Vol. 165, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1989 Pages 182-188

SOLUBLE DERIVATIVES OF THE ß AMYLOID PROTEIN PRECURSOR OF ALZHEIMER'S DISEASE ARE LABELED BY ANTISERA TO THE ß AMYLOID PROTEIN

Mark R. Palmert[1,2], Sandra L. Siedlak[1], Marcia Berman Podlisny[3], Barry Greenberg[4], Earl R. Shelton[5], Hardy W. Chan[5], Marianne Usiak[1,2], Dennis J. Selkoe[3], George Perry[1], and Steven G. Younkin[1,2,*]

[1]Division of Neuropathology, Institute of Pathology, and [2]Department of Pharmacology, Case Western Reserve University School of Medicine, Cleveland OH 44106

[3]Department of Neurology (Neuroscience), Harvard Medical School, and Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA 02115

[4]The Upjohn Company, Kalamazoo, MI 49001

[5]Syntex Research, Division of Syntex (USA) Inc., Palo Alto, CA 94303

Received October 3, 1989

SUMMARY: The amyloid deposited in Alzheimer's disease (AD) is composed primarily of a 39-42 residue polypeptide (ßAP) that is derived from a larger ß amyloid protein precursor (ßAPP). In previous studies, we and others identified full-length, membrane-associated forms of the ßAPP and showed that these forms are processed into soluble derivatives that lack the carboxyl-terminus of the full-length forms. In this report, we demonstrate that the soluble ~125 and ~105 kDa forms of the ßAPP found in human cerebrospinal fluid are specifically labeled by several different antisera to the ßAP. This finding indicates that both soluble derivatives contain all or part of the ßAP sequence, and it suggests that one or both of these forms may be the immediate precursor of the amyloid deposited in AD.

One of the pathologic hallmarks of Alzheimer's disease (AD) is the deposition of amyloid in senile plaques and in cerebral and meningeal vessels. This amyloid is composed primarily of a 39-42 residue polypeptide (ß amyloid protein, ßAP) (1-3) that is derived from a much larger protein referred to as the ß amyloid protein precursor (ßAPP) (4-7). The ßAPP gene, which is located on the long arm of chromosome 21 (4-7), has been shown to produce at least six different mRNAs (8-13). The three major ßAPP mRNAs, which encode proteins of 695, 751, and 770 amino acids, are produced through alternative splicing of two adjacent exons (8). One of these exons encodes a 19-amino acid domain with homology to the MRC OX-2 antigen found on the surface of neurons and thymocytes (14); the other encodes a 56-amino acid domain, reported to inhibit trypsin *in vitro* (8), that is highly homologous to the Kunitz family of serine protease inhibitors. In each of the three major ßAPP mRNAs, the ßAP is encoded as an internal peptide which extends from the extracellular region (~28 amino acids) into the putative membrane-spanning domain (~11-14 amino acids) (5,15) where it ends ~60 amino acids proximal to the carboxyl terminus of the full-length ßAPP (see Fig. 1). It appears, therefore, that proteolytic cleavage of the ßAPP on both the amino and carboxyl sides of the ßAP is necessary to generate the ßAP found in amyloid deposits.

*To whom correspondence should be addressed.

ABBREVIATIONS: **AD**, Alzheimer's disease. **ßAP**, ß amyloid protein. **ßAPP**, ß amyloid protein precursor. **ßAP$_{x-y}$**, peptide at designated location in the ßAP. **ßAPP$_{x-y}$**, peptide at designated location with the ßAPP. **KPI**, Kunitz protease inhibitor.

