Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 745–749, January 1989
Neurobiology

Identification of cellular and extracellular sites of amyloid precursor protein extracytoplasmic domain in normal and Alzheimer disease brains

(senile plaques/neurons/glia/corpora amylacea)

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Communicated by Irwin Fridovich, October 27, 1988 (received for review September 22, 1988)

ABSTRACT **Information concerning the distribution of various subdomains of the amyloid precursor protein (APP) in brain may illuminate aspects of the normal metabolism of this membrane-associated protein, as well as putative abnormal processing that may occur in Alzheimer disease (AD). We prepared affinity-purified antibody, P2, against an extracytoplasmic APP site and applied it, along with monoclonal antibodies to the β-peptide, or A4 region, in conjunction with selective cytochemical staining methods, to control and AD tissues. The following was noted: (*i*) in contrast to A4 epitopes, which are easily demonstrable primarily in extracellular senile plaques of AD patients, the extracytoplasmic P2 antigen was found in association with neurons, glia, and blood vessels in both normal and AD prefrontal cortex; (*ii*) a subset of senile plaques contained both A4 and P2 antigens; (*iii*) in some instances, P2 antigen occurred as an extracellular deposit in the absence of A4; (*iv*) the P2 antigen, but not A4, was also associated with corpora amylacea. In addition to identifying the unique cellular distribution of the APP extracytoplasmic antigen, the results support the view that a segment of this domain undergoes processing and deposition at extracellular sites, including a subset of senile plaques.**

The structure of Alzheimer amyloid precursor protein (APP) cDNA has been determined (1–5) and shown to correspond to a protein of 695 amino acids that has features in common with glycosylated cell-surface receptors (1). Of the APP domains, the A4 (or β-peptide) region consisting of ≈42 amino acids appears to extend from the extracytoplasmic domain to the transmembrane site (1). Immunologic and biochemical data have firmly established that in the Alzheimer disease (AD) brain the A4 polypeptide is a major component of cerebrovascular and senile plaque amyloid (6–11). However, no detailed information has been available concerning the cellular or extracellular localization of the extracytoplasmic domain (ECD) of the APP and its distribution with regard to A4 and senile plaques.

The present report addresses the issue of localization of the ECD by means of an affinity-purified antibody, P2, raised to a unique peptide within the extracytoplasmic region of the APP. Both control and AD brains were stained with P2, anti-A4 monoclonal antibodies (mAbs) (10), and anti-glial fibrillary acidic protein, in addition to selective cytochemical counterstains. We report here that an extracytoplasmic APP domain has unique features in both control and AD brains that distinguish it from A4. We report, further, that certain senile plaques of the AD brain contain the extracytoplasmic P2 antigen in addition to the A4 domain and that the P2 antigen alone can accumulate extracellularly.

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A portion of these data were reported previously in preliminary form (12).

MATERIALS AND METHODS

Source of Brains. Postmortem brains with the neuropathological diagnosis of AD were obtained from the Massachusetts Alzheimer's Disease Research Center, Neuropathology Core at the Massachusetts General Hospital, through the courtesy of E. T. Hedley-Whyte and S. M. de la Monte; and the McLean Hospital Brain Tissue Resource Center, courtesy of E. D. Bird. Controls came from the same sources.

Preparation of Antiserum. A polypeptide containing amino acid residues 413–429 (P2 antigen) of the APP (1) was prepared by methods previously described (10). The polypeptide was determined to have a unique sequence as indicated by searches facilitated by BIONET (IntelliGenetics) data bases. Initial injection of rabbits was as follows: 5 mg of P2 antigen was combined with 100 μg of protein adjuvant (Sigma), 0.5 ml of phosphate buffer (100 mM, pH 7.4), plus 1 ml of Freund's complete adjuvant. Animals were then injected at monthly intervals without protein adjuvant in Freund's incomplete adjuvant. A second series of rabbits were injected with P2 antigen conjugated to edestin (Sigma). P2 peptide (10 mg) was solubilized in formic acid followed by the addition of dimethylformamide and water. The pH was brought to 4.5 with NaOH and the peptide was incubated with 20 mg of edestin plus 10 mg of *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDC, Bio-Rad). The reaction proceeded overnight at 25°C. The conjugate was dialyzed against 0.1 M phosphate buffer, lyophilized, and used for injections after it was suspended in 0.5 ml of phosphate buffer plus 1 ml of Freund's complete adjuvant; 5 mg of P2 edestin was used per injection. Further injections utilized Freund's incomplete adjuvant.

