Alzheimer's Disease and Control Brain Contain Soluble Derivatives of the Amyloid Protein Precursor That End within the β Amyloid Protein Region†

Jennifer M. Pasternack,‡ Mark R. Palmert,‡ Marianne Usiak,‡ Rong Wang,§ Heidi Zurcher-Neely,∥ Patricia A. Gonzalez-DeWhitt,∥ Michael B. Fairbanks,∥ Tobun Cheung,‡ Deborah Blades,‡ Robert L. Heinrikson,∥ Barry D. Greenberg,∥ Robert J. Cotter,§ and Steven G. Younkin*,‡

Division of Neuropathology, Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106, Middle Atlantic Mass Spectrometry Laboratory, Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and Upjohn Laboratories, Kalamazoo, Michigan 49001

Received June 29, 1992

ABSTRACT: The 39–43 amino acid β amyloid protein (Aβ) that deposits as amyloid in the brains of patients with Alzheimer's disease (AD) is encoded as an internal sequence within a larger membrane-associated protein known as the amyloid protein precursor (APP). In cultured cells, the APP is normally cleaved within the Aβ to generate a large secreted derivative and a small membrane-associated fragment. Neither of these derivatives can produce amyloid because neither contains the entire Aβ. Our study was designed to determine whether the soluble APP derivatives in human brain end within the Aβ as described in cell culture or whether AD brain produces potentially amyloidogenic soluble derivatives that contain the entire Aβ. We find that both AD and control brain contain nonamyloidogenic soluble derivatives that end at position 15 of the Aβ. We have been unable to detect any soluble derivatives that contain the entire Aβ in either the AD or control brain.

Alzheimer's disease is characterized by deposition in the central nervous system of amyloid composed of a 39–43 amino acid polypeptide referred to as the β amyloid protein (Aβ) (Glenner & Wong, 1984; Masters et al., 1985). Several groups (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987) have isolated cDNAs that encode the Aβ as part of a larger protein called the amyloid protein precursor (APP). The precursor mRNA is alternatively spliced to encode 3 main forms: APP_{695}; APP_{751}, which contains a 56 amino acid domain that is highly homologous to the Kunitz family of protease inhibitors (KPI domain); and APP_{770}, which contains the KPI domain and an additional 19 amino acid domain (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). In all 3 forms, the Aβ is encoded as an internal peptide that begins 99 residues from the carboxyl terminus of the APP, so cleavage on both sides of the Aβ region appears to be required for generation of the Aβ deposited as amyloid.

Both KPI-containing and KPI-free forms of the APP are normally cleaved in the vicinity of the Aβ by an unknown enzyme referred to as the "APP secretase"; this cleavage produces a large soluble derivative (Schubert et al., 1988; Palmert et al., 1989a; Wiedemann et al., 1989) and a small membrane-associated fragment (Oltersdorf et al., 1989). In their study of human embryonic kidney 293 cells transfected with APP_{695} or APP_{751} expression constructs, Esch et al. (1990) showed that the secreted APP derivative ends at position 15 of the Aβ (Figure 1B) and the corresponding membrane-associated fragment begins at position 17, implying that cleavage occurs between amino acids 15 and 17 of the Aβ. Wang et al. (1991) subsequently showed that CHO cells transfected with APP_{770} expression constructs produce secreted derivatives ending at both positions 15 and 16. This finding suggests that the secretase normally cuts at $Aβ_{16}$ with later removal of the lysine at position 16 by an exopeptidase. Secreted APP derivatives ending at $Aβ_{15}$ and $Aβ_{16}$ were also found by Anderson et al. (1991) in untransfected PC12 cells in which a neuronal phenotype was induced by treatment with nerve growth factor, and by Lowery et al. (1991) in Sf9 insect cells infected with baculovirus expressing APP_{751}. Since secreted derivatives that end at $Aβ_{15}$ and $Aβ_{16}$ contain only part of the Aβ region, they are nonamyloidogenic.

Although it is possible that APP processing is similar in most or all cells that express it, whether naturally or after transfection, the fact that amyloid deposits only in the central nervous system of certain species suggests that processing of the APP in human brain, and particularly in AD brain, could be quite different from processing in other cells. The present study was designed to determine whether APP-soluble derivatives in AD brain are generated by cleavage within the Aβ, as in cell culture, or by an altered cleavage that produces potentially amyloidogenic soluble derivatives.

