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12. We prepared the Y-oligomer substrate by labeling the 5′ end of an appropriate oligonucleotide and annealing with its complements. Oligonucleotides were purified by electrophoresis in a denaturing polyacrylamide gel before use. Fifty picomoles of the appropriate oligonucleotide were ^{32}P-labeled at their 5′ termini by use of T4 polynucleotide kinase and 25 μCi of [γ-^{32}P]adenosine 5′-triphosphate. The labeled oligonucleotide was annealed with an excess (150 to 250 pmol each) of unlabeled complementary strands in 10 mM tris-HCl, pH 7.5, 1 mM EDTA, and 0.1 M NaCl. The mixture was heated to 80°C for 2.5 min, incubated for 20 min each at 50°, 45°, 40°, and 37°C, cooled slowly to room temperature, and then chilled to 4°C. The Y-oligomer substrate was separated from unannealed and partially annealed products by electrophoresis in a 15% native polyacrylamide gel. The region of the gel corresponding to the Y-oligomer was excised and soaked overnight in 0.5 M ammonium acetate and 10 mM magnesium acetate. The supernatant fluid from the soaked gel fragment was filtered through a 0.45-μm cellulose acetate membrane, then concentrated in a Centricon-10 column (Amicon), and washed with 20 × volume of 10 mM tris-HCl, pH 7.5, 1 mM EDTA, and 0.1 M NaCl. Analysis of the purified labeled substrate by gel electrophoresis showed that more than 95% of the radioactivity was associated with the Y-oligomer.
13. Reactions (20 μl) contained a final concentration of 20 mM Hepes, pH 7.5, 10 mM $MnCl_2$, 20 mM NaCl, 1 mM dithiothreitol, 0.1 mM CHAPS, 0.05% Nonidet P40, 0.1 pmol of Y-oligomer substrate, and 2.8 pmol of HIV-1 integrase. The mixture was incubated for 60 min at 37°C. The reaction was stopped by addition of 18 mM EDTA, pH 8.0, proteinase K (300 μg/ml), and 0.1% SDS, and then incubated for an additional 30 min at 37°C. HIV-1 integrase was purified from *E. coli* expressor cells by a procedure modified from that of Sherman and Fyfe (*8*). The construction of the HIV-1 integrase expression plasmid and purification of integrase will be described elsewhere (*20*).
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15. HIV-1 integrase purified from yeast expressor cells was a gift from A. D. Leavitt and H. E. Varmus, University of California, San Francisco. Viral particles prepared from HIV-1–infected cells were provided by Frederick Cancer Research Facility, National Cancer Institute. *Escherichia coli* DNA gyrase was provided by A. Kornberg, Stanford University; IHF, Fis protein, phage λ Int protein, and Gin protein of phage Mu were provided by N. R. Cozzarelli, University of California, Berkeley.
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Potentially Amyloidogenic, Carboxyl-Terminal Derivatives of the Amyloid Protein Precursor

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The 39– to 43–amino acid amyloid β protein (βAP), which is deposited as amyloid in Alzheimer's disease, is encoded as an internal peptide that begins 99 residues from the carboxyl terminus of a 695– to 770–amino acid glycoprotein referred to as the amyloid β protein precursor (βAPP). To clarify the processing that produces amyloid, carboxyl-terminal derivatives of the βAPP were analyzed. This analysis showed that the βAPP is normally processed into a complex set of 8- to 12-kilodalton carboxyl-terminal derivatives. The two largest derivatives in human brain have the entire βAP at or near their amino terminus and are likely to be intermediates in the pathway leading to amyloid deposition.

IN HUMAN CEREBROSPINAL FLUID AND brain, and in medium conditioned by cultured cells, there are large, secreted βAPP derivatives (*1–4*). These secreted NH_2-terminal derivatives are produced by a cleavage within the βAP (*5, 6*) that also produces small cell-associated COOH-terminal fragments. Because they contain only part of the βAP, the derivatives produced by this cleavage cannot produce amyloid.

