_2$ with Cathepsin D—A 6-µg sample of Ac-Orn-GGVVIATVI-Orn-NH$_2$ was digested with 1 µg (0.008 units) of human cathepsin D in 80 µl of 50 mM sodium citrate, pH 3.5, for 2 h at 37 °C. The enzyme to substrate molar ratio was 1:200. Following digestion, the sample was immediately analyzed by RP-HPLC on a Hewlett Packard HP 1050 automatic LC system equipped with a Vydac C18 (0.46 × 15 cm) column equilibrated in 0.1% (v/v) trifluoroacetic acid. Elution was with a linear acetonitrile gradient (1%/min) at room temperature. Absorbance was monitored at 215 nm.

Digestion of FLAG-βAPP156-bFGF with Cathepsin D—A 3-µl sample of purified FLAG-βAPP156-bFGF (90 µg) was diluted to 5 ml with 50 mM sodium citrate, pH 3.5. A 55-µg sample (0.83 unit) of bovine cathepsin D was added to the substrate to yield a final enzyme to substrate molar ratio of 1:5. The mixture was allowed to digest at 37 °C for variable time periods. A 2-h digestion period was routinely used after it was found that the major product peaks were present in greatest abundance after this time period. After digestion, a 10-µl sample was subjected to capillary RP-HPLC separation using an HP1050 LC system adapted for capillary HPLC separations as described in Guzzetta et al. A constant LC flow rate setting of 0.2 ml/min provided a column flow rate of 8 µl/min. The capillary column used in these experiments was a 25 cm × 0.32-mm inside diameter, 5-µm Vydac C18 column (300 Å) (Microtech Scientific, Sunnyvale, CA). The column was equilibrated with 0.1% trifluoroacetic acid and eluted with acetonitrile at room temperature. Absorbance was monitored at 215 nm using a Kratos/ABI 757 UV detector (Applied Biosystems, Foster City, CA) equipped with a capillary UV flow cell (LC Packings, Inc., San Francisco, CA).

Western Blot Analysis of βAPP156 after Digestion with Cathepsin D—Peak fractions collected off the RP-HPLC were dried down, redissolved in Laemmli sample buffer, and separated on a 16.5% Tris-HCl-Tricine polyacrylamide gel. Following electrophoresis, the gel was transferred to a polyvinylene difluoride membrane, blocked with blocking buffer (4% (v/v) fish gelatin, 2% (v/v) nonfat dry milk in PBS) for 1 h, then transferred to blocking buffer containing a 1:300 dilution of BC-1 polyclonal antisera (17). The filter was then developed with an anti-rabbit Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA) and reacted with the horseradish peroxidase substrate, 3,3'-diaminobenzidine (Vector Laboratories).

Amino Acid Sequence and Mass Spectral Analysis—Peak fractions from the cathepsin D digestion of βAPP156 were collected off a RP-HPLC column (Vydac C4, 0.46 × 15 cm), dried, and subjected to Edman degradation using an Applied Biosystems 477A protein sequencer in-line with a 120A phenylthiohydantoin analyzer (Applied Biosystems, Foster City, CA). Alternatively, to reduce sample loss and to enhance sensitivity, capillary RP-HPLC fractions were collected directly onto Porton filters (Beckmen Instruments) and subjected to sequence analysis using an ABI 470A gas-phase sequencer in-line with an Hewlett Packard HP1090 HPLC. This latter technique allowed the direct sequencing of as little as 2–5 pmol of sample.

HPLC-mass spectral (LC/MS) analysis was performed using an SSQ 7000 Finnigan MAT mass spectrometer equipped with a Finnigan electrospray source. The spray voltage was set at 4500 volts. For source collision-induced dissociation experiments, the scans were cycled through the following experiments: scan 1, 100-2000 m/z with a scan.

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2 A. E. Mackay, A. Ehrhard, M. Moniatte, C. Guenet, C. Tardif, C. Tarnus, O. Sorokine, B. Heintzelmann, C. Nay, J.-M. Remy, J. Higaki, A. Van Dorsseleer, J. Wagner, C. Danzin, and P. Mamont, manuscript in preparation.

