Demonstration of a 72 K β -protein precursor fragment in Alzheimer and normal aged brain

FUYUKI KAMETANI, KIKUKO TANAKA, SEIICHI HAGA, MAROTO SATOH, DAVID ALLSOP and TSUYOSHI ISHII

Laboratory of Molecular Biology, Psychiatric Research Institute of Tokyo, Kamikitazawa, Setagayaku, Tokyo 156, Japan

ABSTRACT

To identify the components of amyloid precursor protein (APP) in brain, studies were performed using an immunoblotting method on Alzheimer and control brains with antibodies to synthetic peptides consisting of subsequences of APP. Immunoblots of brain homogenates detected an immunoreactive protein with a molecular weight of 72 K. There was no difference in the patterns of immunoreactive bands when comparing normals and Alzheimer's disease. This protein was recognized by both anti- β -peptide and anti-APP C-terminal antibodies, suggesting that this is a stable intermediate that is further processed by proteolysis to form amyloid.

The deposition of amyloid is considered as the most characteristic hallmark in the brains of patients with Alzheimer's disease (AD) or Down's syndrome in middle age. The isolation and sequence analysis of β -protein (or A4) found in cerebrovascular amyloid (3) and in senile plaque core amyloid (10) has allowed identification of cDNA clones encoding its precursor protein (APP) (6). The predicted sequence of APP suggests a 695 residue glycosylated cell-surface protein with a single membrane-spanning domain (6). Some forms of the APP mRNA contain (due to alternative splicing) a Kunitz-type protease inhibitor sequence interpolated at residue 289 (7). The presence of mRNA which encodes APP has been confirmed in several organs including nervous tissue (4, 16). *In situ* hybridization studies indicate that many neurons in the brain contain APP mRNAs, but the patterns of localization do not seem to correlate closely with those of amyloid deposition (1, 2, 9). The presence of various molecular weight forms of APP in brain has been reported (13-15).

However, the exact posttranslational processing mechanisms which generate amyloid fibrils from APP are still unknown. To help to resolve this question, we have produced antibodies to synthetic peptides consisting of subsequences of APP. In a previous study, we examined sections of the brain using immunohistochemical methods (5). In this paper, we present evidence for the presence of a 72 K immunoreactive protein in AD and normal elderly brain extracts. This protein was recognized by both anti- β -peptide and anti-C-terminal antibodies, and so may represent a stable intermediate which accumulates prior to the conversion of APP into amyloid in the AD brain.

Two peptides were synthesized according to the sequence deduced from cDNA of APP. The method of production and confirmation of the specificity of the antibodies have been described elsewhere (5). Briefly, R37 and β -peptide are identical in sequence to residues 681-695 and 597-620 of the APP respectively (6). Polyclonal antisera were raised in rabbits

Fig. 1 Two-dimensional gel immunoblots of AD brain soluble fraction with anti- β -peptide and anti-C-terminal R37. A: anti- β -peptide, B: anti- β -peptide absorbed by β -peptide, C: gold colloid staining after immunoblotting with anti- β -peptide, D: anti-C-terminal R37, E: anti-C-terminal R37 absorbed by R37, F: gold colloid staining after immunoblotting with anti-C-terminal R37. IEF, isoelectric focusing; SDS, SDS-PAGE. Arrows (major) and arrowhead (minor) indicate specifically labeled spots.

against these peptides coupled to keyhole limpet hemocyanin through an additional C-terminal cysteine by activation with *m*-maleimido-benzoyl-*N*-hydroxysuccinimide ester (5).

Postmortem brains from 4 AD (aged 82, 84, 86, 88) and 2 non-AD normal subjects (aged 57, 73) were used. Frontal cortical gray matter (2–3 g wet weight) was homogenized with a Teflon glass homogenizer in 4 vol of 8 M urea/2% Nonidet P-40/5% mercaptoethanol/0.1 mM phenylmethyl sulfonyl fluoride solution and sonicated for 30 sec. After centrifugation at 23,000 *g* for 45 min, supernatant fractions were diluted with the sample buffer and analyzed using a minigel apparatus by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (8) and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (11). The precipitates were extracted with 99% formic acid at room temperature for 24 h, and the extracts were lyophilized and suspended in the sample buffer for SDS-PAGE. The separated proteins were transferred to Immobilon (Millipore, Bedford, MA) (17). Immunoreac-

tive proteins were detected by immunochemical staining using the avidin-biotin complex procedure with a kit and the method recommended by Vector Labs., Burlingame, CA, U.S.A. The method of Schapira and Keir (12) was used to detect all proteins transferred to the Immobilon membranes.

