Candidate γ-Secretases in the Generation of the Carboxyl Terminus of the Alzheimer's Disease βA4 Amyloid: Possible Involvement of Cathepsin D†

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ABSTRACT: βA4 amyloid peptide, the main constituent of amyloid plaques and cerebrovascular amyloid deposits associated with Alzheimer's disease, derives from a large precursor protein (APP) by the action of β- and γ-secretases, the unidentified endoproteases which release the amino and carboxyl termini of βA4, respectively. Several γ-secretase cleavage sites exist which yield the more soluble (1−39/40) forms of βA4 and the longer forms (1−42/43) which have a greater tendency to aggregate into amyloid plaques. γ-Secretase activity may therefore be critical in amyloid formation. In this study, a synthetic peptide which encompasses the various γ-secretase cleavage sites was used as a substrate to probe proteases of various classes and specificities. Elastase, collagenase, and cathepsin D cleaved at the amyloidogenic sites (after Ala^{42} or after Thr^{43}) to release the carboxyl termini of the aggregating forms. In addition, collagenase and pepsin released the carboxyl terminus of the more soluble forms. Human brain fractions enriched in lysosomes contained a proteolytic activity that cleaved the substrate at the amyloidogenic site(s). This activity was more active at acidic pH and was inhibited by pepstatin, two characteristics of the lysosomal aspartyl proteinase cathepsin D. The same lysosomal fractions were found to contain APP carboxyl-terminal fragments which are potentially amyloidogenic. These were degraded, only in acidic conditions, by an endogenous protease activity inhibited by pepstatin. Thus, a cathepsin D-like activity from human brain is a candidate for APP γ-secretase(s).

The principal constituent of Alzheimer's disease amyloid plaques is βA4,[1] a 4.5 kDa protein which is derived by proteolytic cleavage from a larger precursor, the amyloid precursor protein (APP) (Kang et al., 1987). APP is a member of a family of integral membrane proteins with many of the features of cell-surface receptors (Kang et al., 1987; Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988; König et al., 1992). APP is encoded by a single gene which is subject to alternate splicing of three exons: exon 7, a Kunitz-serine protease inhibitor domain; exon 8, an OX-2-related domain; and exon 15, a juxta-membranous domain which may control a glycosyltransferase acceptor site (Thinakaran & Sisodia, 1994; Hartmann and Beyreuther, unpublished observations). The βA4 domain encompasses the carboxyl terminus of the extracellular domain and part of the transmembrane domain.

The proteolytic enzymes which excise βA4 from APP are yet to be defined (for a review see Evin et al., 1994). A large proportion of APP undergoes constitutive secretion by cleavage within the βA4 domain (Sisodia et al., 1990; Esch et al., 1990) by a so-called α-secretase, although a minor proportion of APP can be released from the membrane by cleavage at or near the βA4 amino terminus by a protease activity termed β-secretase (Seubert et al., 1993). Numerous studies using different cell systems provide conflicting conclusions as to whether β-secretase is part of the normal secretory pathway or of the recycling/degradative pathway. Targeting of APP to the β-secretase pathway may be regulated by several factors such as genetic mutations, increased synthesis of APP, or aberrant expression of APP isoforms (Zhong et al., 1994).

Little is known about the proteolytic activity that releases the βA4 carboxyl terminus by cleaving the transmembrane domain (referred to as γ-secretase). There is some evidence that cleavage by the γ-secretase might be a critical event in amyloid formation. The secretion by normal cells (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992) and the presence of βA4 in the cerebrospinal fluid of healthy individuals, not afflicted by Alzheimer's disease (Seubert et al., 1992), signify that βA4 production is not of itself a pathological event. However, there are structural differences between the forms of βA4 which are found in the amyloid plaque cores and those which are normally secreted. The carboxyl terminus of the secreted peptide is Val^{40} (Seubert et al., 1992), whereas the amyloid plaque core constituent is two, or sometimes three or four residues longer, ending at

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[1] Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Ac-12, acetyl-Tyr-Gly-Val-Val-Ile-Ala-Thr-Val-Ile-Val-Ile-Thr; βA4, the amyloidogenic cleavage product from APP; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; E-64, *trans*-epoxysuccinyl-L-leucylamido-4-guanidinobutane; PHT, 1,10-phenanthroline; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography; TPCK, *p*-tosyl-L-phenylalanine chloromethyl ketone; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Tris, tris(hydroxymethyl)aminomethane.

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Ala[42], Thr[43], or Val[44] (Masters et al., 1985; Miller et al., 1993; Wisniewski et al., 1994). The presence of these extra residues at the carboxyl end of βA4 appears to be a determining factor for β-sheet formation and aggregation (Burdick et al., 1992). The release of the longer peptides might prove to be characteristic of Alzheimer's disease and represent a dysfunction of γ-secretase or processing by a different proteolytic pathway. Some pathogenic mutations in the APP gene (Goate et al., 1991; Chartier-Harlin et al., 1991; Murrell et al., 1991) occur near the γ-secretase cleavage site (at codon 717, using APP_{770} nomenclature; position 46, using the numbering of the βA4 sequence). It has recently been shown that these mutations alter γ-secretase cleavage (Suzuki et al., 1994). However, it remains unclear whether the pathogenic mutations alter APP insertion in the phospholipid bilayer and thereby direct APP toward an amyloidogenic pathway, or whether they interfere directly in the cleavage process itself by changing the specificity of the γ-secretase substrate and rendering a minor secondary cleavage more efficient. Although γ-secretase cleavage occurs in the transmembrane region of APP, it is still unclear whether cleavage occurs within the membrane or after dissociation from the membrane. There is evidence that γ-secretase cleavage may occur in the endocytic compartment in transfected CHO cells which express APP_{751} (Koo & Squazzo, 1994), but other data obtained with COS-1 cells transfected with an APP_{695} construct suggest that βA4 may be released in a secretory pathway (Busciglio et al., 1993), which would imply the presence of γ-secretase in this compartment. These contradictory results might signify the existence of several γ-secretase activities which operate in alternative pathways. They might also imply that APP proteolytic processing varies with cell lines and transfection protocols.