3 A. W. Guzzetta, L.-L., Kang, and H.-T. N. Truong, manuscript in preparation.
Fig. 2. Cleavage products generated by cathepsin D digestion of FLAG-βAPP156-bFGF. A 90-µg sample of FLAG-βAPP156-bFGF was digested with bovine cathepsin D in 5 ml of approximately 50 mM sodium citrate, pH 3.5, at 37 °C for 2 h. Following digestion, a 500-µl aliquot of the digestion mixture was separated by RP-HPLC as described under "Materials and Methods." Curve A, digestion products generated by digesting FLAG-βAPP156-bFGF with cathepsin D at an enzyme to substrate molar ratio of 1:5; curve B, FLAG-βAPP156-bFGF alone; curve C, cathepsin D alone.

Duration of 2 s, octapole offset 15 volts; scan 2, 100-2000 m/z with a scan duration of 2 s, octapole offset 25 volts; and scan 3, 300-2000 m/z with a scan duration of 2 s, octapole offset 3 volts. For non-collision-induced dissociation experiments, the quadrupole was scanned from 300 to 2000 m/z with a scan duration of 3 s.

RESULTS

Digestion of Synthetic Peptide with Cathepsin D—To determine whether cathepsin D is capable of cleaving at the γ-secretase site, a synthetic peptide spanning residues 37–45 of βA4 (Ac-Orn-GGVIATVI-Orn-NH2) was subjected to digestion with human liver cathepsin D. The digestion products were separated by RP-HPLC, and the resulting chromatogram is shown in Fig. 1. The full-length peptide substrate eluted with a retention time of 14.7 min. This peak was well separated from two product peptides that eluted with retention times of 8.2 and 11.4 min. Mass spectral analysis of these two products identified the 8.2-min peak as TVI-Orn-NH2 (MH + mass = 445.3) and the 11.4 min peak as Ac-Orn-GGVI (MH + mass = 671.4). Thus, the intact peptide substrate was cleaved by cathepsin D predominantly (>95% of total digestion) between Ala42 and Thr43. The peaks eluting with retention times of 2, 3.5, and 12.5 min are background peaks (perhaps buffer components) not related to the peptide substrate or any of the products formed during the digestion. In addition to the Ala42-Thr43 cleavage, the mass spectral analysis of the digestion revealed a minor (<5%) product peptide formed by a cleavage between Thr43 and Val44 (data not shown). Identical results were obtained using bovine cathepsin D as well, thus cathepsin D is capable of cleaving this synthetic peptide predominantly carboxy-terminal to Ala42 with a minor cleavage after Thr43.

Digestion of FLAG-βAPP156-bFGF with Cathepsin D—Although the synthetic peptide Ac-Orn-GGVIATVI-Orn-NH2 can be used to assay cathepsin D cleavage at a γ-secretase-like site, caution should be displayed when interpreting this cleavage activity as true γ-secretase activity, since a synthetic peptide might not display all the primary, secondary, and tertiary structural elements of a natural protein substrate. A more appropriate substrate for γ-secretase is full-length βAPP which presumably contains many of the structural requisites for protease recognition. However, since full-length βAPP is a large, complex protein consisting of multiple domains and glycosylation sites, the use of this large protein as a secretase substrate is not straightforward. Therefore, we decided to simplify this protein by omitting much of the extracellular domain. The final βAPP substrate used in these experiments (FLAG-βAPP156-bFGF) (Fig. 2) were analyzed by Western blot with a polyclonal anti-σera (BC-1) specific for the carboxyl terminus of βAPP. The seven major peaks obtained by RP-HPLC of cathepsin D cleaved FLAG-βAPP156-bFGF (Fig. 2) were analyzed by Western blot with a polyclonal anti-σera (BC-1) specific for the carboxyl terminus of βAPP. The samples were run on a 16.5% Tris-HCl-Tricine gel, transferred to polyvinylene difluoride, blotted with BC-1, and developed as described under "Materials and Methods." Lane 1, the purification and characterization of this protein and its proteolytic products, an 8-amino acid FLAG epitope and a 10-amino acid bFGF epitope were engineered at the amino and carboxyl termini, respectively.