0006-291X/89 $1.50

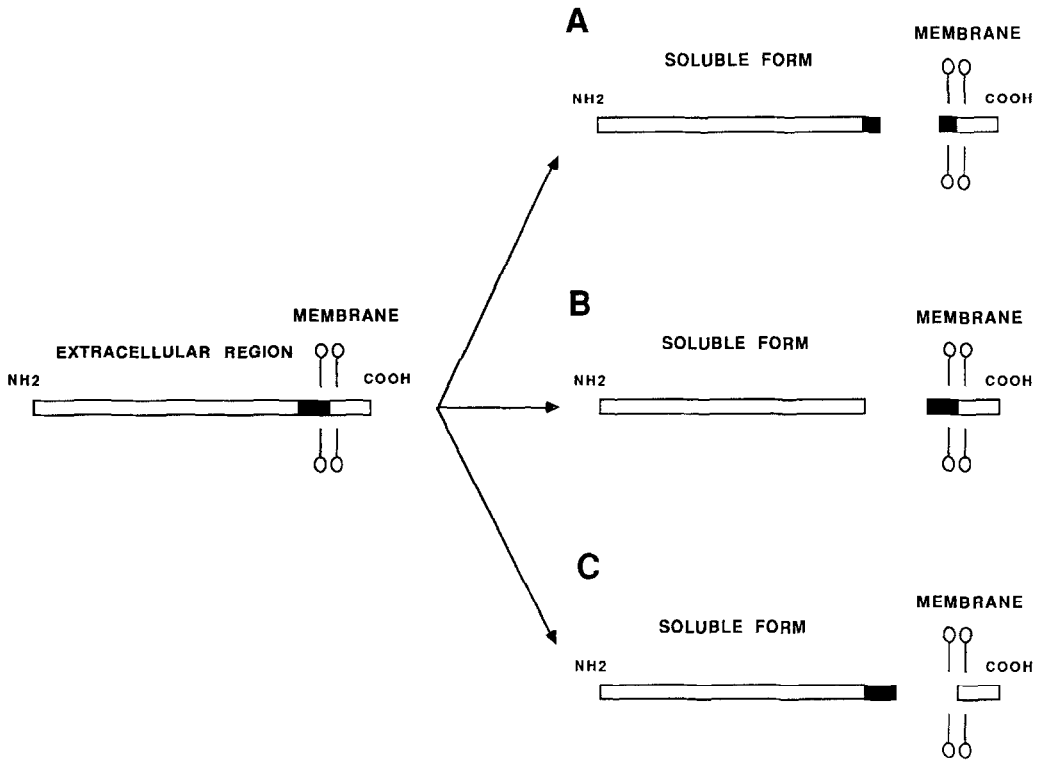

Fig. 1 Cleavage sites within full-length βAPP that could produce the large, soluble derivatives in human brain and CSF. The solid bar in each schematic indicates the position of the βAP. **LEFT.** Full-length membrane-associated βAPP; **A.** Cleavage within the βAP peptide; **B.** Cleavage on the amino side of the βAP; **C.** Cleavage on the carboxyl side of the βAP. In a previous study, we showed that the soluble KPI-containing (~125 kDa) and KPI-free (~105 kDa) βAPP derivatives in human brain and CSF are ~5-10 kDa smaller than the full-length membrane-associated forms from which they are derived, that these derivatives do not require detergent for solubilization, and that they are not labeled by antisera that readily detect the carboxyl terminus of the full-length membrane-associated forms (22). These observations indicate that the soluble forms are produced by a cleavage that removes a ~5 to 10 kDa fragment containing the carboxyl terminus and presumably some or all of the membrane-spanning domain from the full-length molecule. This cleavage must occur in the vicinity of the βAP because the fragments extending from the carboxyl terminus of the βAPP to the carboxyl and amino terminus of the βAP have predicted molecular weights of ~6.5 and ~11 kDa respectively. Our data showing that soluble βAPP derivatives are labeled by antisera to the βAP indicate that the proteolytic event producing these derivatives usually occurs within the βAP as shown in A or on its carboxyl-terminal side as shown in C. It should be noted that, in either the AD or control brain, the cleavage that generates the soluble derivatives could be heterogeneous and occur in more than one of the locations illustrated above.

We and others have identified a set of ~110-135 kDa membrane-associated proteins that represent full-length forms of the βAPP (16-19) and have shown that these membrane-associated forms are processed into soluble derivatives (14,20-22). Specifically, we (22) have identified two soluble derivatives of the βAPP that are readily detected in human brain and cerebrospinal fluid (CSF) and that lack the carboxyl terminus of the full-length membrane-associated forms from which they are derived: a ~125 kDa derivative that contains the Kunitz protease inhibitor (KPI) domain and a ~105 kDa form that lacks this insert. We have confirmed that these two proteins are βAPP derivatives by purifying them from human CSF and directly sequencing their amino termini (22).