To determine if any βAP-bearing, COOH-terminal derivatives are present in human cerebral cortex, we prepared membrane-associated proteins from cortex and passed them over an affinity column made with antibody to $βAPP_{672-695}$ (anti-C_{24}) (*7*). The affinity-purified proteins were then separated by tris-tricine SDS–polyacrylamide gel electrophoresis (PAGE) (*8*), a system that clearly resolves small proteins. The anti-C_{24} column retained full-length forms (Fig. 1A, bracket) and five putative COOH-terminal derivatives (~11.8, ~11.4, ~10.9, ~9.6, and ~8.7 kD) (Fig. 1A, arrows) that were (i) specifically labeled by anti-C_{24} (Fig. 1B, lanes 1 and 2) and (ii) readily detected in brains from patients with Alzheimer's disease (AD) (Fig. 1A) and in control brains (Fig. 1C). As shown in Fig. 1F (lanes 6 and 7), an antibody to $βAPP_{649-664}$ specifically immunoprecipitated the same five 8- to 12-kD proteins immunoprecipitated by antibody to $βAPP_{676-695}$ (anti-C_{20}) (lane 5) or anti-C_{24} (Fig. 1, A and B). The specific recognition of these proteins by antisera against two nonoverlapping epitopes in the COOH-terminal region of the βAPP ($βAPP_{649-664}$ and $βAPP_{676-695}$) provides strong evidence that they are all COOH-terminal βAPP derivatives. When separated by conventional tris-glycine SDS-PAGE, the five 8- to 12-kD proteins migrated above the 14-kD marker as a doublet of ~15 and ~17 kD (Fig. 1D) similar to putative COOH-terminal βAPP derivatives previously described in human brain (*9*) and transfected cultured cells (*10–12*). Thus, the tris-tricine gel system was essential for demonstrating the complex set of COOH-terminal βAPP derivatives in human brain.

To determine whether some of the 8- to 12-kD COOH-terminal derivatives are large enough to contain the entire βAP, we compared these derivatives with a protein, produced by using rabbit reticulocyte lysate, that corresponds to the 100–amino acid segment at the βAPP COOH-terminus (*7*). This protein ($MβAP_1$-COOH) contains the entire βAP and begins with the methionine preceding the βAP sequence. $MβAP_1$-COOH comigrated with the second largest of the five COOH-terminal derivatives (Fig. 1E, lanes 1 and 2). Thus, comparative assessment by SDS-PAGE indicates that the ~11.8- and ~11.4-kD proteins are large enough to contain the entire βAP and therefore are potentially amyloidogenic.

To assess this possibility further, we used an antibody to $βAP_{1-40}$ (SGY2134) to immunoprecipitate COOH-terminal derivatives, which were assessed on immunoblots with anti-C_{20}. SGY2134 immunoprecipitated the two largest COOH-terminal derivatives but failed to immunoprecipitate the smaller COOH-terminal derivatives (Fig. 1F, lane 1). This immunoprecipitation was produced in large part by antibodies to the NH_2-terminal region of the βAP, because it was markedly attenuated by absorption with

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$\beta AP_{1\text{-}15Cys}$ or $\beta AP_{1\text{-}17GlyGlyCys}$ (Fig. 1F, lanes 2 and 3). Immunoprecipitation was completely abolished by absorption with $\beta AP_{1\text{-}40}$ (lane 4). Similar analyses with three other antisera to the βAP (Fig. 1G) confirmed that the two largest derivatives were specifically labeled by antibodies to the NH_2-terminal region of the βAP. The size of the ~11.8- and ~11.4-kD proteins compared to that of synthetic $M\beta AP_1$-COOH (Fig. 1E) and the observation that both are specifically recognized by antisera to opposite ends as well as the middle of the 99-residue COOH-terminal (βAP_1-COOH) region (Fig. 1, F and G) indicate that these proteins are COOH-terminal derivatives of the βAPP that contain the entire βAP.