EXPERIMENTAL PROCEDURES

Purification of APP Soluble Derivatives. Gray matter grossly dissected from human cerebral cortex was obtained at autopsy and flash-frozen in liquid nitrogen. The gray matter was then homogenized using a ratio of 1 g of tissue to 5.3 mL of buffer [0.05 M Tris-HCl, pH 7.6, 0.1 M NaCl, 0.15 M EDTA (ethylenediaminetetraacetic acid), 1.0 μg/mL leupeptin, 10 μg/mL aprotinin, 1.0 μg/mL TLCK (tosyl-L-lysine chloromethyl ketone), 2 mM PMSF (phenylmethanesulfonyl fluoride), and 0.1 μg/mL pepstatin A]. The homogenate was centrifuged at 30 000 rpm for 1 h ($w^2t = 3.55 \times 10^{10}$). The pellet was discarded, and lipids were removed from the supernatant using Sero-Clear (Calbiochem) according to instructions. The supernatant was used immediately or stored at −80 °C.

† R.W. and R.J.C. were supported by Grants DIR 89-14549 and DIR 90-16567 from the National Science Foundation. Mass spectral analyses were carried out at the Middle Atlantic Mass Spectrometry Laboratory, an NSF Shared Instrumentation Facility.

* Address correspondence to this author.
‡ Case Western Reserve University.
§ The Johns Hopkins University School of Medicine.
∥ Upjohn Laboratories.