Affinity Purification of Antibodies. P2 antigen was coupled to AH Sepharose 4B (Pharmacia) as follows: 20 mg of peptide was dissolved in 99% formic acid, dimethylformamide, and H_2O (1:6:4). The mixture was adjusted to pH 4.5 with 5 M NaOH. AH Sepharose 4B gel (2 ml) was prepared in the same solution. EDC was added to a final concentration of 0.1 M. The reaction proceeded for 24 hr at room temperature. Rabbit serum was passed through the P2 column. The antibodies were eluted from the gel with 0.2 M glycine, 0.15 M NaCl (pH 2.5), quickly neutralized with 2 M Tris (pH 8.0), concentrated by precipitation with 50% ammonium sulfate, resuspended,

Abbreviations: APP, amyloid precursor protein; AD, Alzheimer disease; ECD, extracytoplasmic domain; mAb, monoclonal antibody; CA, corpora amylacea; DAB, diaminobenzidine; GFAP, glial fibrillary acidic protein; PAS, periodic acid/Schiff.

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and dialyzed against 100 mM sodium phosphate (pH 7.4). The reactivity of antisera derived from immunizations with P2 alone or P2 conjugated to edestin gave the same immunocytochemical results. Therefore, sera were pooled and the combined antibodies are referred to as P2 antisera. Typically, 6 ml of serum led to the recovery of 1 ml of affinity-purified antibodies. This volume was used for dilution studies to assess the potency prior to using P2 for immunocytochemistry, as indicated in *Results*. Diluted antisera were assessed by the nitrocellulose immunoblot method, as detailed previously (10).

Tissue Staining. Formalin-fixed tissue was cryopreserved with 30% sucrose and cut on a cryostat at 40 μm. Tissue was processed with the avidin–biotin–horseradish peroxidase complex (Vector) that was visualized with diaminobenzidine (DAB) and H_2O_2, as described elsewhere (13). For double immunostaining, either rhodamine- or fluorescein-conjugated goat anti-IgGs (Cappel) were used (14). To increase the sensitivity of the reaction, a three-step procedure was used. Incubation with the primary antibody overnight at room temperature was followed by a 2-hr incubation with the appropriate biotinylated anti-IgG (Jackson Immunologicals) diluted 1:100 in Tris-buffered saline (TBS) plus 0.5% Triton X-100 (TBST) and 2% bovine serum albumin. This was followed by a 2-hr incubation with either Texas Red or fluorescein, each of which was conjugated to streptavidin (Jackson Immunologicals) diluted 1:100 in the same buffer. Antiserum to glial fibrillary acidic protein (GFAP) was purchased from Dako and has been described (14). Antibodies to the A4 region of the APP were characterized in detail elsewhere (10). For thioflavin S staining, tissue sections were immersed in a 1% solution of the dye (obtained from Sigma) in 70% ethanol for 30 min followed by several washes in 70% ethanol and a final rinse in 20 mM Tris, pH 7.4/0.15 M NaCl (TBS). All immunofluorescent sections were viewed with a Zeiss epifluorescent microscope equipped with the appropriate filters for either fluorescein or rhodamine. Thioflavin S staining was observed with fluorescein filters and Texas Red was observed with rhodamine filters.

Periodic Acid/Schiff (PAS) Staining. The procedure was modified from an earlier method (15). Unstained tissue sections, or those that had been processed for immunocytochemistry, were mounted on slides coated with 1% gelatin and air dried. Slides were hydrated and immersed in periodic acid for 5 min, rinsed in distilled water, and immersed in Schiff reagent for 15 min. Slides were then subjected to sulfurous (15) and distilled water rinses and were then air dried and coverslipped.

Methods of Plaque Quantitation for Table 1. Quantitation of plaques was carried out by the following procedures. (*i*) P2 and A4 antigen in thioflavin-positive plaques: A section of frontal cortex from an AD brain was immunostained with P2 antibody or anti-A4 mAbs using the avidin–biotin method, with DAB as the chromogen. After mounting and air drying, the slides were counterstained with thioflavin S. Thirty-five thioflavin-positive plaques were identified and examined in each of three brains for the presence or absence of P2 and A4 antigen. The average values are reported. (*ii*) P2 antigen in A4-positive plaques: Cortical sections from three AD brains were immunostained with P2 antibody by the avidin–biotin method, as before, as well as with anti-A4 mAbs that were visualized with fluorescently labeled secondary antibody. Thirty-five plaques that were immunopositive for A4 were examined in layers I–VI in each of the three brains and judged to be positive or negative for P2 antigen. The percentage of A4-positive plaques containing P2 was calculated for each brain and an average for the three brains was determined. (*iii*) A4 antigen in P2-positive deposits: Sections from two AD brains were immunostained with P2 antibody using a fluorescently labeled secondary antibody and with anti-A4 mAbs by the avidin–biotin procedure, as before. Eighteen P2-positive accumulations were identified and examined for the presence or absence of A4 antigen. The average number of P2-positive plaques containing A4 was calculated.