In an attempt to characterize γ-secretase, we have studied how proteases of various classes and specificities process synthetic peptides homologous to the γ-secretase cleavage site as models of APP processing. We have also studied the endogenous proteases of human brain fractions for their ability to generate the carboxyl terminus of βA4 amyloid.

MATERIALS AND METHODS

Materials. Amino acid derivatives and peptide synthesis reagents were from Auspep (West Melbourne, Victoria, Australia) and from Pharmacia-LKB (Uppsala, Sweden). Affi-Gel 102 was obtained from Bio-Rad (Richmond, CA) and Sep-Pak cartridges from Millipore (Bedford, MA). 1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide reagent, pepsin (from porcine stomach, 1:10 000), collagenase (EC 3.4.24.8, high purity, from *Achromobacter iophagus*), elastase (type IV, from porcine pancreas), and cathepsin D (from bovine spleen) were from Sigma-Aldrich (Castle Hill, NSW, Australia), papain was from Boehringer (Boehringer-Mannheim, Germany), and α-chymotrypsin (from bovine pancreas) was from Calbiochem-Novabiochem (Alexandria, NSW, Australia).

Synthesis of Peptides and Ac-12 Substrate Gel. A peptide, Ac-Tyr-Gly-Val-Val-Ile-Ala-Thr-Val-Ile-Val-Ile-Thr (Ac-12), which corresponds to residues 709−719 of APP_{770}, the analog Ac-Tyr-Gly-Val-Val-Ile-Ala-Thr-Val-Ile-Ile-Ile-Thr, and a series of standard peptides, Ac-Tyr-Gly, Ac-Tyr-Gly-Val, Ac-Tyr-Gly-Val-Val, Ac-Tyr-Gly-Val-Val-Ile, and Ac-Tyr-Gly-Val-Val-Ile-Val, were synthesized manually, as reported elsewhere (Evin et al., 1995). Iodination was carried out by the chloramine-T method (Hunter & Greenwood, 1962). Typically, 10 μg aliquots of peptide, suspended in 0.1 M phosphate buffer, pH 7.4, were labeled with 0.5 mCi of $Na^{125}I$. Peptide tracers were purified by extraction on C-18 Sep-Pak cartridges and elution by 75% acetonitrile in 1% trifluoroacetic acid. ^{125}I-labeled peptides (0.2 mCi) were conjugated to 10 mL of Affi-Gel 102 for 48 h, at 20 °C, using an excess of 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide. The gel was extensively washed with 0.1% (v/v) Triton X-100, and then with 0.05 M Tris-HCl, pH 7.4, and stored at 4 °C.

Assay for Proteolytic Activity. Pepsin and cathepsin D were assayed in 0.1 M citrate buffer, pH 3.2. Elastase and papain were assayed in 0.1 M Tris-HCl buffer, pH 8.0. Collagenase activity was assayed in 50 mM Tricine buffer, pH 7.4, containing 10 mM $CaCl_2$, 200 mM NaCl, and 50 μM $ZnCl_2$.

For each assay, 50 μL aliquots of Ac-12 substrate gel were distributed in 1.5 mL microfuge tubes, washed with 1 mL of assay buffer (0.1 M, Tris-HCl, pH 7.4), and pelleted by centrifugation, and the buffer was discarded. This procedure was repeated three times or until radioactivity above background was not found in the wash buffer. The gel pellets were resuspended in 200 μL of assay buffer before adding 200 μL of enzyme dilution, or brain homogenate fraction. The incubation was allowed to proceed for various periods of time (1−18 h), at 37 °C, with rocking or rotary shaking. The total radioactivity in each incubation was counted, then the tubes were centrifuged (10000*g* for 5 min), and a 200 μL aliquot of each supernatant fraction was counted. Ratios of released versus total counts were calculated, and a nonspecific value corresponding to a mean of incubations where brain homogenate was replaced with assay buffer was subtracted. All incubations were in duplicate, and each assay was repeated at least three times.

Reverse-Phase HPLC and TLC. The peptide products were separated on a Brownlee Aquapore RP-300 column (4.6 × 100 mm) equipped with a Brownlee New Guard C-8 cartridge (3.2 × 15 mm) using Applied Biosystems HPLC equipment (Applied Biosystems−Perkin Elmer, Burwood, Australia). A linear gradient of 0−60% acetonitrile in 0.1% trifluoroacetic acid was developed over 40 min, at a flow rate of 1 mL/min. Elution was monitored for absorbance at 214 nm. Peptides were identified by comparing their retention times with peptide standards. For TLC separation, enzyme incubation mixtures were dried and redissolved in 5−20 μL of 75% acetonitrile in 1% trifluoroacetic acid. Samples and standards (1−2 μL; about 1000 cpm) were loaded onto RP-8 plates (Merck, Darmstadt, Germany) which were developed with 38% 2-propanol in 1% trifluoroacetic acid. The plates were dried, sealed in plastic bags, exposed to a BAS-IIIS imaging plate for 1−2 h, and analyzed using a BAS-1000 FUJIX phosphorimager (Fuji, Japan).