0006-2960/92/0431-10936$03.00/0

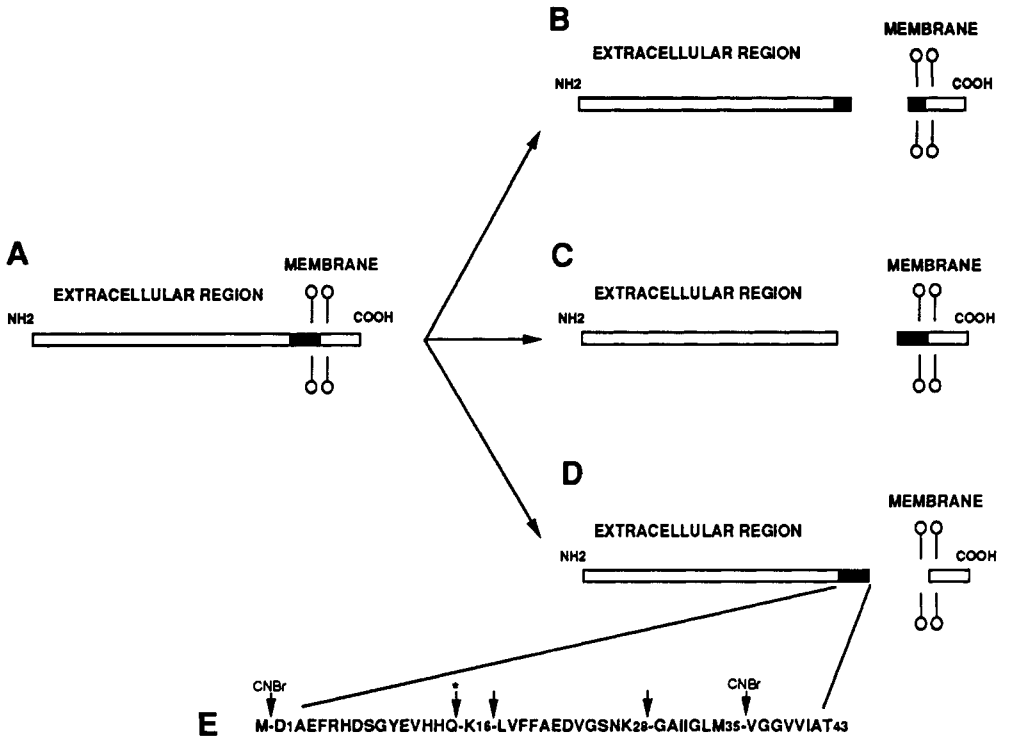

FIGURE 1: Cleavage sites within the APP that could produce the soluble derivatives. The solid bars represent the Aβ region. (A) Full-length APP. (B) Cleavage within the Aβ region produces nonamyloidogenic soluble derivatives and membrane-associated fragments. (C) Cleavage at the Aβ amino terminus. (D) Cleavage at the Aβ carboxyl terminus. In a previous study, Palmert et al. (1989b) showed that the soluble APP derivatives in human CSF are labeled by antisera to $A\beta_{1-17}$, so the cleavage that produces these derivatives must be as depicted in (B) or (D). The current study demonstrates that AD and control brain contain soluble derivatives that end within the Aβ as shown in (B). However, neither of these studies completely rules out the possibility that the cleavage that generates soluble derivatives is heterogeneous and occasionally occurs in more than one of these locations. (E) Amino acid sequence of the Aβ showing CNBr cleavage sites and the three carboxyl termini (15, 16, and 28) identified in baculovirus (arrows). We could identify only the carboxyl terminus at $A\beta_{15}$ (asterisk) in AD and control brain.

Supernatants were thawed quickly in a 37 °C water bath and brought to 47.5% ammonium sulfate over a 30-min period followed by a 30-min equilibration, both at 4 °C with gentle stirring. The mixture was spun at 10000*g* for 30 min, and the pelleted proteins were resuspended in a minimal volume of buffer 1, pH 7.6 (30 mM phosphate buffer, 1.0 μg/mL leupeptin, 1.0 μg/mL TLCK, and 0.1 μg/mL pepstatin A), and desalted using PD-10 columns (Pharmacia). The desalted solution was loaded on an Affi-gel blue (Bio-Rad) column (preequilibrated with buffer 1, pH 7.0) at a flow rate of 1 mL/min. Contaminating proteins were eluted with buffer 1 plus 0.2 M NaCl, pH 7.0, and the APP-containing eluant was collected with buffer 1 plus 0.4 M NaCl, pH 6.5. The column was cleaned with 2.0 M NaCl followed by 2.0 M guanidine hydrochloride. The Affi-gel eluant was pelleted at 80% ammonium sulfate, resuspended in buffer 2 (15 mM phosphate, pH 7.4, and 0.15 M NaCl plus inhibitors), and desalted as described above.

The desalted solution was added to the dextran sulfate column in buffer 2, pH 7.4, and the resulting slurry was rocked overnight at 4 °C. The column was then allowed to pack and rinsed with additional buffer. The APP fraction was collected in 15 mM phosphate buffer, pH 7.4, 0.8 M NaCl, 1.0 μg/mL leupeptin, 1.0 μg/mL TLCK, and 0.1 μg/mL pepstatin A, pelleted at 90% ammonium sulfate, resuspended in 20 mM phosphate buffer, pH 6.8, without inhibitors, and desalted as described above. The solution was then brought to 0.05% Tween-20 and filtered (0.22 μm).

The filtrate was loaded on a Pharmacia Mono Q HR 5/5 FPLC column using a Pharmacia 50-mL Superloop and a Dionex HPLC system. A linear gradient was made from 100% buffer A (20 mM phosphate, pH 6.8, and 0.05% Tween-20) to 100% buffer B (buffer A brought to 1.0 M NaCl) over 50 min at 1 mL/min. The APP-soluble derivatives eluted at 56–60% buffer B. Purity and yield were assessed by silver staining (Morrissey, 1981) and immunoblotting with anti-N (Palmert et al., 1988). Purified protein was concentrated and desalted using a Centricon 30 (Amicon) and stored at −80 °C.

Dextran Preparation. Dextran columns were prepared according to the method of Van Nostrand and Cunningham (1987). Columns can be used only twice and are regenerated between uses by washing with 15 mM phosphate buffer/1.2 M NaCl, pH 7.4, followed by rocking overnight at 37 °C in 15 mM phosphate buffer/0.15 M NaCl, pH 7.4, containing 1% Tween.

Baculovirus Protein Preparation. Protein expression and purification are described in detail in Lowery et al. (1991). To make recombinant baculovirus expressing APP_{751}, a DNA fragment containing the full-length APP_{751} cDNA sequence was subcloned into the baculovirus transfer vector and cotransfected with baculoviral DNA into Sf9 cells (Summers & Smith, 1987). Sf9 cells infected with the resultant recombinant baculovirus (Sf9-Bac_{751}) produced soluble APP derivatives similar to those seen in brain. Serum-free conditioned media were harvested 66 h after infection and clarified by centrifugation (1000*g* for 15 min) and filtration through a 45-μm filter. Protease inhibitors (1 mM PMSF, 5 mM benzamidine, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A) were added to prevent degradation.