The relative sizes of the membrane associated and soluble forms of the βAPP indicate that the proteolytic event generating the soluble derivatives must occur in the vicinity of the βAP (see legend Fig.

1). This cleavage could occur within the βAP peptide itself, producing a large soluble derivative and a small membrane-associated fragment that are not amyloidogenic (Fig. 1A). If the cleavage producing soluble forms normally occurs in this location, then either its location changes in AD or this cleavage is not directly involved in generation of the βAP and amyloid deposition. Alternatively, cleavage could occur on the amino side of the βAP, leaving the βAP attached to a small, membrane-associated protein from which amyloid might be derived (See Fig. 1B and refs. 15,16), or on the carboxyl side of the βAP, leaving the βAP attached to an extracellular protein which could be further processed to release the βAP (Fig. 1C).

To distinguish among these possibilities, we used a series of antibodies that recognize different regions of the βAPP, including the βAP, to determine more precisely the location of the proteolytic event that generates the soluble βAPP derivatives. Our results show that several different antisera to the βAP label both the ~125 and the ~105 kDa βAPP derivatives in AD and control CSF. Thus, in human brain, full-length βAPP is normally cleaved either within the βAP or on its carboxyl-terminal side. This finding eliminates the possibility that cleavage usually occurs on the amino-terminal side of the βAP, and it suggests that βAP-bearing soluble derivatives may be the source of the βAP deposited as amyloid in AD.

MATERIALS AND METHODS

The synthetic peptides homologous to amino acids 18-35 (βAPP_{18-35}) and 573-595 ($\beta APP_{573-595}$) in the Kang sequence (5) as well as the peptide corresponding to the the first 17 amino acids of the βAP (βAP_{1-17}) were synthesized at The Upjohn Company using an Applied Biosystems 430A synthesizer. A Gly-Gly-Cys carboxyl-terminal extension was added to each peptide for spacing and conjugation to porcine thyroglobulin which was used as the carrier protein during production of rabbit antisera. The monoclonal antibody corresponding to the first 10 amino acids of the βAP (anti-βAP_{1-10}) was provided by George Glenner and DuPont. Specificity of labeling with these four antisera was confirmed by peptide absorption. Fifty μg of unconjugated peptide was incubated at 4 C overnight with 1 μl of antiserum diluted (as noted in figure legends) in Tris buffered saline containing 0.05% Tween 20 and 1 mg/ml bovine serum albumin (BSA). After centrifugation at 16,000 x g for 5-10 min, the supernatant was applied to blots.

The synthetic peptides corresponding to amino acids 284-299 ($\beta APP_{284-299}$, Cys-EEVVRVPTTAASTPDA) and to the first 13 residues of the βAP [βAP_{1-13}, DEAFRHDSGY(E->Q)VH-Cys] were prepared at Syntex Research. The "Cys" denotes a terminal Cys added to the sequence to permit coupling to the keyhole limpet hemocyanin used during production of rabbit antisera; the (E->Q) represents a change in peptide sequence as compared to the published protein sequence. These antisera were affinity-purified by immobolizing the antigen to epoxy-activated Sepharose-6B, binding the sera to the resin, and eluting the bound antibodies with 0.1 M glycine buffer, pH 2.8, containing 1 M KCl. Fractions were brought to a final pH of 7.5 using 1 M Tris-acetate, pH 8.4 and adjusted to 1 mg/ml BSA. Specificity of labeling with these two antisera was also demonstrated by peptide absorption in which 500 ng of peptide was combined with 5 μl of antisera in a BSA-phosphate buffered saline solution.