Similar derivatives were identified in human embryonic kidney (293) cells stably transfected with βAPP_{695} transgenes (*13, 14*). These cells (Fig. 2A) showed the expected augmentation of full-length *N*- (~91 kD) and *N*- and *O*-glycosylated (~110 kD) forms of βAPP (*3, 12*). With longer exposure of the fluorograms (Fig. 2B), they also showed augmentation of a set of 8- to 12-kD proteins that were immunoprecipitated by anti-C_{20}. Thus, in transfected 293 cells as in human brain there is a complex set of COOH-terminal βAPP derivatives.

In the transfected cells, full-length βAPP was metabolized as reported (*3, 14*). Labeled amino acids delivered in a 10-min pulse (Fig. 2A) were incorporated into *N*-glycosylated forms (~91 kD), that were *O*-glycosylated by 20 min, and labeling of the *N*- and *O*-glycosylated forms (~110 kD) was maximal at 60 min. The 8- to 12-kD proteins (Fig. 2B) were not labeled until well after full-length βAPP had been labeled. Once labeled, the more abundant, smaller derivatives in the 8- to 12-kD set persisted for 180 min (Fig. 2B) when most of the labeled full-length forms (Fig. 2A) had disappeared. Thus, in cultured cells, the 8- to 12-kD COOH-terminal derivatives are generated by cellular processing of full-length forms and not by artifactual proteolysis during extraction.

Two additional studies were done (*15*) to eliminate the possibility that the ~8- to 12-kD COOH-terminal derivatives in human brain are produced artifactually. These studies showed that these derivatives (i) are not produced during the postmortem interval because there is an identical set of 8- to 12-kD proteins in freshly killed rat brain, and (ii) are not generated during extraction because radiolabeled full-length βAPP added as a tracer prior to homogenization is not proteolyzed to COOH-terminal derivatives.

COOH-terminal βAPP derivatives in the temporal region of eight control and ten AD subjects were compared on a single immunoblot, but no differences were apparent (not shown, but see Fig. 1C).

To determine whether any of the 8- to 12-kD βAPP derivatives are selectively expressed in brain, we compared homogenates of several brain regions and peripheral tissues (Fig. 3A). Each brain region (lanes 1 through 5) had roughly equivalent levels of the various 8- to 12-kD derivatives, whereas each peripheral tissue (liver, kidney, small intestine, and muscle), like transfected 293 cells, had relatively low levels of the larger potentially amyloidogenic forms. Thus the selective deposition of amyloid in brain may be related to the comparatively high level of βAP-bearing COOH-terminal derivatives in this tissue.

In homogenates, an additional ~5.8-kD protein (possibly a doublet) was detected in various brain regions but not in peripheral tissues (Fig. 3A, arrow). This protein appears to be an authentic COOH-terminal βAPP derivative, because it is specifically labeled both by anti-C_{20} and the antibody to

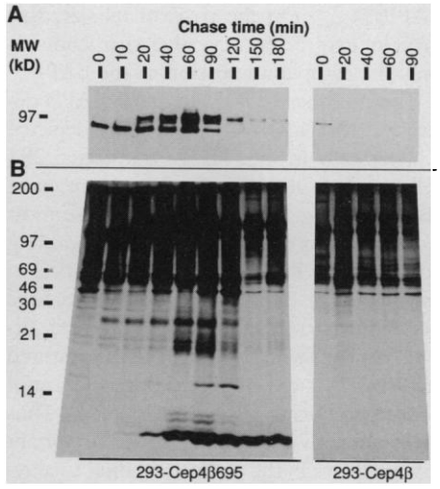

Fig. 2. βAPP processing in transfected (CEP4β695) and control (CEP4β) 293 cells. Fluorogram exposure: (**A**) 4 hours (**B**) 96 hours. Ten to 16% tris-tricine SDS-PAGE. For details see (*14*). Note the 14- to 30-kD proteins that are augmented in transfected 293 cells in (B). Similar human brain proteins are specifically labeled by anti-C_{24} (Fig. 1). These proteins could be additional βAP-bearing COOH-terminal derivatives or aggregates of the 8- to 12-kD proteins (*11, 16*).