Sf9-Bac_{751} APP-soluble derivatives were purified by DEAE-Sepharose, dextran sulfate, phenyl-Sepharose, and then Sephacryl S-200 HR column chromatography. To minimize proteolysis, protease inhibitors were added to all but the final

sizing column, and all steps were performed at 4 °C. Purity was assessed by quantitative amino acid analysis.

CNBr Digestion and Peptide Separation. Purified human brain or baculovirus APP-soluble derivatives (approximately 250 μg) were dried and then digested with CNBr (100-fold excess by weight) in 70% formic acid for 24 h at room temperature, diluted 1:10 in water, and dried in a speed-vac concentrator. The resulting peptides were resuspended in 25% trifluoroacetic acid (TFA, Pierce) immediately before being loaded on a Beckman HPLC. Peptides were separated on a C18 RPLC column (Vydac) with a 0–100% acetonitrile gradient (0–16% over 200 min, 16–50% over 200 min, 50–100% over 50 min) in the presence of 0.05% TFA at a flow rate of 200 μL/min.

ELISA. RPLC fractions of interest were detected by ELISA. Aliquots of fractions were diluted to 100 μL in 15 mM phosphate buffer/0.15 M NaCl, pH 7.2 (PBS), and were adsorbed to flat-bottomed poly(vinyl chloride) microtiter wells (Dynatech Laboratories) at 4 °C overnight. Wells were then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Antiserum 1280, a rabbit antiserum against $A\beta_{1-40}$ generously provided by D. Selkoe, was then added to the wells (50 μL of a 1:1500 dilution in 1% BSA/PBS) and allowed to interact with antigen overnight at 4 °C. After washing of the wells with PBS, alkaline phosphatase-coupled goat anti-rabbit antiserum (Promega) was added for 5 h at 4 °C. Finally, after another wash with PBS, substrate (*p*-nitrophenyl phosphate, Sigma) was added at room temperature. The absorbance at 405 nm was then determined for each well with an automated ELISA reader (BioTek).

Plasma Desorption Mass Spectrometry. Purified RPLC peptide fractions were analyzed on a BIO-ION Nordic, AD (Upsalla, Sweden), Model BIN 10K plasma desorption time-of-flight mass spectrometer. Peptide fractions were speed-vacuum-dried and dissolved in 50 μL of 0.1% TFA; 2.5 μL of the resulting solutions was deposited on a nitrocellulose-coated sample foil and rinsed with an additional 2.5 μL of 0.1% TFA to remove alkali ions and other impurities. Sample foils were then analyzed by PDMS at an accelerating voltage of 20 kV to a preset count of 3 million counts.

RESULTS

Soluble APP derivatives from human brains obtained at autopsy (postmortem intervals of 4–7 h) were purified as described under Experimental Procedures. The silver-stained ~125- and ~105-kDa secreted forms obtained in a typical purification are shown in Figure 2. To determine where the soluble derivatives in the AD and control brain terminate, we compared the CNBr digest of soluble derivatives purified from the AD and control brain with the digest of the derivatives secreted by Sf9 cells infected with baculovirus expressing APP_{751} (Sf9-Bac_{751}). In a previous study with Lowery et al. (1991), we showed that virtually all of the derivatives secreted by Sf9-Bac_{751} end at $A\beta_{15}$ or $A\beta_{16}$. If any of the soluble APP derivatives produced by the AD or control brain are potentially amyloidogenic forms that contain the entire Aβ, then CNBr digestion will produce $A\beta_{1-35}$ (Figure 1E) instead of the carboxyl-terminal $A\beta_{1-15}$ and $A\beta_{1-16}$ peptides that are produced by digestion of the derivatives secreted by Sf9-Bac_{751}.

