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Cleavage of Amyloid β Peptide During Constitutive Processing of Its Precursor

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The amyloid β peptide (AβP) is a small fragment of the much larger, broadly distributed amyloid precursor protein (APP). Abundant AβP deposition in the brains of patients with Alzheimer's disease suggests that altered APP processing may represent a key pathogenic event. Direct protein structural analyses showed that constitutive processing in human embryonic kidney 293 cells cleaves APP in the interior of the AβP, thus preventing AβP deposition. A deficiency of this processing event may ultimately prove to be the etiological event in Alzheimer's disease that gives rise to senile plaque formation.

ALZHEIMER'S DISEASE (AD) IS A neurodegenerative disorder characterized by the presence of extracellular senile plaques and intracellular neurofibriliary tangles in the brains of affected individuals. The central core of senile plaques is comprised of the 4-kD AβP, which is a small fragment of the 90- to 130-kD glycosylated, membrane-bound APP (*1–3*). Three forms of APP that contain AβP are derived from a single gene by alternative mRNA splicing. These putative AβP precursors contain 770 (APP_{770}), 751 (APP_{751}), and 695 (APP_{695}) amino acids and differ from one another primarily by the presence (APP_{770} and APP_{751}) or absence (APP_{695}) of a Kunitz-type protease inhibitor insert (*4–6*).

Proteolytic processing of APP results in the secretion of a large (>100 kD), soluble, NH_2-terminal APP fragment (*3, 7*), identified as protease nexin II (*8*) and the generation of an approximately 11-kD membrane-associated COOH-terminal fragment (*2*). Direct microsequencing has shown that the NH_2-terminus of the secreted APP fragment begins with APP(Leu^{18}) (*9, 10*) and immunochemical studies suggest that the COOH-terminus of the membrane-bound COOH-terminal fragment contains all or nearly all of the COOH-terminal APP sequence (*2, 9*). Immunochemical studies have suggested that some or all of the AβP sequence may be present in the secreted NH_2-terminal APP fragment (*11*), but the exact location of the proteolytic cleavage in APP that gives rise to the NH_2- and COOH-terminal APP fragments has not been identified. To answer this question we have purified these fragments from human embryonic 293 cells stably transfected with cDNA constructs encoding full-length forms of APP_{751} and

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Table 1. Structural characterization of the COOH-terminus of the secreted APP_{695} and APP_{751} forms. Each secreted protein (5 nmol) (*16*) was reduced, treated with pyridylethylene, and cleaved with CNBr. The resulting fragments were purified by RPLC (*13*). The COOH-terminal CNBr fragment was identified in each case by amino acid analysis (**A**) and microsequencing (**B**) with a model 420 amino acid analyzer and a model of 477A gas-liquid phase sequencer (Applied Biosystems), respectively. Fab mass spectrometry of each COOH-terminal CNBr digestion fragment yielded protonated molecular mass ions of 1826.72, which are identical with the calculated mass of the predicted COOH-terminal AβP(1–15) peptide. In (A), 25 and 39 pmol of the COOH-terminal CNBr digestion fragments from the secreted forms of APP_{695} and APP_{751}, respectively, were hydrolyzed and analyzed. In (B), 85 and 128 pmol of the COOH-terminal CNBr digestion fragments from the secreted forms of APP_{695} and APP_{751}, respectively, were subjected to microsequence analyses. PTH-AA, phenylthiohydantoin amino acid.

A Amino acid compositions

Amino acid	Amino acid ratios		
	APP_{695}	APP_{751}	AβP (1–15)
Asp	2.19	1.98	2
Glu	3.42	3.35	3
Ser	0.85	0.91	1
Gly	1.12	1.19	1
His	2.97	2.92	3
Arg	1.12	1.06	1
Thr	0	0	0
Ala	0.98	1.05	1
Pro	0	0	0
Tyr	0.69	0.74	1
Val	0.79	0.89	1
Met	0	0	0
Cys	0	0	0
Ile	0	0	0
Phe	0.88	0.93	1
Lys	0	0	0

B Microsequence analyses

Cycle	PTH-AA	Yields (pmol)	
		APP_{695}	APP_{751}
1	Asp	47.5	73.7
2	Ala	76.5	130.6
3	Glu	55.4	64.7
4	Phe	57.1	87.6
5	Arg	73.2	156.5
6	His	26.1	46.5
7	Asp	37.2	67.3
8	Ser	11.5	18.6
9	Gly	31.1	48.0
10	Tyr	29.7	46.2
11	Glu	22.7	38.3
12	Val	18.3	35.1
13	His	10.3	20.7
14	His	12.3	23.9
15	Gln	5.0	11.0

APP_{695} and have structurally characterized the amino acid sequence around this processing site.