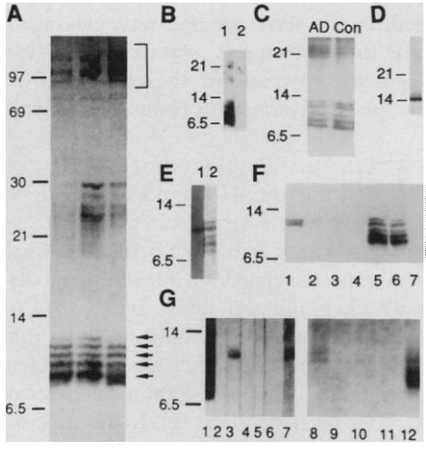

Fig. 1. Carboxyl-terminal βAPP derivatives in human brain. Ten to 16% tris-tricine SDS-PAGE [except for (D)]. Anti-C_{24} and anti-C_{20} produced the same results in immunoprecipitation and immunoblotting. For details see (*7*). (**A**) Three preparations of membrane proteins from individuals with AD, affinity-purified and immunoblotted with anti-C_{24}. (**B**) Anti-C_{20} affinity-purified proteins immunoblotted with anti-C_{20} (lane 1) or anti-C_{20} absorbed with C_{24} (lane 2). (**C**) Cortical membrane preparations from an AD and a control brain, immunoprecipitated and immunoblotted with anti-C_{20}. (**D**) Anti-C_{24} affinity-purified proteins separated by 5 to 20% tris-glycine SDS-PAGE and immunoblotted with anti-C_{24}. (**E**) $M\beta AP_1$-COOH autoradiogram (lane 1) compared with anti-C_{24} affinity-purified and labeled proteins (lane 2). (**F**) Epitope identification. Anti-C_{20} immunoblot of affinity-purified proteins immunoprecipitated with antibody to $\beta AP_{1\text{-}40}$ (SGY2134) (lane 1), antibody to $\beta AP_{1\text{-}40}$ absorbed with $\beta AP_{1\text{-}15Cys}$ (lane 2), antibody to $\beta AP_{1\text{-}40}$ absorbed with $\beta AP_{1\text{-}17GlyGlyCys}$ (lane 3), antibody to $\beta AP_{1\text{-}40}$ absorbed with $\beta AP_{1\text{-}40}$ (lane 4), anti-C_{20} (lane 5), antibody to $\beta APP_{649\text{-}664}$ (lane 6), and antibody to $\beta APP_{649\text{-}664}$ absorbed with $\beta APP_{649\text{-}664}$ (lane 7). (**G**) Identification of βAP-bearing derivatives by immunoblotting (lanes 1 to 7) and immunoprecipitation (lanes 8 to 12). Anti-C_{24} affinity-purified proteins were labeled with anti-C_{24} (lane 1), anti-C_{24} absorbed with C_{24} (lane 2), antibody to $\beta AP_{1\text{-}40}$ (DS1280) (lane 3), antibody to $\beta AP_{1\text{-}40}$ absorbed with $\beta AP_{1\text{-}15Cys}$ (lane 4), antibody to $\beta AP_{1\text{-}40}$ absorbed with $\beta AP_{1\text{-}17GlyGlyCys}$ (lane 5), antibody to $\beta AP_{1\text{-}40}$ absorbed with $\beta AP_{1\text{-}40}$, (lane 6), and antibody to $\beta AP_{1\text{-}17}$ (lane 7). Anti-C_{20} immunoblot of affinity-purified proteins immunoprecipitated with antibody to $\beta AP_{1\text{-}42}$ (lane 8), antibody to $\beta AP_{1\text{-}42}$ absorbed with $\beta AP_{1\text{-}15Cys}$ (lane 9), antibody to $\beta AP_{1\text{-}42}$ absorbed with $\beta AP_{1\text{-}17\text{-}GlyGlyCys}$ (lane 10), and antibody to $\beta AP_{1\text{-}42}$ absorbed with $\beta AP_{1\text{-}40}$ (lane 11). (Lane 12) Anti-C_{20} immunoblot of the protein used for immunoprecipitation in lanes 8 to 11.