Since the major product peaks were the most abundant after a 2-h digestion, FLAG-βAPP156-bFGF was digested with bovine cathepsin D for this amount of time, and the resulting digest was separated by RP-HPLC (Fig. 2). The large amount of enzyme used here was determined to generate efficient cleavage of the substrate within a short time period where the aggregation and precipitation of the recombinant substrate was not a problem. Carboxy-terminal fragments of βAPP, including βAPP156, are well known to aggregate (9, 13, 18). When the digestion was carried out at lower enzyme to substrate ratios for extended time periods, a considerable amount of substrate was found to precipitate and resulted in much lower cleavage efficiency. A series of product peaks were generated and the seven primary product peaks were collected for Western blot analysis using BC-1 anti-σera. The resulting Western blot (Fig. 3) identified an ~5-kDa carboxy-terminal fragment of FLAG-βAPP156-bFGF present in product peaks 6 and 7 that contains the cytosolic BC-1 epitope. This fragment is of interest since, based on the known amino acid sequence of
FLG-bAPP156-bFGF

\[
\begin{align*}
&\text{DYKDDDDKLEVPLDDLNWHSFGAALSVPANTENEVEPVDARAPAADRGLTRPGSGLTNIKTEISEVK} \\
&\text{(U)}
\end{align*}
\]

\[
\begin{align*}
&\text{MDAEPHRDSGYEVHQQKLVPFAEDVSNGKGAIGLVMGGVVIATVITLVMKLKKQYTSIHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFPEQMQNAPPCHFKDP} \\
&\text{(U)}
\end{align*}
\]

\[\text{SP100-FLAG}\]

FIG. 4. Schematic representation of the major cathepsin D cleavage sites in FLG-bAPP156-bFGF and in bAPP100-FLAG. The amino acid sequences for the FLG-bAPP156-bFGF and bAPP100-FLAG substrates are shown. Large arrows denote major cleavage sites. Unique cathepsin D cleavage sites in urea-denatured FLG-bAPP156-bFGF are labeled (U). Small arrowheads denote minor bAPP100-FLAG cleavage sites determined by mass spectral analysis.

FLG-bAPP156-bFGF, the cleavage of this substrate at the γ-secretase site predicts a BC-1 reactive carboxyl-terminal fragment of this approximate size. Additional Western blot analysis of these fractions using antibodies directed toward other regions of βAPP confirmed that this ~5-kDa fragment is a carboxyl-terminal fragment of βAPP formed by a proteolytic cleavage at or near the predicted γ-secretase site. In addition, digestion with human cathepsin D gave identical results (data not shown).

All seven major HPLC peaks from this digest were collected separately over several runs to isolate enough material for amino acid sequence analysis. The primary sequence obtained for each of the seven fractions is shown in Table I, and a diagram of the resulting cleavage sites is shown in Fig. 4. FLG-bAPP156-bFGF was cleaved by cathepsin D at four primary sites. The amino acid sequence obtained for the ~5-kDa fragment containing the BC-1 epitope present in fraction 7 shows that this fragment was produced by a cleavage between Leu\(^{49}\) and Val\(^{50}\) of bAPP156. This site is within the putative transmembrane domain of βAPP, but 7–9 amino acid residues downstream of the carboxyl terminus of the βA4 sequence. Fraction 1 consisted of a FLAG-bAPP156-bFGF fragment possessing a carboxyl-terminal of βAPP fragment formed by cleavage at Phe\(^{53}\)–Phe\(^{54}\). The amino acid sequence of this fragment was also observed in fractions 3, 4, and 6. Fraction 2 contained a FLG-bAPP156-bFGF fragment generated by a cleavage at Asp\(^{62}\)–Ala\(^{63}\) within the cytoplasmic domain of βAPP, downstream of the putative transmembrane domain. Double amino acid sequences were detected in fractions 4, 5, and 6. Common among these fractions was the amino-terminal FLG fragment sequence of FLG-bAPP156-bFGF, indicating that these fractions contained, in part, amino-terminal fragments of the substrate. Fraction 5 also contained a unique secondary sequence generated by a cleavage between Asp\(^{44}\) and Ser\(^{43}\) of the substrate. Full-length (intact) βA4 was not detected by this method of analysis.