The 9-kD COOH-terminal fragments from both APP_{751}- and APP_{695}-transfected 293 cells were purified (see legend to Fig. 1) with essentially the same results. Immunoblot analysis with an antibody directed against the COOH-terminal sequence of APP [anti-APP_{695} (590–695)] (*12*) was used to assay chromatography fractions for the presence of the 9-kD fragment. This fragment migrates aberrantly on SDS-polyacrylamide gels with an apparent molecular size of 10 to 14 kD.

The purification protocol took advantage of the strong tendency of the 9-kD fragment to elute as a high molecular weight (MW) aggregate on gel filtration columns in the absence of strong detergent. Precipitated protein obtained from the formic acid–solubilized cells was chromatographed sequentially over two identical gel filtration columns, first in the absence (Fig. 1A) and then in the presence of 0.1% SDS (Fig. 1B). The high MW aggregate containing the 9-kD fragment from the first column was dissociated in 1% SDS and eluted as a low MW protein in the second chromatography step. The high degree of purification obtained from these two gel filtration columns is evident from their elution profiles. Further purification was achieved by reversed-phase liquid chromatography (RPLC) in 0.1% SDS (Fig. 1C). Fractions containing the 9-kD fragment with minimal quantities of surrounding contaminants were pooled (Fig. 1C) and concentrated by vacuum centrifugation.

The 9-kD fragments in the pooled RPLC fractions were purified by preparative SDS–polyacrylamide gel electrophoresis. Microsequencing was done on the fragments obtained. Two major sequences were observed for the APP_{751}- and APP_{695}-derived samples: the NH_2-terminal sequence of ubiquinol cytochrome c reductase (*13*) and the sequence of AβP beginning at AβP(Leu^{17}) (Fig. 2, A and B).

The location of this processing site in the interior of the AβP sequence was confirmed by identifying the COOH-terminus of the secreted, soluble fragments of APP_{695} and APP_{751}. This was accomplished by cleaving the secreted proteins with cyanogen bromide (CNBr) and structurally characterizing their respective COOH-terminal CNBr digestion fragments by amino acid analyses, microsequencing, and mass spectrometry (Table 1). The resulting data were in complete agreement and showed that the secreted forms of both APP_{695} and APP_{751} terminate at AβP(Gln^{15}) in the interior of the AβP sequence.

Thus, the secreted forms of APP_{695} and APP_{751} terminate at AβP(Gln^{15}), whereas the NH_2-terminus of the residual membrane-bound 9-kD COOH-terminal APP fragments begins at AβP(Leu^{17}). AβP(Lys^{16}) is most probably excized by a basic exopeptidase after initial proteolytic cleavage of APP. The existence of both carboxypeptidase B (*14*) and aminopeptidase B (*15*) activities capable of specifically removing single basic