PAS-Positive CA-Containing P2 Antigen. A section of frontal cortex from an AD brain and a section of hippocampus from a second AD brain were stained by the PAS method and then processed for immunocytochemical localization of P2 antigen by the avidin–biotin technique, as before. In frontal cortex (layers I–VI), 100 PAS-positive CA were examined and those containing P2-positive reaction product were scored. In hippocampus, 123 PAS-positive CA were examined by the same method (see *Results*). The diameter of CA was determined by standard micrometric procedures. The procedure was repeated with anti-A4 mAbs (10).

RESULTS

P2 Antisera: Characterization and Application to Control and AD Brains. Antisera were raised to extracytoplasmic APP amino acids 413–429 (1). The latter region was chosen for study since database searches identified this sequence as a unique polypeptide without appreciable homology with other known proteins. Immunoblotting experiments indicated that affinity-purified P2 antisera prepared under the stated conditions detected 1 μg or less of antigen at dilutions of 1:100 to 1:5000. Under the same experimental conditions, P2 antisera did not detect A4 antigen, and neither preimmune serum nor anti-A4 mAbs reacted with P2 antigen as assayed by the immunoblotting procedure.

In contrast to anti-A4 mAbs (10), P2 stained normal control prefrontal cortex and reacted with both neuronal and apparent nonneuronal elements (Fig. 1*A*). A portion of the latter appeared as well-circumscribed spheres of varying diameters (see below). Counterstaining with cresyl violet verified that the DAB reaction product was localized to cortical cells with the morphology of neurons, particularly, but not exclusively, the larger pyramidal cells (Fig. 1*B*). Also, unlike anti-A4 antibody reactivity, blood vessels of normal control brains that were not thioflavin positive were readily immunostained by P2 (Fig. 1*C*). Four control brains exhibited similar staining patterns.

Immunostaining of five AD cortices with P2 revealed striking similarities to the control cases since both neuronal and other elements were visualized (compare Fig. 1 *A* and *D*). Similar observations were made on neurons of hippocampus and amygdala, which also exhibited a homogeneous distribution of the antigen over cell bodies and large dendrites (Fig. 1*D Insets*). Preimmune serum subjected to the affinity-purification procedure was without appreciable immunoreactivity, as indicated by the matched tissue sections of Fig. 1 *E* and *F*. Preincubation of P2 with the immunogen (20 μg/ml) eliminated the characteristic discrete staining of both cellular and noncellular elements in matched sections. Preincubation with A4 peptide (20 μg/ml) had no effect on P2 immunostaining.

In a previous report, we noticed discrete lamination of A4-positive deposits in the AD cortex (10). While the distribution of P2 was not uniformly related to cortical lamellae, similar to previous observations, there was a noticeable tendency of P2 reaction product to be more heavily concentrated in supragranular layers.

P2 Antigen Is Associated with Astrocytes. Certain of the smaller cells detected by P2 appeared nonneuronal. This possibility was verified by an AD cortical section that was doubly stained with anti-GFAP and P2 using an immunofluorescence technique and an immmunoperoxidase method, respectively (Fig. 2 *A* and *B*).

P2 Antigen Is Associated with a Subset of Senile Plaques. To determine whether or not senile plaques contained the P2