Subcellular Fractionation of Brain Homogenates. All steps were performed on ice, and all centrifugations were carried out at 4 °C, in a Sorvall RC-5B centrifuge equipped with a Sorvall SS-34 rotor (Dupont, Wilmington, DE). In a typical preparation, a piece of human brain cortex (8.2 g) was dissected to remove most of the white matter and blood vessels. The grey matter was minced in 40 mL of 0.32 M sucrose buffer, pH 7.0, containing 1 mM EDTA (buffer A),

homogenized manually with a Dounce homogenizer, and then centrifuged at 270*g* for 5 min, to remove nuclei and cellular debris. The supernatant was centrifuged at 3000*g* for 10 min, and the pellet was resuspended in 20 mL of buffer A and centrifuged at 480*g* for 10 min to remove more cellular debris. Pellet resuspension and washing were repeated three times. At the last time, the resuspended pellet was centrifuged at 3000*g* for 10 min. The washed pellet was resuspended in 2 mL of buffer A, layered onto 35 mL of 27% Percoll (Pharmacia-LKB, Uppsala, Sweden) in buffer A, and centrifuged at 48000*g* for 1 h. (Percoll was made isotonic by 9/10 dilution with 2.5 M sucrose, containing 10 mM EDTA, and then diluted to 27% with buffer A.) The brownish lysosome-rich fraction was separated from a fraction of lower density which contained membrane vesicles, myelin debris, and synaptosomes. The 3000*g* supernatant was centrifuged at 20000*g* for 1 h. The pellet was rich in plasma membrane vesicles and is referred to as plasma membrane fraction, while the supernatant is referred to as cytosolic fraction.

For characterization by electron microscopy, samples were fixed overnight, at 4 °C, by addition of 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Samples were washed in the same buffer and further fixed in 1% OsO_4 for 1 h, at room temperature, followed by washing with water, dehydration in acetone, and embedding in Epon-Araldite epoxy resin (50:50). Thin sections were stained with uranyl acetate and lead citrate and examined in a Siemens 102 electron microscope.

Western Blots. Tris−glycine and Tris−Tricine SDS−polyacrylamide gels were prepared and used in a Bio-Rad Mini Protean II electrophoresis apparatus (Bio-Rad Laboratories, North Ryde, NSW, Australia). Samples were boiled for 5 min in Laemmli SDS sample buffer prior to loading. The Tris−glycine gels were run at 50 mA for 40 min and the Tris−Tricine gels at 40 mA for 150 min. Proteins were transferred onto 0.2 μm nitrocellulose ProBlot membrane (Bio-Rad) overnight, at a constant 150 mA current. Membranes were blocked for 1 h with 3% BSA in Tris-HCl (0.1 M), pH 7.4, containing NaCl (0.25 M), and then incubated with antibodies 6E10 (monoclonal anti-βA4 1−16 antibody, purchased from the Institute for Basic Research in Developmental Disabilities, New York), 369 (rabbit polyclonal anti-APP cytoplasmic domain, kindly provided by Dr. S. Gandy, Cornell University Medical College, New York, NY), or 93/3 (a rabbit polyclonal antibody against the sequence 751−770 of APP_{770} raised in our laboratories, using a keyhole limpet hemocyanin conjugate of the corresponding synthetic peptide). Detection was performed using a secondary antibody conjugated to alkaline phosphatase (Promega, Madison, WI) and Fast-Red/naphthol AS-MX phosphate as substrate. Molecular weights were calculated from calibration curves obtained with molecular weight standards (Rainbow Markers from Amersham Australia, North Ryde, NSW, Australia).

Digestion of a Recombinant APP Protein. C-97, a protein that encompasses residues 672−770 of APP, was produced in yeast. The *Eco*RI fragment from APP_{695} (codons 1795−2851, according to Kang et al., 1987) was cloned into the *Eco*RI site of the *Pichia pastoris* expression vector pHILSI (Invitrogen, San Diego, CA). Plasmid DNA was prepared and linearized with *Bgl*II. Spheroplasts of *P. pastoris* strain GS115 were prepared and transformed with the above DNA (Cregg et al., 1985). Clones expressing C-97 were identified by growing cultures in shaker flasks and preparing cell extracts (Sreekrishna et al., 1989). C-97 protein was characterized by Western blotting, as described in the Results section. Unpurified yeast lysate was incubated at 37 °C, for 1−18 h, in the presence of bovine cathepsin D (in 0.1 M citrate, pH 4.0, or in 0.1 M phosphate, pH 6.0) or porcine pancreatic elastase (in 0.1 M Tris-HCl, pH 8.0). Enzymatic digestion was stopped by adding Laemmli SDS sample buffer and boiling for 5 min. The digests were analyzed by Western blotting.