In order to determine whether any structural features of FLG-bAPP156-bFGF might dictate cathepsin D cleavage of this protein, a second experiment was performed in which the substrate was first denatured in 8 m urea prior to digestion with cathepsin D. RP-HPLC of the digest (Fig. 5) and amino acid sequence analysis of the resulting products (Table II) identified three of the four primary cleavage sites described above. In addition, two other cleavage sites were identified. The first was 3 amino acid residues amino-terminal to the βA4 domain (Glu\(^{−3}\)–Val\(^{−2}\)), and the second was just inside the βA4 domain (Glu\(^{5}\)–Phe\(^{4}\)). Since these cleavages were not detected in the previous digestion of nonadenatured bAPP156, the physical state/structure of the substrate appears to influence the susceptibility of bAPP156 to cathepsin D cleavage near the amino terminus of the βA4 domain.

Digestion of bAPP100-FLAG with Cathepsin D—A more appropriate protein substrate for determining the role of cathepsin D as a γ-secretase is the carboxyl-terminal 100 residues of βAPP (βAPP100) since a γ-secretase cleavage of this carboxyl-terminal fragment of βAPP should directly generate a βA4-like peptide independent of a β-secretase cleavage (17). The βAPP100-FLAG substrate used in this study contains the last 100 amino acid residues of βAPP751, starting at Met\(^{6}\) at the amino terminus of βA4 and continuing to the carboxyl terminus of βAPP751. Previous cell culture results showed that the expression of a similar fragment in COS cells generates large amounts of soluble βA4, indicating that this βAPP fragment contains all the requisite information for a γ-secretase cleavage (18, 25). In addition, the final βAPP100-FLAG substrate contains an 8-amino acid FLAG epitope at the carboxyl terminus to facilitate the purification of this recombinant protein.

Pure βAPP100-FLAG was digested with human liver cathepsin D at an enzyme to substrate molar ratio of 1:30 for 2 h, at 37 °C. As with the FLG-bAPP156-bFGF substrate, these conditions prevented extensive aggregation and precipitation of the substrate prior to digestion. The 2-h time point was again chosen for further analysis, since the major products were in the most abundance at this time. The digest was separated by


RP-HPLC on a capillary LC system. The resulting chromatogram is shown in Fig. 6. The improved resolution and sensitivity of this system allowed for the detection and collection of 15 major product peaks which were individually collected for both amino acid sequence and electrospray MS analysis. The results are summarized in Table III.

Reliable amino-terminal sequences were obtained for 12 of the 15 fractions collected. While most fractions showed one primary sequence, fractions eluting late in the gradient possessed multiple sequences; the two most abundant sequences are shown. To fully characterize the fragments present in each fraction, electrospray MS analysis was also performed, resulting in MH + masses for 10 of the 15 fractions collected. Single mass values were obtained for fractions 1–11 (no MS data was obtained for fractions 1, 4, 6, 12, and 15), while fractions 13 and 14 contained multiple fragments as detected by MS analysis. Source collision-induced dissociation for many of these fragments helped to definitively identify the fragments present in each fraction. The amino acid sequences obtained by MS analysis were in good agreement with those obtained by direct sequencing and taken together, provided a complete identification of the βAPP100 residues comprising each fragment (Table III). These fragments can be categorized according to their carboxy-terminal residues. By doing so, four groups of fragments were observed, ending with either Phe19, Asp68, Phe93, or Lys107. These related fragments were thus formed by proteolytic cleavages at three primary sites within APP100-FLAG (the Lys107-containing fragments originated from the preexisting carboxyl terminus of βAPP100-FLAG). The heterogeneity observed at the amino-terminal end of these carboxy-terminally related fragments indicates that minor cleavages adjacent to or near the primary these cleavage sites occurred. Together, these cleavages identify specific regions of βAPP that are particularly susceptible to cathepsin D cleavage. Based on the amino-terminal residue of the fragment present in fraction 8, one additional primary proteolytic cleavage site was identified at Leu49–Val50, located 7–9 amino acid residues downstream of the BA4 domain.