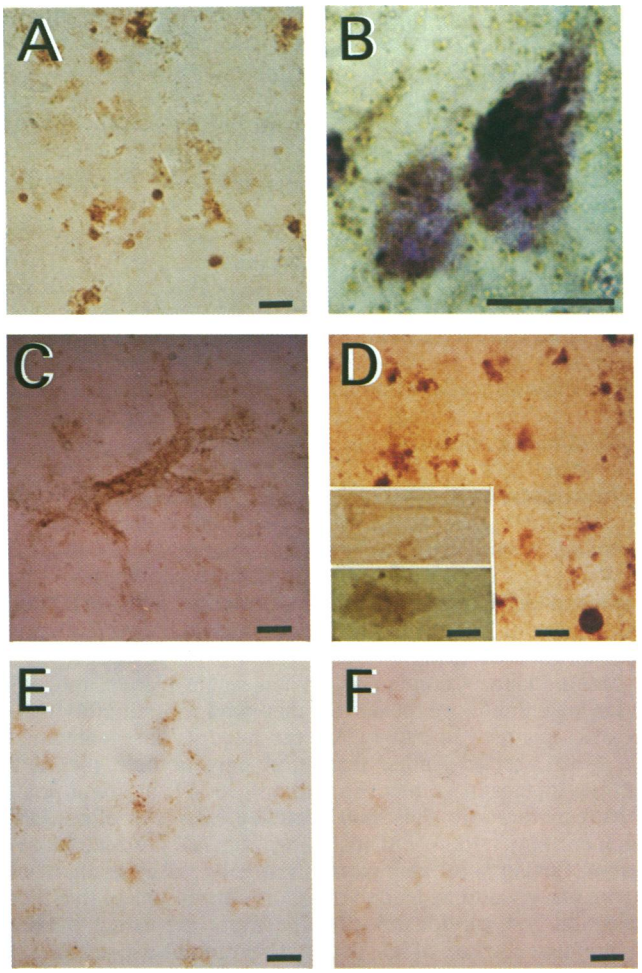

FIG. 1. Immunostaining of normal control and AD brain sections using affinity-purified P2 antibody. (*A*) Normal control prefrontal cortex. (*B*) High magnification of a section of prefrontal cortex counterstained with cresyl violet, in which large pyramidal neurons are shown. (*C*) A blood vessel in the same brain as shown in *A*. (*D*) AD prefrontal cortex. (*Upper Inset*) AD hippocampal neuron and processes. (*Lower Inset*) Neuron of AD amygdala. (*E* and *F*) Matched sections of prefrontal cortex of a normal control brain stained with either P2 (*E*) or affinity-purified preimmune serum (*F*). (Bars = 20 μM.)

antigen, AD cortical sections were immunoprocessed and then counterstained with thioflavin S. Certain of the thioflavin S-positive plaques (e.g., Fig. 2*C*) contained heavy deposits of P2 reaction product (Fig. 2*D*). The distribution and morphologic appearance of P2 antigen in plaques varied from discrete foci, as shown, to a more diffuse appearance.

The relationship between sites of A4 deposition in senile plaques and the distribution of P2 antigen was examined by double-immunostaining experiments using a mixture of anti-A4 mAbs (4E12, 5E2, and 10H3; ref. 10) and P2 antisera applied to AD cortical sections. Plaques containing the A4 region of the APP precursor (fluorescent label, Fig. 2*E*) were identified that also contained the P2 region of the extracytoplasmic domain (DAB label, Fig. 2*F*). The same results were obtained with A4 and P2 antibodies when double-immunofluorescence methods were used.

Quantitation of P2 and A4 Antigens in Plaques. The presence of an extracytoplasmic APP domain in senile plaques was explored in more detail by quantitative studies applied to additional AD brains. As shown in Table 1 (Exp. 1), those plaques identified by thoiflavin S staining were uniformly A4 positive, and the vast majority (89.5%) of the thioflavin S-positive plaques also contained the P2 antigen. We then