The antiserum raised to amino acids 45-62 in the Kang sequence (anti-βAPP_{45-62}) has been described previously (17,22) as has the procedure for purifying the soluble βAPP derivatives from human CSF (22). The soluble derivatives used in the immunoblots shown in Fig. 2 were purified from 319 mls of CSF collected at autopsy from three histologically proven AD patients [ages = 77, 82, and 77 yrs; post-mortem intervals (PMI) = 3.5, 5.5, and 2.5 hrs]. The pure proteins used for Fig. 3 were obtained from CSF collected at autopsy from three other AD patients (ages = 96, 78, and 77 yrs; PMI = 7, 4, and 6.5 hrs). Two hundred fifty μl of crude CSF from an AD patient (age = 74 yrs; PMI = 4 hrs) was exchanged into 1 mM phosphate buffer, pH 7.4 and concentrated 20 fold for use in lane 9 of Fig. 3.

Immunoblot analysis was performed on nitrocellulose blots of 5-15% SDS-polyacrylamide gels using 5% defatted milk to block non-specific binding of primary anitbodies and either goat anti-rabbit IgG coupled to alkaline phosphatase (Promega) or goat anti-mouse IgG,M, and A coupled to horseradish peroxidase (Cappel) for immunodetection.

RESULTS AND DISCUSSION

Fig. 2 shows immunoblots of the ~125 and ~105 kDa derivatives of the βAPP purified from AD CSF and labeled with an antiserum to amino acids 18-35 in the Kang sequence (anti-βAPP_{18-35}). This

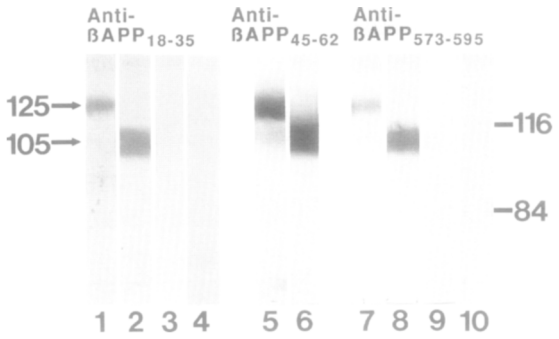

Fig. 2. Immunoblots of soluble ~125 and ~105 kDa βAPP derivatives purified from human CSF and labeled with antisera to peptides proximal to the βAP. Lanes 1-4 show labeling with anti-βAPP_{18-35} (1:100): lane 1, ~125 kDa form; lane 2, ~105 kDa form; lane 3, ~125 kDa form, antibody absorbed with βAPP_{18-35}; lane 4, ~105 kDa form, antibody absorbed with βAPP_{18-35}. Lanes 5 (~125 kDa) and 6 (~105 kDa) show labeling with anti-βAPP_{45-62} (1:100). Lanes 7-10 show labeling with anti-$\beta APP_{573-595}$ (1:100): lane 7, ~125 kDa form; lane 8, ~105 kDa form; lane 9, ~125 kDa form, antibody absorbed with $\beta APP_{573-595}$; lane 10, ~105 kDa form, antibody absorbed with $\beta APP_{573-595}$. Gel is 5-15% SDS-polyacrylamide; bars show molecular weights in kDa.

antiserum detected both proteins, and the labeling was specific because it could be abolished by absorption with the βAPP_{18-35} peptide. This finding is consistent with our previous sequence analysis (22) which demonstrated that the amino termini of these two proteins begin at residue 18 of the Kang sequence (5), after removal of the signal peptide identified by Dyrks *et al.* (15). As a positive control, adjacent lanes of the blot were labeled with anti-βAPP_{45-62}, an antiserum to amino acids 45-62 in the Kang sequence, which we have shown specifically labels the ~125 and ~105 kDa derivatives (22). In addition, we labeled the blot with an antiserum raised against residues 573-595 in the Kang sequence (anti-$\beta APP_{573-595}$) and found, as demonstrated in Fig. 2, that this antiserum specifically detected both proteins. These data and our previous sequence analysis indicate that the soluble derivatives begin at residue 18 and extend at least to within the 573-595 domain, just proximal to the βAP which begins at residue 597 of the Kang sequence.