The peptides produced by CNBr digestion were separated by reverse-phase liquid chromatography (RPLC) using a C18 column. As expected, digests of the derivatives from AD brain, control brain, and Sf9-Bac_{751} gave similar profiles (Figure 3). In our previous study with Lowery et al. (1991), we showed

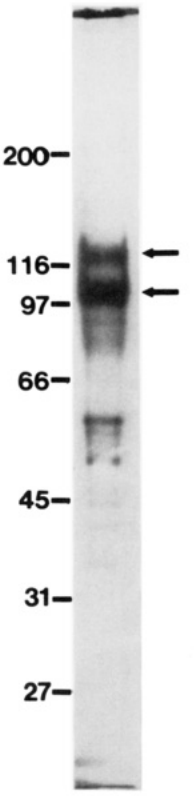

FIGURE 2: Silver stain of soluble APP derivatives purified from AD brain. Note that the purification produced a mixture of ~125- and ~105-kDa derivatives (arrows) with very little contaminating protein.

that $A\beta_{1-15}$ and $A\beta_{1-16}$, identified by sequencing, amino acid composition, and mass spectrometry, elute from the C18 column as a doublet with $A\beta_{1-16}$ eluting slightly before $A\beta_{1-15}$ as illustrated in Figure 3A (arrow). Digestion of the soluble derivatives from both the AD and control brain produced a peptide with the same retention time as $A\beta_{1-15}$ (Figure 3B,C, arrows), and plasma desorption mass spectrometry (Figure 4) showed that this peptide had the expected mass (1826.8 Da) for $A\beta_{1-15}$. Thus, we have shown that human AD and control brain contain soluble derivatives that end at $A\beta_{15}$. Mass spectrometric analysis of fractions adjacent to those containing $A\beta_{1-15}$ failed to detect any $A\beta_{1-16}$. Thus, we have been unable to detect soluble derivatives ending at $A\beta_{16}$.

Since we were particularly interested in determining whether any of the soluble derivatives in human brain are potentially amyloidogenic, we looked carefully in our digests for $A\beta_{1-35}$, the peptide that would be produced by CNBr cleavage of any soluble derivatives that contain the entire Aβ (Figure 1E). To determine the retention time of $A\beta_{1-35}$, we digested synthetic $A\beta_{1-40}$ with CNBr to produce $A\beta_{1-35}$, separated the CNBr digest by RPLC, and confirmed the identity of $A\beta_{1-35}$ by plasma desorption mass spectrometry. We then carefully analyzed the corresponding as well as nearby fractions of the brain digests by mass spectrometry, and we were unable to detect any $A\beta_{1-35}$. Thus, we have been unable to detect any soluble derivatives containing the entire Aβ in human brain, and our data suggest that these derivatives, if they are present at all, are much less abundant than derivatives that end at $A\beta_{15}$. In our study with Lowery et al. (1991) of the derivatives secreted by Sf9-Bac_{751}, we identified a longer carboxyl-terminal peptide, probably $A\beta_{1-28}$ (Figure 3A, arrowhead), in CNBr digests, but this peptide could not be detected by mass spectrometry of the corresponding fractions of the brain digest.

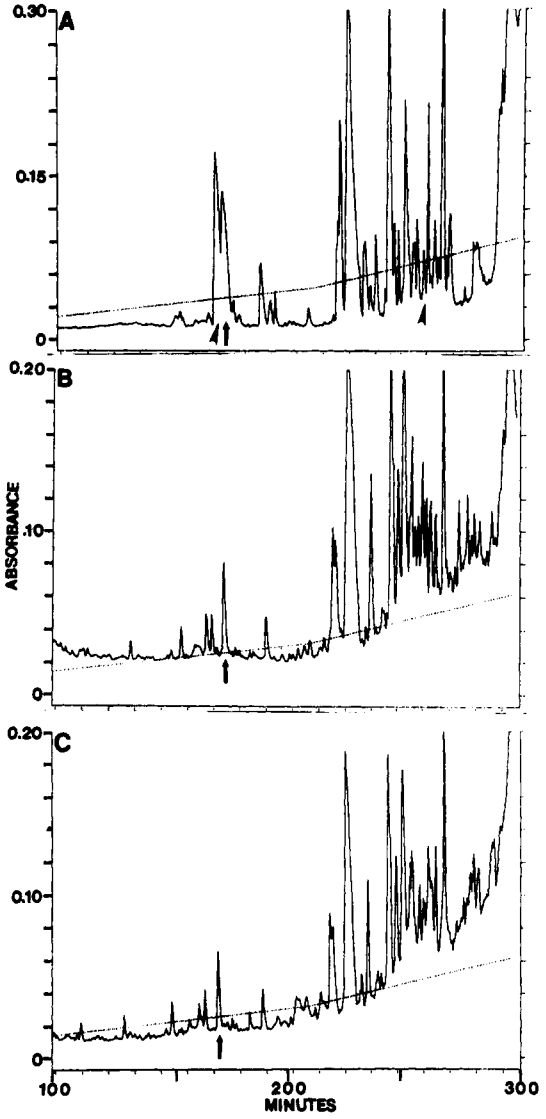

FIGURE 3: C18 chromatograms of CNBr-digested soluble APP derivatives. (A) Sf9-Bac$_{751}$. (B) Control brain. (C) AD brain. Arrows indicate fractions containing Aβ_{1-15}. Arrowheads indicate Aβ_{1-16} and a longer peptide, probably Aβ_{1-28}, identified in Sf9-Bac$_{751}$ but not detected in brain.