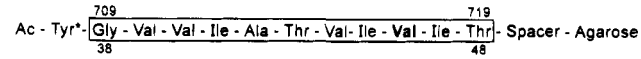

FIGURE 1: Structure of Ac-12 substrate. Ac-12 substrate is a peptide fragment of APP with the given sequence, which spans the γ-secretase cleavage site(s). The peptide is acetylated at its amino terminus (indicated by Ac), radiolabeled (as indicated with *) by 125-iodination of the Tyr residue, and it is coupled at its carboxyl terminus to agarose through a six-carbon spacer. Peptide numbering is according to APP_{770} (upper line) and to βA4 sequences (lower line). The residue which is mutated in some AD families (codon 717 mutation) is shown in bold.

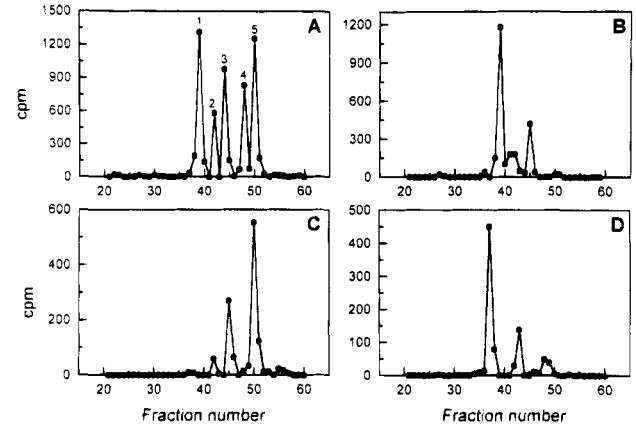

FIGURE 2: HPLC analysis of Ac-12 substrate digests. Panel A shows separation of standard ^{125}I-labeled peptides which represent Ac-12 digestion products, on a C-18 reverse-phase column developed with a 0−100% gradient of acetonitrile in 0.1% trifluoroacetic acid in 50 min, at a flow rate of 1 mL/min. 0.5 mL fractions were collected and counted for radioactivity. 1: Ac-Tyr-Gly; 2: Ac-Tyr-Gly-Val; 3: Ac-Tyr-Gly-Val-Val; 4: Ac-Tyr-Gly-Val-Val-Ile; 5: Ac-Tyr-Gly-Val-Val-Ile-Ala. Panels B−D show analysis in the same conditions of Ac-12 substrate digests produced by pepsin (B), elastase (C), and collagenase (D).

RESULTS

Digestion of a Synthetic Peptide Model, Ac-12, by Selected Proteases. The substrate Ac-12 was prepared using a synthetic peptide which encompasses residues 709−719 of APP_{770}, spanning the γ-secretase cleavage sites (Figure 1). The peptide also contains residue 717 found to be mutated in some AD families. To circumvent problems inherent to the poor solubility of the peptide in aqueous buffers, a solid-phase radiometric assay was developed in which the peptide, after N-acetylation and ^{125}I-labeling, was conjugated to agarose through its carboxyl terminus via a C_6 spacer. Substrate digestion was monitored by the release of radioactivity from the gel. The proteolytic products were separated by reverse-phase HPLC or TLC and compared with peptide standards, as previously described (Evin et al., 1995).

We studied the digestion of Ac-12 by selected proteases which are known to cleave preferentially hydrophobic and aliphatic amino acid sequences (Figure 2 and Table 1). The

Table 1: Quantitation (%) of Ac-12 Substrate Cleavage Resulting from Digestion by Various Proteases, As Determined by HPLC Analysis of the Digest Products on a Reverse-Phase C-18 Column

protease	Gly^{38} ↑	Val^{39} ↑	Val^{40} ↑	Ala^{42}/Thr^{43} ↑
chymotrypsin (10 μg/mL)	100	0	0	0
papain (0.15 unit/mL)	100	0	0	0
pepsin (10 μg/mL)	60.2	18.4	21.4	0
collagenase-like activity (200 μg/mL)	68.1	0	20.9	10.9
elastase (100 μg/mL)	0	12.0	30.6	57.4
cathepsin D (200 μg/mL)	0	33.9	0	66.1

cleavage site preferred by most proteases was before Val^{39}, at the site most remote from the solid support, and this seems to be mainly due to the design of the substrate itself. We also found that when a large excess of protease was used or when incubation times were prolonged, the dipeptide was the only product detected as it is likely to be the ultimate degradation product of the various peptides released from the gel. Cleavages of the substrate at the γ-secretase sites were observed (Figure 2 and Table 1). The main cleavage site of elastase was at the Ala^{42}-Thr^{43} peptide bond, with a secondary cleavage at Val^{40}-Ile^{41} (Figure 2C). The main cleavage site of collagenase was at Val^{39}-Val^{40}, with secondary cleavages at Val^{40}-Ile^{41} and Ala^{42}-Thr^{43} (Figure 2D). Pepsin gave three products resulting from cleavages at Gly^{38}-Val^{39}, Val^{40}-Ile^{41}, and Val^{39}-Val^{40} (Figure 2B). Another aspartyl protease, cathepsin D, gave two products, a main one with similar chromatographic mobility as the standard Ac-YGVVIA and the minor product that corresponds to cleavage at Val^{39}-Val^{40}. Chymotrypsin and papain only cleaved Ac-12 at Gly^{38}-Val^{39}, before the γ-secretase cleavage sites. Thus, among the proteases used for this study, proteases of three classes, i.e., the serine protease elastase, the aspartyl protease cathepsin D, and the metalloprotease collagenase, were able to cleave the substrate at a site corresponding to the carboxyl terminus of the aggregating form of βA4. The proteases able to cleave the substrate at the site corresponding to the carboxyl terminus of the secreted more soluble form of βA4 are elastase, collagenase, and pepsin. In addition, collagenase, elastase, pepsin, and cathepsin D could also cleave at the site corresponding to the carboxyl terminus of the form of βA4 reported to be present in cerebrovascular amyloid deposits.