The major cleavage sites found within βAPP100-FLAG were compared to those identified for FLAG-βAPP156-bFGF and were in good agreement (Fig. 4), despite the fact that, in contrast to the Ac-Orn-GGVVIATI-Orn-NH2 peptide substrate, neither the Ala42–Thr43 nor the Thr43–Val44 sites were cleaved in the recombinant protein substrates. Three major cleavage sites identified in βAPP100-FLAG (Leu49–Val50, Asp68–Ala69, and Phe93–Phe94) were present in FLAG-βAPP156-bFGF. However, the cleavage site observed at Phe69–Phe70 within the BA4 domain of βAPP100-FLAG was not detected for FLAG-βAPP156-bFGF. It is possible that, in FLAG-βAPP156-bFGF, this site is either inaccessible to cathepsin D, due to a conformational difference between the two substrates, or is exposed and cleaved, but the resulting products were not present in great enough abundance for detection and analysis.

The majority of the cathepsin D cleavage sites identified include a hydrophobic amino acid residue at the P-1 position. This is consistent with the known specificity of cathepsin D for such amino acids (39). In addition, cleavages frequently occurred on the carboxyl side of acidic residues. This is not surprising since, under the acidic conditions used in these assays, acidic side chains may be protonated and uncharged, rendering them better targets for cathepsin D recognition. Although other potential cathepsin D cleavage sites exist in both FLAG-βAPP156-bFGF and βAPP100-FLAG substrates, cleavages at these sites were not observed, again suggesting that the structure of the native substrate molecule is important in dictating preferred cleavage sites of βAPP.

**DISCUSSION**

The γ-secretase cleavage site located at the carboxyl terminus of the BA4 domain is situated within the transmembrane domain of the βAPP molecule. Because of the high degree of hydrophobicity associated with synthetic peptides that span this region, the design of reliable in vitro assays has been
difficult and has required alternative methods for handling hydrophobic substrates (42, 43). Despite these challenges, studies have identified protease activities that can cleave peptide substrates spanning the γ-secretase site of βAPP. Recently, an endogenous aspartic acid protease activity was observed in human brain homogenates that cleaved a synthetic peptide spanning residues 711–716 of βAPP770 at Ala 42 and at Thr 43. This activity closely resembled that of purified cathepsin D (43). A similar observation was reported using an immobilized synthetic peptide spanning a larger region around the γ-secretase site (42). The ability of cathepsin D to form potentially amyloidogenic carboxyl-terminal fragments from a full-length βAPP695 precursor protein (44) and to further proteolytically cleave amyloidogenic carboxyl-terminal βAPP fragments (42) provides evidence that this enzyme plays an important role in βAPP processing. Based on the known specificity of cathepsin D toward hydrophobic amino acids (39), the fact that cathepsin D is an abundant aspartic protease in human brain tissue (30) and that membrane associated forms of cathepsin D are known to exist (37, 38), this enzyme is ideally suited for the proteolytic attack of membrane-bound proteins such as βAPP. Hence, it is a strong candidate for γ-secretase. We decided to investigate in greater detail the role of cathepsin D as a potential γ-secretase by using both synthetic peptides and native protein substrates that span the γ-secretase site of βAPP. Improved methods for the handling and analysis of hydrophobic peptides and proteins lead to the identification of precise cathepsin D cleavage sites within the βAPP molecule.