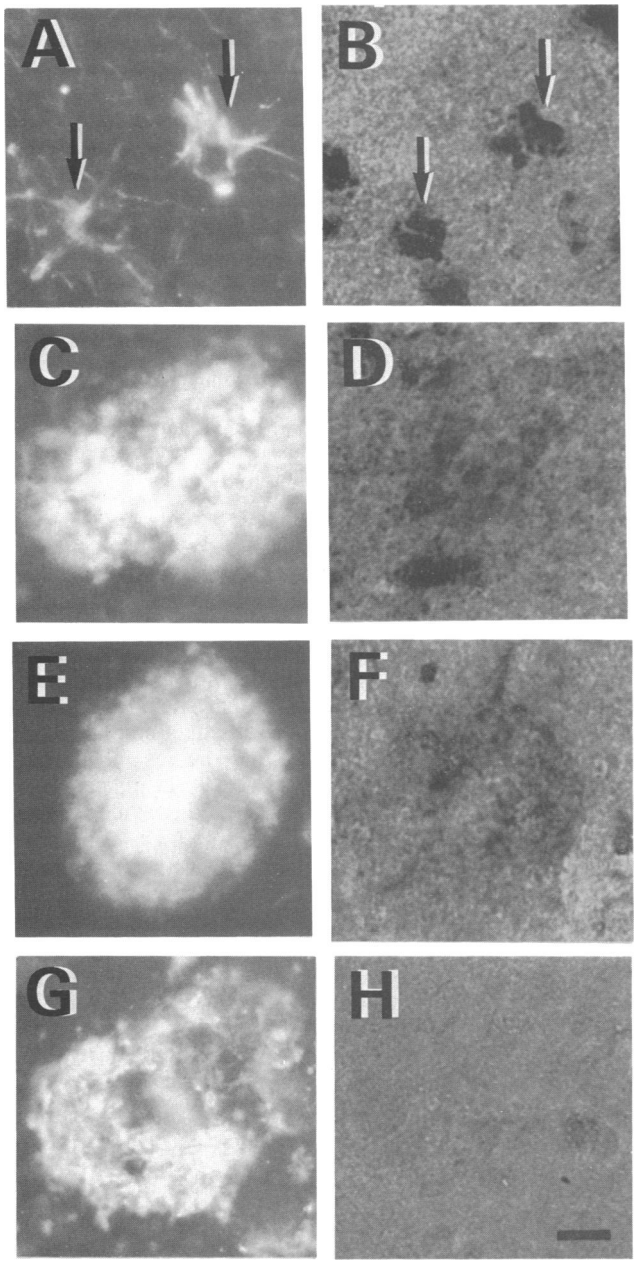

FIG. 2. Immunostaining of AD brain sections with P2, anti-GFAP, anti-A4, and thioflavin S. (*A*) Prefrontal cortex of an AD brain fluorescently stained with antibody against GFAP. Arrows denote astroglia. (*B*) P2 DAB stain of the same section shown in *A* indicating that the same cells were detected in both cases. (*C*) Thioflavin S staining of a plaque in the prefrontal cortex of an AD brain. (*D*) P2 DAB staining of the same plaque shown in *C*. (*E*) Anti-A4 mAb fluorescent staining of a plaque in the prefrontal cortex of an AD brain. (*F*) P2 stain of the same plaque shown in *E*. (*G*) P2 fluorescent stain of the prefrontal cortex of an AD brain. (*H*) Anti-A4 mAb DAB staining of the same area shown in *G*. (Each panel photographed at the same magnification; bar = 20 μm.)

examined the quantitative distribution of P2 and A4 antigens within plaques. Consistent with the previous results, double-immunostaining studies revealed that 88% of plaques identified by A4 mAbs were also P2 positive (Table 1, Exp. 2). In some AD cortical sections, irregularly shaped extracellular accumulations of P2 antigen were observed. Of these, 75% also contained the A4 epitope (Table 1, Exp. 3). However, the remaining sites appeared to contain the P2 antigen as the exclusive extracellular deposit. Comparative examples are shown in Fig. 2 *G* and *H*. Similar P2 deposits were also seen in control brain tissue.

Table 1. Distribution of P2 and A4 antigens in senile plaques

	% of plaques	*n*
Exp. 1		
A4 positive	100	105
P2 positive	89.5	105
Exp. 2	88	105
Exp. 3	75	18

Three separate experiments were carried out. In Exp. 1, senile plaques of three AD brains were identified by thioflavin S staining and the average number of A4- and P2-positive plaques was counted and is expressed as percentage of the total. Exp. 2, three AD brains were surveyed. Plaques that were identified by anti-A4 immunostaining were examined for P2 antigen; the average value of the latter is expressed as percentage of the total exhibiting A4 reactivity. For Exp. 3, two AD brains were used. Sites that were identified by P2 antibody immunostaining were examined for A4 antigen content and the average value of the latter is indicated as percentage of those exhibiting P2 reactivity. *n* values indicate the number of plaques examined in each experiment.

P2 Antigen Is Associated with CA. We observed that immunostaining of cortical and hippocampal sections with P2 frequently revealed the appearance of spherical bodies containing reaction product at their periphery and/or within the core (Fig. 3*A*). The size (usually 10–12.5 μm diameter) and morphologic appearance of these elements, as well as their concentration at subpial and perivascular sites, led to speculation that they represented glycogen-containing CA (16); this was confirmed by PAS staining. The resemblance of P2-positive spheres and PAS-positive CA is indicated by comparison of Fig. 3 *A* and *B*. Corroborating evidence was obtained by double-staining reactions in which P2-positive spheres were also PAS positive in AD cortex and hippocampus (Fig. 3*C*). However, not all PAS-positive CA were P2 positive (Fig. 3*C*, arrow). Although infrequently encountered, P2-positive CA were also observed in the elderly normal brain. Quantitative evaluation of one AD frontal cortex revealed that the P2 antigen was present in 48% of CA examined; in a second AD brain, 85% of hippocampal CA were P2 positive. By contrast, we did not detect the A4 epitope in CA (Fig. 3*D*). However, CA were occasionally observed in close proximity to senile plaques (Fig. 3*D*, arrows).