Digestion of Ac-12 Peptide Ile^{717} Mutant Analog by Various Proteases. A peptide analog to Ac-12 which carries the FAD mutation Ile^{717} (instead of Val in the wild type) was digested by elastase, collagenase, pepsin, chymotrypsin, and cathepsin D. The digestion patterns were very similar to those obtained with the peptide corresponding to APP wild type, and a significant increased production of longer, aggregating peptides was not observed with the mutated form.

Digestion of Ac-12 Peptide by Human Brain Homogenates and Subfractions. When Ac-12 peptide substrate was incubated in the presence of total cytosolic fraction of cortex homogenate (20000*g* centrifugation supernatant), at pH 7.4, the only radioactive product that could be detected and characterized was the amino-terminal dipeptide, possibly due to the presence of numerous proteases in the homogenate, including dipeptidyl aminopeptidases and carboxypeptidases that would degrade peptides released from the gel.

Brain homogenates were subfractionated, and the fractions were incubated with Ac-12 in the presence of various buffers and with different protease inhibitors. Differential centrifugations including Percoll gradient separation yielded a lysosome-rich fraction (Figure 3A) and a fraction rich in plasma membrane vesicles and synaptosomes (Figure 3B), as assessed by electron microscopy. After 1:20 hypotonic dilution to rupture vesicles by osmotic shock, these fractions were assayed with Ac-12 substrate. At pH 5.2, the TLC pattern of the digests (Figure 4A) showed that the lysosome-rich fraction released the dipeptide Ac-YG and a more hydrophobic peptide with mobility similar to the Ac-YGVVIA standard (lane 2), whereas the synaptosome-rich fraction (lane 1) and the cytosolic fraction (lane 3) only released the dipeptide. Release of the hydrophobic product was not detected at pH 7.4, but it was increased at pH 3.2 and was totally inhibited by pepstatin, indicating that the protease involved was an aspartyl protease. The same results were obtained when testing subfractions prepared from four different brains, including two normal controls and two AD. At pH 3.2, both platelet cytosol (Figure 4B, lane 2; prepared according to Li et al., 1994) and brain cytosol (Figure 4B, lane 4) were able to release from Ac-12 a similar hydrophobic peptide by means of a proteolytic activity inhibited by pepstatin (Figure 4B, lanes 3 and 5). The pattern was similar to that obtained from digestion with a commercial preparation of cathepsin D (Figure 4B, lane 1). Thus, it appeared that both brain and human platelets contained a cathepsin D-like activity able to cleave the synthetic substrate at a site that would correspond to the carboxyl terminus of the aggregating form of βA4. The brain cytosolic fraction, when incubated at pH 5.2, showed a single detectable product that corresponded to the Ac-YG standard, and the release of

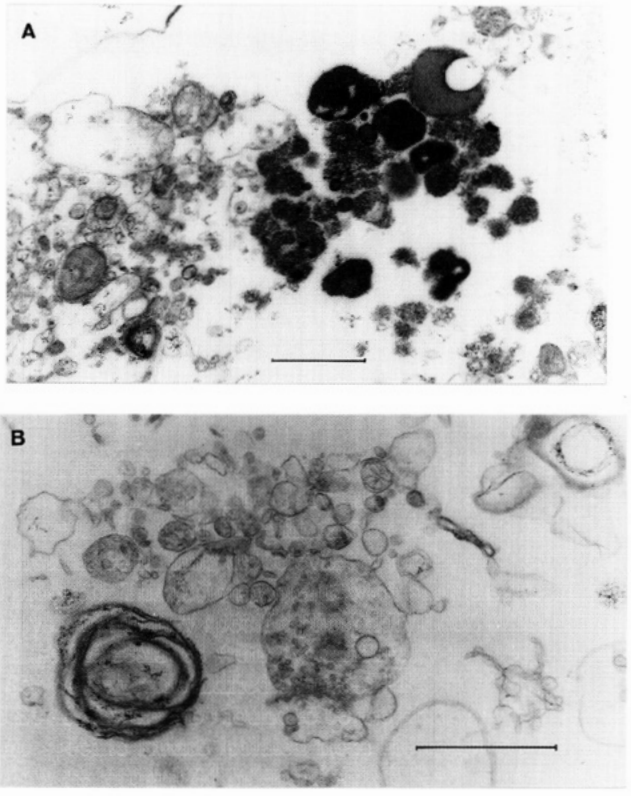

FIGURE 3: Electronmicrographs of subcellular fractions from human brain. Panel A: Lysosomal-rich fraction showing heterogeneous, aggregated lysosomal material and membrane vesicles. Panel B: Lower-density fraction containing assorted membrane vesicles, myelin whorls, and synaptosomes which contain numerous small synaptic vesicles. Bars equal 1 μm.