The results of immunoblots with both anti- β -peptide and anti-C-terminal R37 antibodies are shown in Figs. 1 and 2. In the 8 M urea/2% Nonidet P-40/5% mercaptoethanol soluble fraction, both antibodies recognized apparently identical protein spots with the same isoelectric point and molecular weight of around 72 K as shown in Fig. 1. This identity was tested by detecting all the proteins transferred from the two dimensional gels using a colloidal gold reagent as shown in Fig. 1, C and F (indicated by arrow). From the positions of the immunoreactive spots against the background pattern of transferred proteins, it was apparent that these two antibodies recognized the same protein spot. A minor protein spot of 110 K was also detected with anti-R37.

Table 1 Summary of Reactivities Observed with Antibodies to β -peptide and C-terminal R37

	Anti- β -peptide	Anti-C-terminal R37
AD brain		
Immunoblot		
Soluble fraction	72 K	72 K, 110 K
Insoluble fraction	Smear	Not found
Immunohistochemistry (5)	Amyloid in senile plaques and amyloidotic vessels	Degenerate neurites in senile plaques
Normal brain		
Immunoblot		
Soluble fraction	72 K	72 K, 110 K
Insoluble fraction	Not found	Not found
Immunohistochemistry (5)	Not found	Not found

The antibodies absorbed with the corresponding peptides showed greatly reduced reactivity against the spots described above as shown in Fig. 1, B and E, and these were not detected by the pre-immune serum or by antibodies to

uncoupled keyhole limpet hemocyanin (data not shown). No consistent difference in the pattern or intensity of immunoblots was observed in the soluble fractions of the AD and normal control brains (Table 1). A broad smear with molecular weight < 17 K was detected only with anti- β -peptide in the formic acid extract of AD brain (Fig. 2).

Immunohistochemical studies on cryostat sections of the AD and normal control brains with these antibodies have been described elsewhere (5). The antibody to the C-terminal R-37 peptide stained small clusters of material in senile plaques which were thought to be degenerating neurites. The results of immunohistochemical studies are summarized in Table 1.

The presence of APP mRNA and various forms of APP has been reported in the brain as well as in other organs (4, 5, 13-16). An important question is whether the whole sequence of APP persists in the brain, or whether APP is present in the form of fragments produced by proteolytic processing, and in the latter case where and how fragmentation occurs. In the present study, immunoblot analysis of homogenates of the AD and normal brains disclosed that immunoreactive materials against antibodies to R37 and β -peptide consisted mainly of a 72 K protein with a minor 110 K spot. These results agree with those of Simpson *et al.* (15). These spots may be fragments of APP, although amino acid sequence analysis is required to confirm this.

It was reported by Selkoe *et al.* (13) that an

Fig. 2 One-dimensional gel immunoblots of AD and normal control brain insoluble fraction with anti- β -peptide and anti-C-terminal R37. A: anti- β -peptide, B: anti-C-terminal R37. Lane 1, normal brain; lane 2, AD brain. Only AD brain extracts reacted with anti- β -peptide. No reaction was seen with anti-C-terminal R37.

antibody to the C-terminal portion (676–695) of APP reacted mainly with 110–135 K protein in brain homogenates. However, in our present study, the anti- β -peptide and anti-R37 antibodies mainly recognized a 72 K protein together with a minor 110 K protein in the AD and normal control brain (Fig. 1). Using our antibody to C-terminal R37, Selkoe *et al.* also recognized a strongly reactive 72 K band with minor 110–130 K bands, whereas his antibody to residues 676–695 of APP detected only the 110–135 K protein (see Fig. 1 in Selkoe's paper). Using antibody kindly provided by Dr Selkoe, we have confirmed the latter finding. Whether or not the 72 K protein is a partially processed form or degradation product of the 110–135 K protein described by Selkoe *et al.* (13) is not clear. Regardless, our results suggest that this 72 K protein is a major stable intermediate containing both β and C-terminal regions. Excision of the C-terminal part from this 72 K protein may be the final stage in the pathway to amyloid fibril formation in the AD brain. A previous immunohistochemical study suggests that the C-terminal part remains in the degenerating neurites (5). We are currently attempting to isolate and sequence this and other immunoreactive proteins.