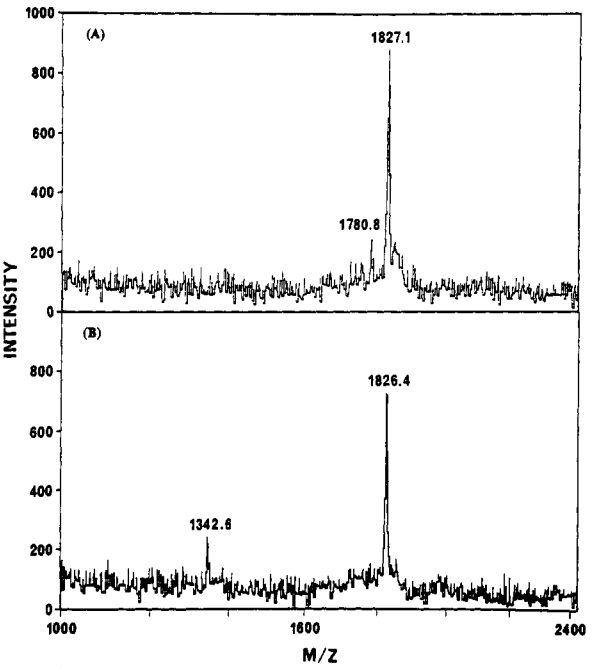

FIGURE 4: Plasma desorption mass spectra of the carboxyl-terminal CNBr fragment of soluble APP derivatives from human brain. (A) Control brain. (B) AD brain.

DISCUSSION

The demonstration that both AD and control brain contain soluble derivatives that end at position 15 of the Aβ indicates that APP secretase activity in brain produces products similar to those found in cell culture systems (Esch et al., 1990; Anderson et al., 1991; Lowery et al., 1991; Wang et al., 1991). Our failure to detect any soluble derivatives containing the full-length Aβ in either the AD or control brain suggests that amyloid is produced by an alternative processing pathway and not by APP secretase.

Recently Estus et al. (1992) have demonstrated that normal processing of the APP in brain produces at least five carboxyl-terminal membrane-associated fragments and that the two largest fragments contain the entire Aβ. A similar set of carboxyl-terminal derivatives was identified in 293 cells transfected with a full-length APP$_{695}$ expression construct. Golde et al. (1992) then examined the transfected cells to identify the cellular processing that produces this complex set of carboxyl-terminal fragments. The results of this study indicate that APP secretase cleaves at a single site, previously shown to be within the Aβ (Esch et al., 1990; Anderson et al., 1991; Lowery et al., 1991; Wang et al., 1991), to produce one secreted derivative and one nonamyloidogenic carboxyl-terminal fragment, whereas endosomal/lysosomal processing produces a complex set of carboxyl-terminal derivatives that includes potentially amyloidogenic forms with the entire Aβ at or near their amino terminus.

Our identification of soluble derivatives ending at Aβ_{15} in both the AD and control brain and our failure to detect soluble derivatives containing the entire Aβ suggest that, in the AD and control brain as in cultured cells, APP secretase clips within the Aβ to prevent amyloid deposition. Alterations in the secretory pathway could, however, play an important role in AD since a decrease in the amount of APP processed in the secretory pathway could increase production of potentially amyloidogenic carboxyl-terminal APP derivatives by increasing the amount of substrate available for the endosomal/lysosomal pathway.

REFERENCES

Anderson, J. P., Esch, F. S., Keim, P. S., Sambamurti, K., Lieberburg, I., & Robakis, N. K. (1991) *Neurosci. Lett. 128*, 126–128.

Autilio-Gambetti, L., Morandi, A., Tabaton, M., Schaetzle, B., Kovacs, D., Perry, G., Greenberg, B., & Gambetti, P. (1988) *FEBS Lett. 241*, 94–98.

Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D., & Ward, P. J. (1990) *Science 248*, 1122–1124.

Estus, S., Golde, T. E., Kunishita, T., Blades, D., Lowery, D., Eisen, M., Usiak, M., Qu, X., Tabira, T., Greenberg, B. D., & Younkin, S. G. (1992) *Science 255*, 726–728.

Glenner, G. G., & Wong, C. W. (1984) *Biochem. Biophys. Res. Commun. 122*, 1131–1135.

Golde, T. E., Estus, S., Younkin, L. H., Selkoe, D. J., & Younkin, S. G. (1992) *Science 255*, 728–730.

Goldgaber, D., Lerman, M. I., McBride, O. W., Saffiotti, U., & Gajdusek, D. C. (1987) *Science 235*, 877–880.

Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K.-H., Multhaup, G., Beyreuther, K., Muller-Hill, B. (1987) *Nature 325*, 733–736.

Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S., & Ito, H. (1988) *Nature 331*, 530–532.

Lowery, D. E., Pasternack, J. M., Gonzalez-DeWhitt, P. A., Zurcher-Neely, H., Tomich, C.-S. C., Altman, R. A., Fairbanks, M. B., Heinrikson, R. L., Younkin, S. G., & Greenberg, B. D. (1991) *J. Biol. Chem. 266*, 19842–19850.

Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L., & Beyreuther, K. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4245–4249.

Morrissey (1981) *Anal. Biochem. 117*, 307–310.

Oltersdorf, T., Ward, P. J., Henrikkson, T., Beattie, E. C., Neve, R., Lieberburg, I., & Fritz, C. (1989) *J. Biol. Chem. 265*, 4492–4497.

Palmert, M. R., Podlisny, M. B., Witker, D. S., Oltersdorf, T., Younkin, L. H., Selkoe, D. J., & Younkin, S. G. (1988) *Biochem. Biophys. Res. Commun. 156*, 432–437.

Palmert, M. R., Podlisny, M. B., Witker, D. S., Oltersdorf, T., Younkin, L. H., Selkoe, D. J., & Younkin, S. G. (1989a) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6338–6342.

Palmert, M. R., Siedlak, S. L., Podlisny, M. B., Greenberg, B., Shelton, E. R., Chan, H. W., Usiak, M., Selkoe, D. J., Perry, G., & Younkin, S. G. (1989b) *Biochem. Biophys. Res. Commun. 165*, 182–188.

Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F., Cordell, B. (1988) *Nature 331*, 525–527.

Robakis, N. K., Wisniewski, H. M., Jenkins, E. C., Devine-Gage, E. A., Houck, G. E., Yao, X.-L., Ramakrishna, N., Wolfe, G., Silverman, W. P., & Brown, W. T. (1987) *Lancet (Feb 14)*, 384–385.

Schubert, D., Schroeder, R., LaCorbiere, M., Saitoh, T., & Cole, G. (1988) *Science 241*, 223–226.

Selkoe, D. J., Podlisny, M. B., Joachim, C. L., Vickers, E. A., Lee, G., Fritz, L. C., & Oltersdorf, T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7341–7345.

Sisodia, S. S., Koo, E. H., Beyreuther, K., Unterbeck, A., & Price, D. L. (1990) *Science 248*, 492–495.

Summers, M. D., & Smith, G. E. (1987) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, pp 10–48, Texas Agricultural Experimental Station, College Station, TX.

Tanzi, R. E., Gusella, J. F., Watkins, P. C., Bruns, G. A. P., St. George-Hyslop, P., Van Keuren, M. L., Patterson, D., Pagan, S., Kurnit, D. M., & Neve, R. L. (1987) *Science 235*, 880–884.

Tanzi, R. E., McClatchey, A. I., Lamperti, E. D., Villa-Komaroff, L., Gusella, J. F., & Neve, R. L. (1988) *Nature 331*, 528–530.

Van Nostrand, W. E., & Cunningham, D. D. (1987) *J. Biol. Chem. 262*, 8505–8514.

Wang, R., Meschia, J. F., Cotter, R. J., & Sisodia, S. S. (1991) *J. Biol. Chem. 266*, 16960–16964.

Wiedemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J. M., Masters, C. L., & Beyreuther, K. (1989) *Cell 57*, 115–126.