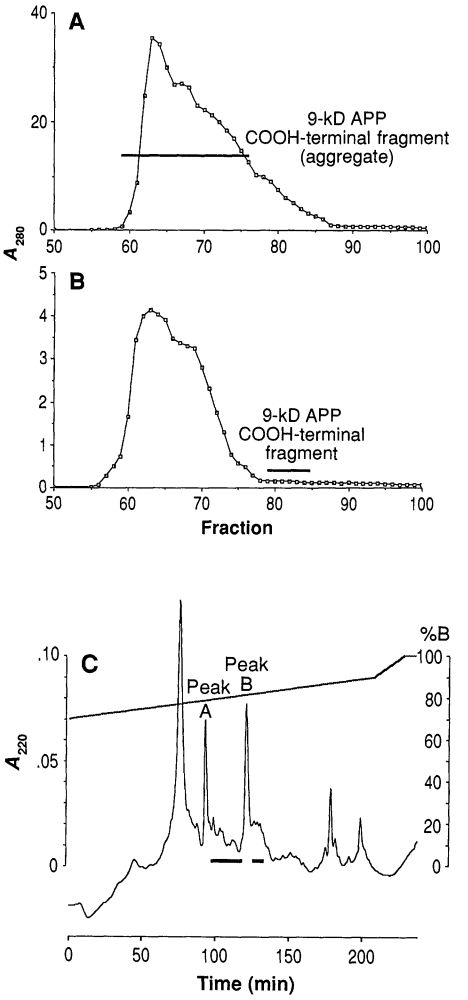

Fig. 1. Purification of the 9-kD APP membrane-bound COOH-terminal fragment from APP-transfected 293 cells. Ten roller bottles (1800 cm^2 Falcon no. 3035) of 293 cells that had been stably transfected with APP (*2*) (approximately 5×10^8 cells per bottle) were cultured in 1 mM sodium pyruvate, 25 mM Hepes, 2 mM glutamine, Dulbecco's minimum essential medium (DMEM), and 10% fetal calf serum for 10 days. The cells were dislodged with PBS + 10 mM EDTA, pelleted by centrifugation, and extracted twice with 10 ml of PBS + 10 mM EDTA + phenylmethylsulfonyl fluoride (PMSF) (0.2 mM), and leupeptin (20 μg/ml) per roller bottle. The cell pellet was solubilized with 16 ml of formic acid containing pepstatin (10 μg/ml) and lipid was partially removed with two extractions of 100 ml of dichloromethane:formic acid (95:5). The solubilized cell pellet was chromatographed over a Sephacryl HR S-100 column (2.5 by 160 cm) that had been equilibrated in 10% formic acid (**A**). Fractions containing the 9-kD COOH-terminal APP fragment were pooled and dialyzed against 2 liters of 0.1M tris-HCl, pH 8.0, and 0.1 mM EDTA. The precipitated protein was collected by centrifugation, dissolved in 15 ml of 10 mM tris-HCl, pH 8.0, 50 m*M* NaCl, and 1.0% SDS and rechromatographed over a Sephacryl HR S-100 column (2.5 by 160 cm) that had been equilibrated in 10 mM tris-HCl, pH 8.0, 50 mM NaCl, and 0.1% SDS (**B**). The partially purified 9-kD APP fragment was then chromatographed on a Vydac C4 (0.46 by 15 cm) reversed-phase column with a solvent system containing 0.1% trifluoroacetic acid (TFA), 0.1% SDS, and 20% methanol (buffer A). The protein was eluted at a rate of 0.20 ml/min with a 200-min linear gradient from 70 to 90% buffer B (buffer B is made of buffer A containing 70% acetonitrile). (**C**) RPLC fractions containing the 9-kD APP COOH-terminal fragment with minimal quantities of the two surrounding protein contaminants (peaks A and B) were pooled as indicated and dried by vacuum centrifugation.

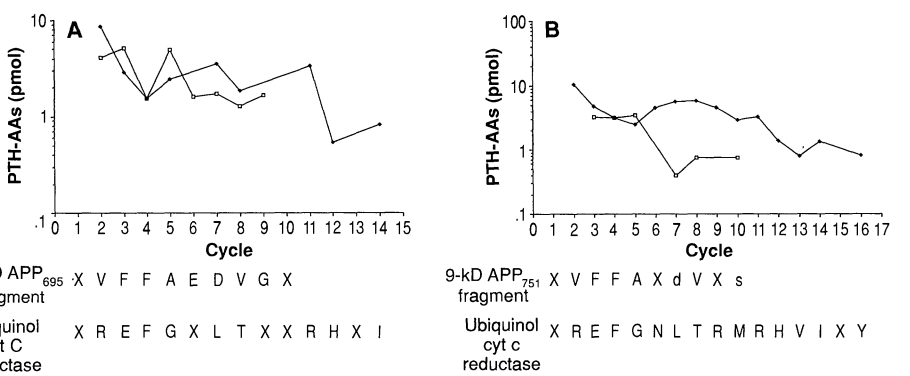

Fig. 2. Microsequence analyses of the electroblotted 9-kD APP COOH-terminal fragments from APP_{695} and APP_{751} stably transfected 293 cells. The RPLC-pooled protein fractions from Fig. 1C were separated by electrophoresis on a 12.5% SDS polyacrylamide gel and electroblotted onto Immobilon membrane (Millipore) for 1 hour at 0.5 amps in 10 mM CAPS, pH 11, 0.01% SDS, and 1% methanol. The 9-kD APP COOH-terminal fragment-containing Immobilon strips were thoroughly washed with distilled H_2O and subjected to microsequencing. The identities and yields of PTH-AAs at each sequencing cycle are shown for the samples prepared from (**A**) APP_{695}-transfected and (**B**) APP_{751}-transfected 293 cells. Unidentifiable residues are shown as X, and tentative identifications are denoted with the lower case single-letter amino acid code.

amino acids from the termini of polypeptides precludes our ability to discriminate whether initial APP processing occurs between AβP(Gln[15]–Lys[16]) or AβP(Lys[16]–Leu[17]).

These data show that a constitutive processing event, mediated by a protease we have called the APP secretase, results in (i) secretion of the ~100-kD soluble NH_2-terminal APP fragment terminating at AβP(Gln[15]) and (ii) the generation of a 9-kD membrane-bound COOH-terminal APP fragment whose NH_2-terminal sequence begins at AβP(Leu[17]). Thus, the APP secretase cleaves APP in the interior of the amyloid peptide sequence, thereby precluding AβP formation and deposition by this pathway in 293 cells.