To determine whether the soluble ~125 and ~105 kDa derivatives extend to within the βAP sequence, we labeled immunoblots of the purified proteins with several antibodies raised against the βAP. Fig. 3 shows that a monoclonal antibody to the first 10 amino acids of the βAP sequence (residues 597-606 in the Kang sequence, anti-βAP_{1-10}) detected both proteins and that this labeling was abolished by absorption with an appropriate peptide. Fig. 3 also demonstrates that an antiserum raised to the first 13 amino acids of the βAP (anti-βAP_{1-13}) and an antiserum to the first 17 residues in the βAP sequence (anti-βAP_{1-17}) specifically detected both proteins. Although not shown here, two other βAP antibodies--an antiserum against the first 38 amino acids of the βAP (anti-βAP_{1-38}) and an antiserum raised to isolated meningovascular amyloid (ref. 23)--also specifically detected the purified ~125 and ~105 kDa proteins. Although five of the βAP antisera that we used recognized the purified ~125 and ~105 kDa proteins, not all antisera raised to domains within the βAP detected the two derivatives. In fact, six other antisera that recognize amyloid deposits in AD brain (four raised to βAP peptides and two against purified βAP) failed to stain the soluble forms of the βAPP, presumably because these sera recognize βAP epitopes that are present in fibrillar amyloid deposits but not in the native forms of the βAPP.

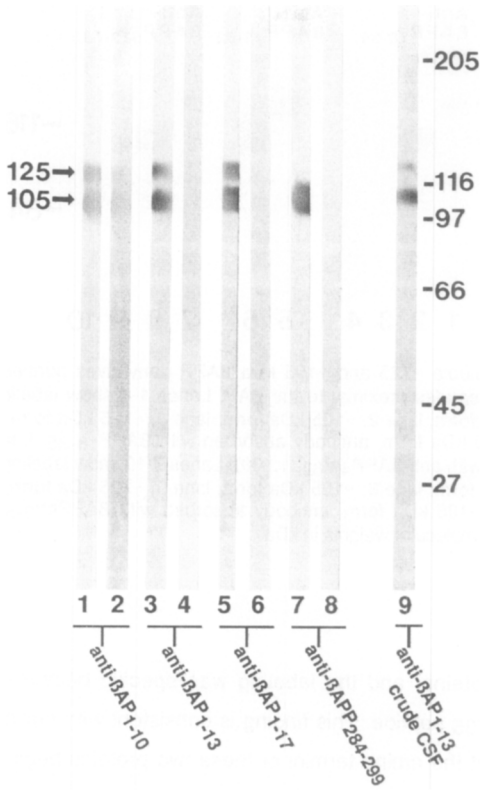

Fig. 3. Immunoblots of soluble ~125 and ~105 kDa βAPP derivatives purified from human CSF and labeled with antisera to regions within the βAP and to a $βAPP_{695}$-specific junctional peptide. Lane 1, anti-$βAP_{1-10}$ (monoclonal, 1:10); lane 2, anti-$βAP_{1-10}$ absorbed with $βAP_{1-42}$; lane 3, anti-$βAP_{1-13}$ (affinity-purified, 1:20); lane 4, anti-$βAP_{1-13}$ absorbed with $βAP_{1-13}$; lane 5, anti-$βAP_{1-17}$ (1:100); lane 6, anti-$βAP_{1-17}$ absorbed with $βAP_{1-17}$; lane 7, anti-$βAPP_{284-299}$ (affinity-purified, 1:20); lane 8, anti-$βAPP_{284-299}$ absorbed with $βAPP_{284-299}$; lane 9, crude CSF from an AD patient stained with anti-$βAP_{1-13}$ (affinity-purified, 1:20). Gel is 5-15% SDS-polyacrylamide; bars show molecular weights in kDa.

We have also examined the βAPP derivatives in crude CSF from several AD and control patients using anti-$βAP_{1-13}$ and anti-$βAP_{1-17}$. Anti-$βAP_{1-17}$ detected the two derivatives in their unpurified form in 3 AD cases and 3 controls, and as illustrated in the last lane of Fig. 3, the affinity-purified anti-$βAP_{1-13}$ also recognized the ~125 and ~105 kDa proteins in crude CSF.