We first used a synthetic peptide (Ac-Orn-GGVVIAVTVI-Orn-NH2) to determine if cathepsin D can cleave a sequence of amino acid residues that spans the putative γ-secretase site. The primary cathepsin D cleavage sites within this peptide were found to be carboxyl-terminal to Ala 42 (55% of total digestion) and Thr 43 (5% of total digestion) while cleavage at Val 40 was not observed. Thus, consistent with the earlier studies described above (42, 43), cathepsin D is capable of cleaving a synthetic peptide substrate at biologically relevant sites corresponding to the longer (1–42 and 1–43) and potentially more pathogenic forms of βA4 (3). Digestion with either human or bovine cathepsin D gave identical results, indicating that the cleavage specificity of this enzyme is conserved across species. Caution must be displayed, however, when interpreting these results to mean that cathepsin D is γ-secretase since the Ac-Orn-GGVVIAVTVI-Orn-NH2 peptide used in these studies contains flanking ornithine residues that could influence cathepsin D cleavage. Furthermore, as with other synthetic peptides, a short sequence of amino acids may lack many of the structural features of the natural βAPP substrate that might be important in directing protease cleavage. This concern was addressed by using recombinant βAPP proteins as substrates for cathepsin D.

Two recombinant βAPP substrates were used in this study. The longer of the two forms, FLAG-βAPP156-bFGF, consists of the last 156 amino acids of βAPP and contains a portion of the extracellular domain in addition to the entire βA4 sequence, the transmembrane domain and the cytoplasmic carboxyl-terminal domain. The second βAPP substrate, βAPP100-FLAG is shorter and only consists of the carboxyl-terminal 100 amino acids of βAPP, starting at Met41 of the βA4 domain. Since the latter substrate has a preformed βA4 amino terminus, an authentic γ-secretase cleavage at the carboxyl terminus of the βA4 domain would directly generate βA4 peptide species identical to those identified in vitro and in vivo, i.e. terminating at positions 39–44. Previous results from this laboratory showed that βA4 is efficiently produced by cells expressing a similar amino-terminally truncated βAPP protein indicating that βAPP100-FLAG possesses all the requisite cleavage sites for βA4 production (18). Unlike the Ac-Orn-GGVVIAVTVI-Orn-NH2 peptide substrate described above, neither FLAG-βAPP156-bFGF nor βAPP100-FLAG was cleaved by cathepsin D directly at putative γ-secretase sites. Thus, in cleaving βAPP protein substrates, cathepsin D does not display a γ-secretase activity. It is plausible that
the flanking ornithine residues present in the synthetic peptide, but not in the recombinant substrates, impart an amino acid sequence that is readily recognized by cathepsin D. Alternatively, structural features of the longer βAPP substrates prevent cathepsin D from recognizing or binding to the putative γ-secretase site. Additional studies are required to prove either concept.

Although cathepsin D failed to show authentic γ-secretase activity on recombinant βAPP substrates (as defined by cleavage at positions 39–44), both FLAG-βAPP156-bFGF and βAPP100-FLAG were readily cleaved by this enzyme at other sites along the sequence including the Phe19–Phe20 site of βAPP100-FLAG located within the βA4 domain. These major cleavage sites combined with adjacent minor cleavage sites, localized specific regions of βAPP that were particularly susceptible to cathepsin D cleavage. The cleavage of βAPP at these sites suggests that cathepsin D might alternatively be involved in the catabolism of βAPP or βA4 (17, 18). The Leu49–Val50 cleavage site located within the putative transmembrane domain, and the Asp68–Ala69 and the Phe93–Phe94 cleavage sites located within the cytoplasmic domain of βAPP were found to be preferred cathepsin D cleavage sites in both recombinant protein substrates.

The similarity in cleavage patterns between FLAG-βAPP156-bFGF and βAPP100-FLAG indicates that cathepsin D cleavage on the carboxyl-terminal side of the βA4 domain is not influenced by the presence of amino acid residues upstream of the βA4 domain. Of the three cathepsin D cleavage sites common to both FLAG-βAPP156-bFGF and βAPP100-FLAG, the Leu49–Val50 is of particular interest since this site is located 7 amino acid residues downstream of the carboxyl terminus of βA4. It is conceivable that cathepsin D might play a role in the generation of βA4 by initially processing βAPP at this downstream site (contingent on prior removal/exposure of this βAPP site from within the membrane), followed by a secondary processing event(s) to liberate the final collection of carboxyl terminus A4 by initially processing βAPP at this downstream site (contingent on prior removal/exposure of this βAPP site from within the membrane), followed by a secondary processing event(s) to liberate the final collection of carboxyl terminus A4 ending at positions 39–44. Such a secondary processing event might involve a carboxypeptidase as discussed previously (46, 47). Whether βA4 (1–40) or (1–42) is ultimately produced might then be regulated by the extent of carboxypeptidase processing (46, 47).