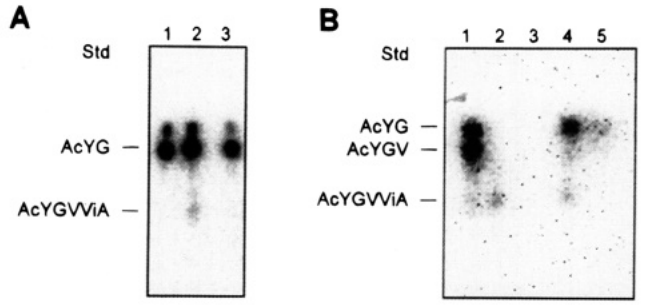

FIGURE 4: TLC analysis of Ac-12 digestion by brain cortex and platelet cytosolic fractions. Panel A shows digestion by brain cortex fractions. Ac-12 substrate aliquots were incubated overnight, at pH 5.2, with subfractions from a control human brain. Peptide products were separated on a C-8 reverse-phase TLC plate (200−1000 cpm/lane) developed with 38% 2-propanol in 1% TFA. Brain subfractions were as follows: lane 1, synaptosome-rich fraction; lane 2, lysosome-rich fraction; lane 3, cytosolic fraction. Panel B shows digestion upon incubation for 2 h at pH 3.2 with commercial bovine cathepsin D (lane 1) or with human platelet cytosol, in the absence or presence of 1 μM pepstatin (lanes 2 and 3, respectively), as compared to incubation with human brain lysosome-rich fraction, in the absence or presence of 1 μM pepstatin (lanes 4 and 5, respectively).

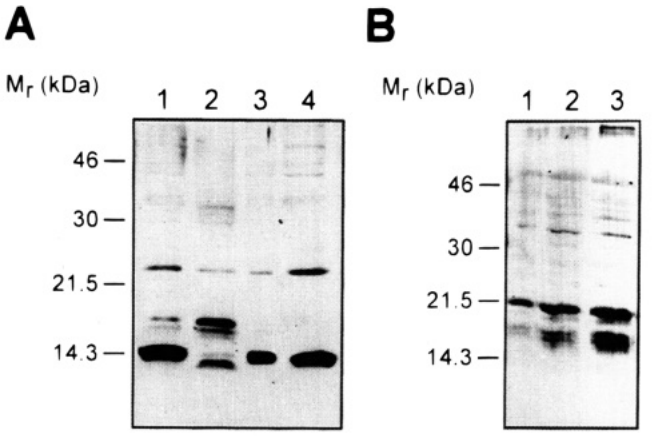

FIGURE 5: Characterization of APP carboxyl-terminal fragments from human brain and processing by endogenous proteases. Immunoblots of 15% SDS−polyacrylamide Tris−glycine gels stained with the anti-APP cytoplasmic domain antiserum 369 (panel A) and the anti-βA4 (1−16) monoclonal antibody 6E10 (panel B). Panel A shows analysis of subcellular fractions from human brain cortex: lane 1, crude lysosomal fraction (total lysate of a 3000*g* pellet prepared by osmotic shock, in the absence of detergent); lane 2, 1% Triton X-100 extract of the 100000*g* pellet obtained from centrifugation of the lysed lysosomal fraction shown in lane 1; lane 3, 100000*g* supernatant of the lysed lysosomal fraction shown in lane 1; lane 4, crude plasma membrane/vesicles fraction lysed by osmotic shock, in the absence of detergent. Panel B shows processing of a crude lysosomal fraction (3000*g*) by endogenous proteases: lane 1, incubation at pH 3.2, for 1 h, in the absence of protease inhibitors; lane 2, incubation at pH 3.2, for 1 h, in the presence of 1 μM pepstatin; lane 3, no incubation.

this digestion product was inhibited by E-64 and by iodoacetamide, two inhibitors of cysteine proteinases (data not shown). This activity in the cytosolic fraction is probably the main lysosomal protease, cathepsin B, which generally cleaves after basic and hydrophobic residues. Indeed, cathepsin B activity was detected in both the lysosomal and the cytosolic fractions using Z-Arg-Arg-4-nitroanilide as a substrate according to Barrett and Kirschke (1981).

Western Blot Analysis of APP Carboxyl-Terminal Fragments and a Study of Their Degradation under Various Conditions. Brain cytosolic and membrane fractions were analyzed by Western blotting using antibody 369, which is directed to the cytoplasmic domain of APP (Buxbaum et al., 1990). A crude lysosomal fraction (3000*g* pellet), after lysis by osmotic shock in the absence of detergent, displayed a set of APP carboxyl-terminal fragments ranging between 24 and 14 kDa (Figure 5A, lane 1). These species were subfractionated into membrane-associated and soluble fractions by ultracentrifugation. The soluble fraction retained some of the 24 kDa species and most of the 14 kDa product (Figure 5A, lane 3). The 24, 17.5, 16, and 13 kDa species were found to be associated with the membrane fraction (Figure 5A, lane 2). A fraction rich in plasma membranes (20000*g* crude pellet obtained by centrifugation of the 3000*g* supernatant) showed only the 24 and 14 kDa bands (Figure 5A, lane 4). Thus, the 17.5 and 16 kDa fragments appear to be characteristic of the lysosomal fraction. These fragments were detected by 6E10 (Kim et al., 1990), an antibody raised against the first 16 amino-terminal residues of βA4 (Figure 5B, lane 3), indicating that they contain the whole βA4 domain and are potentially amyloidogenic. The smaller species were not reactive with 6E10 and thus do not contain intact βA4. The corresponding cytosolic fraction showed the bands at 14−15 kDa as well as a 17.5 kDa species similar to the one observed in the lysosomal fraction (data not shown). These products, particularly the 17.5 kDa species, are likely to be associated with small vesicles which remain in suspension when centrifuging at 20000*g*.