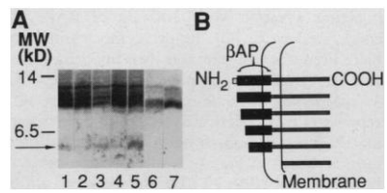

Fig. 3. Carboxyl-terminal βAPP derivatives. (**A**) Homogenates of human frontal cortex (lane 1), frontal white matter (lane 2), temporal cortex (lane 3), cerebellum (lane 4), nucleus basalis of Meynert (lane 5), liver (lane 6), and kidney (lane 7), immunoprecipitated and immunoblotted with anti-C_{20}. For details see (*16*). (**B**) Schematic diagram of COOH-terminal βAPP derivatives. Note that there could be fewer than six separate forms if some derivatives have the same amino acid backbone but different posttranslational modifications [for example, phosphorylation (*17*)].

$\beta APP_{649-664}$. On the basis of its size, this protein may be a derivative that contains only the cytoplasmic region of the βAPP.

The multiple COOH-terminal βAPP derivatives that we have identified are depicted schematically in Fig. 3B. In transfected 293 cells, the smaller two fragments are most abundant (Fig. 2B), and one of these most likely corresponds to the derivative beginning at βAP_{17} (*6*). Proteins essentially identical to the two large βAP-bearing derivatives have been shown to (i) aggregate in vitro (*16*), (ii) form deposits when overexpressed in cultured cells (*10, 18*), and (iii) cause degeneration of neurite-producing PC12 cells (*19*). Thus these derivatives are likely to be important intermediates in the pathway leading to amyloid deposition in AD.

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7. Pellets containing membrane-associated proteins, prepared from cortical gray matter of control subjects or subjects with histologically proven AD (postmortem interval <5 hours) as described (*1*), were homogenized in TBS (150 mM NaCl, 10 mM tris, pH 8.0)-LPT (1 μg/ml leupeptin, 0.1 μg/ml pepstatin, and 1 μg/ml 7-amino-1-chloro-3-tosylamido-2-heptanone) containing 2% Nonidet P-40, 2% Triton X-100, 2 mM EDTA, and 1 mM phenylmethylsufonyl fluoride. After 20 min on ice, homogenates were centrifuged for 1 hour at 100,000*g*, and the supernatant was applied to a protein A–agarose column. The void volume was then passed over an anti-C_{24} column made by dimethylpimelimidate cross-link of 5 ml of antiserum to protein A–agarose (*20*). The bound proteins were washed extensively with TBST-LPT (TBS-LPT containing 0.05% Tween-20), RIPA (150 mM NaCl, 10 mM tris, pH 8.0, 1% Nonidet P-40, 0.5% cholic acid, 0.1% SDS)-LPT, TBST-LPT, and then eluted with 0.1 M glycine-LPT (pH 2.6) in 0.05% Tween-20. The pH of the eluant was neutralized, and it was applied to a second protein A–agarose column. Immunoblotting and immunoabsorptions (50 μg peptide) were carried out as described (*1*). Anti-C_{24} was induced by injecting a rabbit with 100 μg of $\beta APP_{672-695}$ conjugated to keyhole limpet hemocyanin in complete Freund's adjuvant and then injecting biweekly 250 μg $\beta APP_{672-695}$ plus 1 mg of heat killed *Mycobacterium tuberculosis*. Figure 1, A through D, represents βAPP derivatives purified from approximately 0.5 g of human brain. The immunoprecipitations shown in Fig. 1, F and G, were carried out by incubating 10 to 20 μl of the indicated antibody with affinity-purified protein in 600 μl (final volume) RIPA-LPT. $M\beta AP_1$-COOH was generated with a subclone of the transcription vector pSP72 that contained an Eco R1–Mae 1 βAPP cDNA fragment ($\beta APP_{1795-2085}$). Translation was initiated from an ATG site located within oligonucleotide adaptors.
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14. βAPP_{695} was overexpressed in 293 cells by using an episomal replicon (CEP4β) similar to that described by Hambor and co-workers (*13*) except that expression was driven by a cytomegalovirus promoter. Stable lines containing CEP4β or CEP4β-βAPP_{695} were established by transfecting 10^6 cells with lipofectin (BRL) (20 μg/ml) and cesium chloride (10 μg/ml)–purified plasmid DNA in Opti-MEM 1 (BRL) medium. After 4 hours in the lipofectin DNA mix, cells recovered for 24 hours in fresh medium [Opti-MEM 1 plus 5% calf serum, supplemented and iron enriched (BRL)], were trypsinized, and stable lines were selected by 2-week exposure to hygromycin B (Boehringer Mannheim) (100 μg/ml). Stable lines were maintained in hygromycin B (200 μg/ml). Approximately 5×10^6 cells (80% confluent grown at 37°C in 5% CO_2) were rinsed twice with Hanks balanced salt solution and labeled with TRAN^{35}S-LABEL (ICN, 400 μCi/ml [^{35}S]methionine and 60 μCi/ml [^{35}S]cysteine) in 2 ml of methionine and cysteine-deficient DMEM for 10 min. Chases were performed by rinsing twice with serum-free media and incubating in 5 ml of fresh serum-free media for the indicated times. Cells were then lysed in RIPA-LPT (7), the supernatants cleared with protein A–agarose, and the proteins immunoprecipitated with 4 μl of anti-C_{20} and protein A–agarose (*20*).
15. Samples (1.5 g) of adult rat cerebrum or human tissue (control sample, 4 hours after death, flash frozen at autopsy and stored at −80°C until use) were thawed, Dounce-homogenized in RIPA-LPT, cleared with protein A–agarose, immunoprecipitated with 10 μl of anti-C_{20} and 20 μl of protein A–agarose (*20*), separated by 10 to 16% tris-tricine SDS-PAGE, and immunoblotted with anti-C_{20}.
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Processing of the Amyloid Protein Precursor to Potentially Amyloidogenic Derivatives