We have previously shown that anti-C_1 [an antiserum that recognizes the carboxyl terminus of full-length, membrane-associated βAPP (16,17)] fails to detect the ~125 and ~105 kDa soluble proteins in human brain tisue and CSF (22). In the present study, we stained immunoblots of the purified proteins with four additional antisera against the carboxyl terminus of the full-length molecules: one raised to the last 15 residues of the βAPP, two to the last 20 amino acids, and one to the last 30 residues. Consistent with our previous result, each of these antisera readily detected the full-length, membrane-associated forms of the βAPP but none of them recognized the purified soluble derivatives. Thus the soluble derivatives that we have identified lack the carboxyl terminus of the full-length forms and presumably some or all of the hydrophobic membrane spanning domain. Both soluble derivatives do, however, extend to at least within the $βAP_{1-10}$ domain, and both may contain the entire AP sequence.

Finally, we stained the ~125 and ~105 kDa proteins with a "junctional" antiserum designed to recognize only the form of the βAPP that lacks the two alternatively spliced exons encoding the 19 amino acid and the KPI domains (See Material and Methods). As shown in Fig. 3, this antiserum detected only the ~105 kDa protein. This finding is consistent with a previous study (22) in which we demonstrated that the ~125 kDa protein, but not the ~105 kDa derivative, is specifically detected by an antiserum to the KPI domain. Together the two studies demonstrate that the ~125 kDa soluble protein contains the KPI domain whereas the ~105 kDa protein lacks this insert.

De Sauvage and Octave (12) have recently identified a novel βAPP mRNA that would encode a soluble KPI-containing form of the βAPP. This mRNA is similar to the βAPP_{751} mRNA (8-10) but lacks the sequence encoding the 208 amino acids at the carboxyl terminus of βAPP_{751}, a region that includes the βAP and the putative membrane-spanning domain. Our data demonstrating that the ~125 and ~105 kDa proteins are labeled by antisera to $\beta APP_{573-595}$ and to the βAP, regions not encoded in the recently described βAPP mRNA, indicate that these derivatives are produced from full-length βAPP mRNAs and not from the truncated mRNA described by de Sauvage and Octave.

Wiedemann *et al.* (14) have reported that the soluble forms of the βAPP produced by cultured PC12 (rat pheochromocytoma) cells have molecular weights which are ~17-18 kDa less than those of the membrane-associated proteins. Since the predicted size of the fragment extending from the amino terminus of the βAP through the membrane-spanning domain to the carboxyl terminus of the βAPP is ~11 kDa, Weidemann *et al.* (14) concluded that the cleavage producing the truncated soluble forms must occur on the amino-terminal side of the βAP (See Fig. 1B). Such processing would produce small, membrane-associated fragments bearing the βAP, and it would likely be the further processing of these fragments that is required for amyloid deposition in AD. Analysis of soluble forms would therefore be unlikely to reveal the final processing that gives rise to the βAP. Our discovery that all or part of the βAP sequence is present on the soluble derivatives from human CSF radically changes this perspective. This finding indicates that the cleavage producing the ~125 and ~105 kDa derivatives either occurs within the βAP sequence or on its carboxyl side (Fig. 1A, C), and it suggests that one or both of these derivatives may be the immediate precursor of the extracellular amyloid deposited in AD. This possibility, which is consistent with our previous data showing that the soluble forms in human brain and CSF are produced by a ~5-10 kDa truncation (22), is particularly attractive because it would liken AD to the other known amyloidoses in which the amyloid peptides are produced from soluble precursor proteins (24-26).

ACKNOWLEDGMENTS. We thank Satish Sharma and Jim Cornette of the Upjohn Company for antibody preparation and Linda Maggiora and Tom Lobl of the Upjohn Company for peptide synthesis and purification. We would also like to thank George Glenner and DuPont for providing the anti-βAP_{1-10} antiserum. Supported by USPHS grants AG06656 (SGY), MH43444 (SGY),5T32GM0250 (MRP), KO4-AG00415 (GP), AG07775 (GP), AG07911 (LEAD award, DJS), and grants from the the American Health Assistance Foundation (GP) and the State of Ohio (GP,SGY).