Processing of the potentially amyloidogenic products from the lysosomal and cytosolic fractions by endogenous proteases was studied at various pH conditions and in the presence of protease inhibitors and metal ions. The APP carboxyl-terminal fragments were quite stable at neutral pH (data not shown), but they were rapidly processed at acidic pH by a protease activity that was present in both the crude lysosomal fraction (Figure 5B, lane 1) and the cytosolic fraction. This activity was blocked by pepstatin (Figure 5B, lanes 2), showing that processing was due to an aspartyl protease. The APP carboxyl-terminal fragments were stable, at least for an hour at 37 °C, in all other conditions studied (data not shown).

Digestion of C-97, a Recombinant C-Terminal Fragment of APP, by Cathepsin D. A recombinant protein comprising the last 97 amino acids of APP carboxyl terminus, starting at residue 3 of the βA4 domain, was expressed in the *P. pastoris* yeast system. This would represent a carboxyl terminal fragment of APP which results from cleavage near the β-secretase site, and thus, it constitutes a model to study γ-secretase. Yeast lysates were assayed by Western blot, probing with antibodies against βA4 (6E10: to βA4 1−16) and against the cytoplasmic domain (369: to the whole cytoplasmic domain, and 93/3: to the last 15 carboxyl-terminal residues of APP, data not shown). All three antibodies identified the same major band that corresponded to a product of 15 kDa, when run on a Tris−Tricine polyacrylamide gel. This apparent molecular mass is consistent with that reported by others who have prepared similar APP fragments (Maruyama et al., 1990; Dyrks et al., 1992). It is worth noting that antibody 369 reacted with an additional band of about 13.5 kDa, which is not recognized by 6E10 and might represent a product of digestion by α-secretase (not shown).

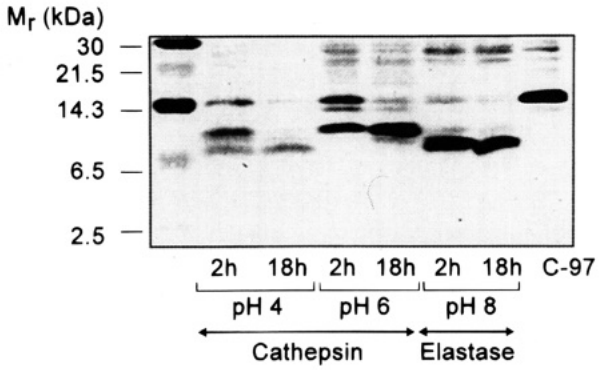

FIGURE 6: Digestion of C-97 APP yeast product by cathepsin D and elastase. Immunoblot of a Tris−Tricine gel (10−15% polyacrylamide) developed with antibody 6E10 (directed to βA4 1−16). C-97 crude protein (3 μg) was digested with cathepsin D (0.028 unit) at pH 4.0 and 6.0, for 2 or 18 h. Incubation with elastase (0.065 unit) was conducted at pH 8.0.

Proteolytic processing of the yeast product by bovine cathepsin D was studied at pH 4.0, which is close to optimal pH for this enzyme's activity, and at pH 6.0, which is closer to the pH of cellular acidic compartments. Antibody 6E10 revealed two products of 10 and 7.5 kDa (Figure 6). The 10 kDa product appeared first and would correspond to removal of about half of the cytoplasmic domain from the carboxyl end. The 7.5 kDa product would have lost most of the cytoplasmic domain. Digestion by elastase released only a 7.5 kDa product of similar electrophoretic mobility as the cathepsin D product. Antibody 369 did not detect any digestion product.

DISCUSSION

Synthetic peptides are often used as substrates for proteinases, but because of low solubility, this approach has rarely been used for APP γ-secretase. Ishiura (1991) used a fluorogenic derivative of a dipeptide which corresponded to residues 41−42 of βA4 (713−715 of APP_{770}) and isolated rat brain prolyl-endopeptidase. Another fluorogenic derivative corresponding to residues 714−716 of APP_{770} was used by Mundy (1994) who isolated, from PC-12 cultured cells, the chymotrypsin-like subunit of the multicatalytic proteinase as a γ-secretase candidate. Ladror et al. (1994) have recently reported that a synthetic peptide derivative which encompasses residues 711−716 of APP_{770} could be digested by a brain acidic proteinase at sites consistent with γ-secretase. For this study, we used a dodecapeptide, Ac-12, which represents residues 709−719 of APP_{770} and which spans several possible cleavage sites for γ-secretase, as well as the sites of mutations corresponding to codons 713 and 717. We analyzed the processing of Ac-12 by purified proteases and by brain homogenates, using a solid-phase radiometric assay.