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The ~120-kilodalton amyloid β protein precursor (βAPP) is processed into a complex set of 8- to 12-kilodalton carboxyl-terminal derivatives that includes potentially amyloidogenic forms with the ~4-kilodalton amyloid β protein (βAP) at or near their amino terminus. In order to determine if these derivatives are processed in a secretory pathway or by the endosomal-lysosomal system, (i) deletion mutants that produce the normal set of carboxyl-terminal derivatives and shortened secreted derivatives were analyzed and (ii) the effect of inhibitors of endosomal-lysosomal processing was examined. In the secretory pathway, cleavage of the βAPP occurs at a single site within the βAP to generate one secreted derivative and one nonamyloidogenic carboxyl-terminal fragment, whereas, in the endosomal-lysosomal system, a complex set of carboxyl-terminal derivatives is produced that includes the potentially amyloidogenic forms.

THE βAPP IS NORMALLY CLEAVED within the 43–amino acid βAP to produce a large secreted derivative ending at amino acid 15 of βAP and a small membrane-associated COOH-terminal derivative beginning at amino acid 17 (*1*). If βAPP secretase cleaves full-length βAPP at additional minor sites, then it could generate the entire set of COOH-terminal derivatives that have been identified (*2*). In this process, a corresponding set of secreted derivatives would be produced. These secreted derivatives of over 100 kD, differing by only several kilodaltons, would be difficult to separate by SDS–polyacrylamide gel electrophoresis (PAGE). To avoid this difficulty and to determine whether βAPP secretase produces multiple secreted derivatives, we made βAPP_{695} deletion constructs (Fig. 1A) similar to those described by Sisodia and co-workers (*3*). These constructs secrete shortened derivatives in which small length differences are readily detectable by SDS-PAGE.

Comparison of control (CEP4β) 293 cells and cells transfected with these deletion constructs showed the production of shortened βAPPs (Fig. 1B). These shortened βAPPs were processed into (i) a set of 8- to 12-kD COOH-terminal derivatives essentially identical to that observed in cells transfected with a βAPP_{695} expression construct (Fig. 1C) and (ii) secreted derivatives that, like secreted βAPP_{695}, were ~10 kD smaller than the full-length forms (compare

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