We thank Mr K. Kato for the photographs. This study was supported in part by the Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, the grants for the Research of Dementia and for Primary Amyloidosis from the Ministry of Health and Welfare, Japan.

Received for publication 1 February 1989; and in revised form 10 February 1989

REFERENCES

1. BAHMANYAR S., HIGGINS G. A., GOLDGABER D., LEWIS D. A., MORRISON J. H., WILSON M. C., SHANKAR S. K. and GAJDUSEK D. C. (1987) Localization of amyloid β protein messenger RNA in brains from patients with Alzheimer's disease. *Science* **237**, 77–80
2. COHEN M. L., GOLDE T. E., USIAK M. F., YOUNKIN L. H. and YOUNKIN S. G. (1988) *In situ* hybridization of nucleus basalis neurons shows increased β -amyloid mRNA in Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **85**, 1227–1231
3. GLENNER G. G. and WONG C. W. (1984) Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **120**, 885–890
4. GOLDGABER D., LERMAN M. I., MCBRIDE O. W., SAFFIOTTI U. and GAJDUSEK D. C. (1987) Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease. *Science* **235**, 877–880
5. ISHII T., KAMETANI F., HAGA S. and SATO M. (1989) The immunohistochemical demonstration of subsequences of the precursor of the amyloid A4 protein in senile plaques in Alzheimer's disease. *Neuropathol. Appl. Neurobiol.* (in press)
6. KANG J., LEMAIRE H.-G., UNTERBECK A., SALBAUM J. M., MASTERS C. L., GRZESCHIK K.-H., MÜLTHAUP G., BEYREUTHER K. and MÜLLER-HILL B. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **325**, 733–736
7. KITAGUCHI N., TAKAHASHI Y., TOKUSHIMA Y., SHIOJIRI S. and ITO H. (1988) Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. *Nature* **331**, 530–532
8. LAEMMLI U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
9. LEWIS D. A., HIGGINS G. A., YOUNG W. G., GOLDGABER D., GAJDUSEK D. C., WILSON M. C. and MORRISON J. H. (1988) Distribution of precursor amyloid- β -protein messenger RNA in human cerebral cortex: Relationship to neurofibrillary tangles and neuritic plaques. *Proc. Natl. Acad. Sci. USA* **85**, 1691–1695
10. MASTERS C. L., SIMMS G., WEINMAN N. A., MÜLTHAUP G., McDONALD B. L. and BEYREUTHER K. (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc. Natl. Acad. Sci. USA* **82**, 4245–4249
11. O'FARRELL P. H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007–4021
12. SCHAPIRA A. H. V. and KEIR G. (1988) Two-dimensional protein mapping by gold stain and immunoblotting. *Anal. Biochem.* **169**, 167–171
13. SELKOE D. J., PODLISNY M. B., JOACHIM C. L., VICKERS E. A., LEE G., FRITZ L. C. and OLTERSDORF T. (1988) β -Amyloid precursor protein of Alzheimer disease occurs as 110- to 135-kilodalton membrane-associated proteins in neural and nonneural tissues. *Proc. Natl. Acad. Sci. USA* **85**, 7341–7345
14. SHIVERS B. D., HILBICH C., MÜLTHAUP G., SALBAUM M., BEYREUTHER K. and SEEBURG P. H. (1988) Alzheimer's disease amyloidogenic glycoprotein: expression pattern in rat brain suggests a role in cell contact. *EMBO J.* **7**, 1365–

- 1370
15. SIMPSON J., BLADON C. M., YATES C. M. and HARMAR A. J. (1988) An antiserum to the C-terminus of the Alzheimer amyloid precursor recognizes a soluble 70 kDa protein. *FEBS Lett.* **237**, 196-198
 16. 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., KUR-
NIT D. M. and NEVE R. L. (1987) Amyloid β protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. *Science* **235**, 880-884
 17. TOWBIN H., STAEBELIN T. and GORDON J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354