Aspartyl, metallo-, and serine proteases were able to release the carboxyl terminus of the secreted form of βA4 from the synthetic substrate. The same types of proteases, particularly elastase, and also the aspartyl protease cathepsin D could cleave after Ala^{42} or Thr^{43} to release the carboxyl terminus of βA4 forms which are found in the amyloid plaques. It was interesting to observe that collagenase can also process the synthetic peptide by cleaving at the γ-secretase sites, since collagenases are found in the extracellular matrix: a collagenase activity acting as γ-secretase could be consistent with the extracellular genesis of βA4. Elastase was shown to cleave the synthetic peptide preferentially after Ala^{42}, and γ-secretase cleavage by a brain serine protease of similar specificity would resemble the processing of TGF-α and other membrane-anchored growth factors (Massagué & Pandiella, 1994). Finally, the finding that cathepsin D can release the carboxyl terminus of the aggregating forms of βA4 is in agreement with the results of Ladror et al. (1994) and would be consistent with βA4 being produced in an acidic compartment of the degradation pathway (Koo & Squazzo, 1994).

A difference was not observed in the processing of two peptide analogs carrying either the wild type Val or the mutated Ile at the position of codon 717. Thus, the mutation may not alter the substrate specificity of APP, at least using the proteases and the techniques employed in this study. However, the 717 mutation may alter the specificity toward the authentic secretase. Suzuki et al. (1994) found an increased release of βA4 from cells transfected with an APP 717 mutant, compared to those transfected with APP wild type. As our results suggest, the mutation may not interfere with the γ-secretase-APP interaction, but instead may alter APP insertion in the phospholipid bilayer and/or its intracellular targeting by increasing the amount of APP passing through the "amyloidogenic" pathway, where it would encounter a γ-secretase activity that produces the longer, more aggregating peptides. Other interpretations would be that our synthetic model approach cannot pick up the effect of the mutation, due to embedding of the mutated residue in the Sepharose matrix or due to the lack of peptide secondary structure.

Human brain fractions enriched in lysosomes show a capacity for cleaving the peptide at an amyloidogenic site. The protease involved was most active at acidic pH and totally inhibited by pepstatin, sharing similarities with the aspartyl protease cathepsin D. Interestingly, these fractions contained APP carboxyl-terminal fragments ranging from 13 to 24 kDa apparent molecular mass (which is in agreement with the data from other authors (Gandy et al., 1992) who used the same 369 antibody to characterize carboxyl-terminal fragments in the Sf9 cell line transfected with APP_{751}). The 13−17 kDa set of carboxyl-terminal fragments would correspond to the 15 and 17 kDa bands observed by Estus et al. (1992) in human brain membrane fractions. Immunocharacterization proved that only the larger species (22−16 kDa) contained the whole βA4 peptide, and these were specifically found in the fractions enriched in lysosomes. The finding of APP carboxyl-terminal fragments in soluble fractions has not been reported before, but similar fragments have been found in human platelets (Q.-X. Li, unpublished observations). In addition, full-length APP with an intact carboxyl terminus has recently been described to be released by several cell lines (Ripellino et al., 1994; Bhasin et al., 1994; Conn et al., 1994). The possible release of soluble amyloidogenic APP fragments from the membrane is directly relevant to amyloid formation since βA4 amyloid is mainly found in the extracellular compartment. Discovering how APP and its amyloidogenic fragments are dissociated from the membrane could contribute to understanding βA4 deposition and aggregation.

The APP carboxyl-terminal fragments were degraded in acidic conditions, by an endogenous aspartyl protease, possibly the lysosomal/endosomal cathepsin D. Since we

have shown that cathepsin D can cleave a synthetic peptide at the carboxyl terminus of the amyloidogenic form of βA4, its role as an APP γ-secretase appears possible and consistent with processing of APP carboxyl-terminal fragments by the endocytic pathway (Cole et al., 1989; Knops et al., 1992; Koo & Squazzo, 1994). Thus, we studied how bovine cathepsin D processed a recombinant APP carboxyl-terminal fragment that would correspond to a β-secretase product. The protein was expressed with the yeast secretion signaling system and was thus membrane inserted. Cathepsin D cleaved the recombinant protein, and two products were detected which resulted from cleavage within the cytoplasmic portion. No βA4 peptide was detected, and this could mean that cathepsin D was not able to access to the γ-secretase site which is in the transmembrane region. Meanwhile, when the experiment was repeated after treating the yeast lysate with a detergent (Triton X-100) to dissociate the protein from the membrane, no change in the digestion pattern was observed (data not shown). Considering that at pH 4.0 most of C-97 and its βA4-containing digestion products were degraded, some cleavage had occurred within the βA4 portion, proving that βA4 is sensitive to proteolysis by cathepsin D. Maybe βA4 could not be detected in our system for this reason, and self-association/aggregation or association with another factor is required to stabilize the peptide and induce resistance to degradation (Norstedt et al., 1994).

Cathepsin D has been shown to process full-length APP into small amyloidogenic fragments (Dreyer et al., 1994). This lysosomal aspartyl proteinase is present in all mammalian species and in most tissues, particularly in the brain, and it may be increased in AD neurons (Cataldo et al., 1991). It is found in endosomes as well as lysosomes (Diment & Stahl, 1985). Besides compatibility with the endocytic pathway and the production of βA4 in early endosomes, cathepsin D could also operate as a γ-secretase extracellularly, since it has been found in the extracellular compartment, in association with amyloid plaques (Cataldo et al., 1991, 1994). It is possible that some neuronal lysosomal contents can be released into the extracellular milieu, thereby providing a βA4 release pathway. The major challenge is to demonstrate whether the pathway actually exists *in vitro* and *in vivo*.

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