The inability of cathepsin D to cleave βAPP at other potential cathepsin D (hydrophobic) sites suggests that the structural configuration of the substrate influences and/or defines susceptibility to proteolytic cleavage. Evidence for this was obtained by digesting urea-denatured FLAG-βAPP156-bFGF with cathepsin D. In addition to three of the cleavage sites common to both βAPP156 and βAPP100 identified above, urea denatured βAPP156 was cleaved at two additional sites near the amino terminus of the βA4 domain, Glu3–Val2 and Glu3–Phe4. The Glu3–Val2 cleavage is 3 amino acids amino-terminal to the Asp3 of βA4, while the Glu3–Phe4 cleavage is 2 amino acids carboxy-terminal to Asp3. Cleavages at these sites suggest that βAPP is folded in such a way that the amino terminus of βA4, under native conditions, is sterically/structurally unavailable to cathepsin D cleavage. Upon denaturation, this portion of βAPP may become more susceptible to proteolytic cleavage.

The Glu3–Phe4 amino-terminal cleavage is consistent with reports that describe forms of βA4 beginning with Phe4 observed to be produced by cultured cells (48, 49). In addition, the Glu3–Val2 cleavage was previously identified using a βA4 peptide substrate that spans residues Ile5–Asp7 (N-dansyl-βAPP(591–601)-amide) (44). This cleavage is consistent with a minor form of βA4 produced by cultured cells, the amino-terminal of which begins with Val2 (21). Thus, the Glu3–Phe4 and the Glu3–Val2 cleavages suggest that cathepsin D may function as a β-secretase. Actual in vitro β-secretase activity by cathepsin D would depend on enzyme:substrate accessibility and on favorable intracellular conditions for cathepsin D activity. The recent finding that cathepsin D is capable of appropriately cleaving peptide substrates containing the Swedish KM-NL mutation is in support of the potential role of this enzyme as a β-secretase (50), although it remains to be seen whether β-secretase cleavage involves a single or multiple proteolytic activities.

βAPP contains a type 1 transmembrane domain and as such is an integral membrane protein. Since the γ-secretase site is located within the transmembrane domain, it must normally be situated within a phospholipid bilayer and hence, protected from proteolytic cleavage. In order for γ-secretase to cleave at this site, it must first become accessible to the enzyme. A number of mechanisms have been proposed to explain how a γ-secretase cleavage may occur in βAPP when this site is buried within the transmembrane domain. These mechanisms include membrane damage or degradation, a shorter transmembrane domain, allowing exposure to cleavage (51), or a less rigid and more permeable membrane (52). All mechanisms potentially explain how the γ-secretase site may become accessible to proteolytic cleavage. For mechanisms not involving membrane damage, what role, if any, the intact membrane has on directing proteolytic cleavage once the γ-secretase site becomes exposed is unknown.

In summary, this study has found that, although cathepsin D possesses many of the features expected of a γ-secretase, this enzyme does not show a precise γ-secretase activity on recombinant βAPP substrates in vitro. Thus, it is unlikely that cathepsin D is a γ-secretase candidate. Our conclusion is supported by results with cathepsin D "knock-out" mice whose cultured neurons efficiently produce βA4. The ability of cathepsin D to readily cleave βAPP at other "nonamyloidogenic" sites, suggests that it might alternatively play a role in βAPP and/or βA4 catabolism. The in vitro assays developed in this report will help in the characterization of other known and novel brain proteases that may be involved in the amyloidogenic processing of βAPP. The identification of the authentic βAPP processing enzyme(s) will pave the way toward the development of therapeutic inhibitors to prevent the formation of βA4.

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