Hypothetical pathogenic mechanisms linking aberrant APP processing to amyloid deposition have tended to emphasize the importance of inappropriate APP cleavage, either extracellularly or intracellularly, at the NH_2-terminus of AβP. However, one can now speculate that such inappropriate cleavage may lead to AβP deposition only in the absence of efficient constitutive APP processing. Thus, the key etiological event in AD may be inefficient secretase cleavage within the AβP sequence. The resulting pathogenic accumulation of some membrane-bound form of APP that cannot be effectively cleared from the cell may thus be the source of amyloid plaque formation.

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16. The secreted forms of APP_{695} and APP_{751} from APP-transfected 293 cells were purified essentially as described (*8*), with the addition of a final RPLC purification step. Briefly, the partially purified protein eluted from DEAE-Sepharose was loaded onto a Vydac C4 column (0.46 by 15 cm, 5 μm) in buffer A (0.18 M triethylammonium phosphate, pH 6.5) and then equilibrated to 20% buffer B (20% buffer A + 80% acetonitrile). The protein was eluted with a linear gradient from 20 to 100% buffer B over 80 min at a rate of 0.2 ml/min. The NH_2-terminal APP fragment eluted at approximately 59% buffer B (F. S. Esch *et al.*, unpublished data).
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Mutation of the Alzheimer's Disease Amyloid Gene in Hereditary Cerebral Hemorrhage, Dutch Type

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An amyloid protein that precipitates in the cerebral vessel walls of Dutch patients with hereditary cerebral hemorrhage with amyloidosis is similar to the amyloid protein in vessel walls and senile plaques in brains of patients with Alzheimer's disease, Down syndrome, and sporadic cerebral amyloid angiopathy. Cloning and sequencing of the two exons that encode the amyloid protein from two patients with this amyloidosis revealed a cytosine-to-guanine transversion, a mutation that caused a single amino acid substitution (glutamine instead of glutamic acid) at position 22 of the amyloid protein. The mutation may account for the deposition of this amyloid protein in the cerebral vessel walls of these patients, leading to cerebral hemorrhages and premature death.

HEREDITARY CEREBRAL HEMORRHAGE with amyloidosis of Dutch type (HCHWA-D) is an autosomal dominant form of amyloidosis. Patients with HCHWA-D develop recurrent intracerebral hemorrhages leading to death by the sixth decade of life (*1*). The disease has been described in four families from two coastal villages in the Netherlands. Three families (136 patients) were from Katwijk and one family (14 patients) was from Scheveningen (*1*). HCHWA-D is characterized by extensive amyloid deposition in the vessel walls of the leptomeninges and cerebral cortex. Parenchymal amyloid accumulations have also been found in some patients in plaques that resemble the immature senile plaques of Alzheimer's disease (AD) and sporadic cerebral amyloid angiopathy (CAA) (*2, 3*). The amyloid fibrils in HCHWA-D are formed by a 39–amino acid amyloid that is similar to the β protein in the amyloid deposits of AD and Down syndrome (DS) (*2, 4–7*).

Sequence analysis of complementary DNA (cDNA) clones that encode the amyloid β protein reveals that it is part of a larger precursor protein proposed to be a cell surface receptor (*8, 9*). Multiple forms of mRNA are derived by alternate splicing (*10*) of a transcript from a single gene located on chromosome 21 (*8, 9*). The primary structure of the 751–amino acid splicing variant of the amyloid β protein precursor is analogous to protease nexin II, a secreted protease inhibitor (*11*).

Genomic DNA and cDNA clones encoding the precursor amyloid β protein from AD, DS, and familial AD patients have been sequenced; the sequences were identical to those obtained from unaffected individuals (*9, 12, 13*). Although the genetic defect causing familial AD was localized to chromosome 21 (*14*), no linkage was found between familial AD and the amyloid β protein precursor gene, suggesting that this gene is not the site of the inherited defect underlying this disorder (*15*).

We hypothesized (*2*) that a defect in the amyloid β protein gene may cause the pathology associated with HCHWA-D. To sequence the amyloid β protein gene in HCHWA-D, we synthesized oligonucleotides based on known intron sequences that flank the two exons encoding the amyloid β protein (exons 14 and 15) (Fig. 1) (*12*). The oligonucleotides were used in the polymerase chain reaction (PCR) (*16*) to amplify the sequences that contain the two exons. The amplified fragments were subcloned into an M13 bacteriophage vector and sequenced by dideoxy chain termination (*17*).

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