REFERENCES

1. Glenner, G. G. and Wong, C. W. (1984) Biochem. Biophys. Res. Commun. **120**, 885-890.
2. Masters, C. L., Simms, G., Weinmann, N. A., Multhaup, G., McDonald, B. L., and Beyreuther, K. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4245-4249.
3. Selkoe, D. J., Abraham, C. R., Podlisny, M. B., and Duffy, L. K. (1986) J. Neurochem. **146**, 1820-1834.

4. Goldgaber, D., Lerman, M. I., McBride, O. W., Saffiotti, U., and Gajdusek, D. C. (1987) Science **235**, 877-880.
5. Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K.-H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) Nature (London) **325**, 733-736.
6. Robakis, N. K., Ramakrishna, N., Wolfe, G., and Wisniewski, H. M. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 4190-4194.
7. 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., and Neve, R. L. (1987) Science **235**, 880-884.
8. Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S., and Ito, H. (1988) Nature (London) **331**, 530-532.
9. Ponte, P., Gonzalez-DeWhitt, P., Schilling J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F., and Cordell, B. (1988) Nature (London) **331**, 525-527.
10. Tanzi, R. E., McClatchey. A. I., Lamperti, E. D., Villa-Komaroff, L., Gusella, J. F., and Neve, R. L. (1988) Nature (London) **331**, 528-530.
11. Mita, S., Sadlock, J., Herbert, J., and Schon, E. A. (1988) Nucl. Acid Res. **16** (19), 9351.
12. De Sauvage, F., and Octave, J. N. (1989) Science **245**, 651-653.
13. Golde, T. E., Estus, S., Usiak, M., Younkin, L. H., and Younkin, S. G., submitted.
14. Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J. M., Masters, C., Beyreuther, K. (1989) Cell **57**, 115-126.
15. Dyrks, T., Weidemann, A., Multhaup, G., Salbaum, J. M., Lemaire, H-G., Kang, J., Muller-Hill, B., Masters, C. L., and Beyreuther, K. (1988) EMBO J. **7**, 949-957.
16. Selkoe, D. J., Podlisny, M. B., Joachim, C. L., Vickers, E. A., Lee, G., Fritz, L. C., and Oltersdorf, T. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7341-7345.
17. Palmert, M. R., Podlinsy, M. B., Witker, D. S., Oltersdorf, T., Younkin, L. H., Selkoe, D. J., and Younkin, S. G. (1988) Biochem. Biophys. Res. Commun. **156**, 432-437.
18. Autillo-Gambetti, L., Morandi, A., Tabaton, M., Schaetzle, B., Kovacs, D., Perry, G., Greenberg, B., and Gambetti, P. (1988) Febs. Letters **241**, 94-98.
19. Takio, K., Hasegawa, M., Titani, K., and Ihara, Y. (1989) Biochem. Biophys. Res. Commun. **160**, 1296-1303.
20. Schubert, D., Schroeder, R., LaCorbiere, M., Saitoh, T., and Cole, G. (1988) Science **241**, 223-226.
21. Schubert, D., LaCorbiere, M., Saitoh, T., and Cole, G. (1989) Proc. Natl. Acad. Sci. USA **86**, 2066-2069.
22. Palmert, M. R., Berman-Podlinsy, M., Witker, D. S., Oltersdorf, T., Younkin, L. H., Selkoe, D. J., and Younkin S. G. (1989) Proc. Natl. Acad. Sci. USA. **86**, 6338-6342.
23. Joachim, C. L., Duffy, L. K., Morris, J. H., and Selkoe, D. J. (1988) Brain Research **474**, 100-111.
24. Cohen, A. S., and Connors, L. H. (1987) J. Pathol. **151**, 1-10.
25. Castano, E. M., and Frangione, B. F. (1988) Lab. Invest. **58** (2), 122-132.
26. Kisilevsky, R. (1988) Can. J. Physiol. Pharmacol. 65, 1805-1815.