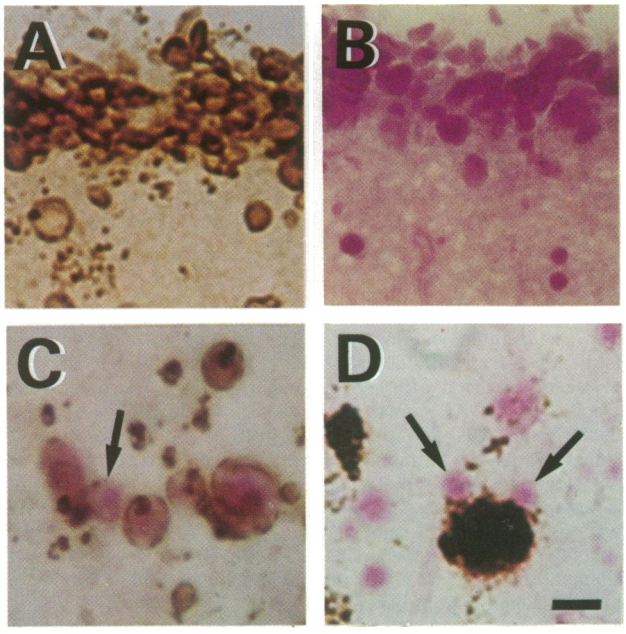

FIG. 3. Staining of CA by both P2 and PAS in an AD brain. (*A*) P2 stain of prefrontal cortex of an AD brain. Note the abundance of P2-positive spheres beneath the pial surface. (*B*) PAS stain of a similar area of the same brain. (*C*) P2 and PAS double stain of CA in hippocampus of an AD brain. The P2 DAB reaction product was observed within and/or around PAS-positive CA. Arrow denotes a sphere that was PAS positive but not detected by P2 (see text). (*D*) Anti-A4 and PAS double stain of prefrontal cortex of an AD brain. Note that anti-A4 does not stain PAS-positive CA. Arrows indicate CA in close proximity to the A4 deposit. (Each panel photographed at the same magnification; bar = 20 μm.)

DISCUSSION

Cellular Distribution of Antigens. APP mRNA is found in nonneural tissues as well as brain (2, 17). However, the A4 peptide is most easily demonstrated by immunologic techniques in extracellular amyloid deposits and affected blood vessels of the AD brain (8–11). These observations suggest that at least a portion of the A4 peptide is normally blocked from reacting with standardly applied immunologic probes at cellular sites. In distinct contrast, P2 antigen corresponding to an extracytoplasmic APP region is readily demonstrated in association with neurons, glia, and blood vessels of both control and AD cases. These findings suggest that there is no apparent difference between nondemented and AD brain with regard to the cellular distribution of the P2 APP domain in prefrontal cortex.

Senile Plaque Components. When senile plaque antigens were examined, we observed that while all thioflavin S-positive plaques contained A4, the majority also contained P2. Thus, senile plaque formation may involve the APP extracytoplasmic domain, in addition to A4, at some point during maturation. This may occur by extrusion of an APP segment larger than, but including, the A4 site and subsequent further processing; alternatively, degeneration of nerve processes may lead to the same consequence. In either case, the A4 peptide may be the more persistent plaque component because of its stability to proteolytic digestion (7) and tendency to aggregate (18).

Other Extracellular P2 Deposits. While the APP ECD may be more readily degraded, to some extent, than the A4 peptide, nevertheless we made the unexpected observation that the P2 antigen accumulated at extracellular sites that were devoid of A4. Quantitative data were presented on AD cases; however, other studies showed similar results for normal brain (unpublished observations). At least a portion of the ECD occurs extracellularly in sufficient abundance to be observed as immunologically detectable accumulations. However, since this type of deposit may not be associated with the molecular pathogenesis of AD, it may represent a normal aspect of APP metabolism.

P2 in CA. CA are spherical PAS-positive bodies found in the central nervous system that are known to increase with age and in degenerative conditions (16, 19). They have been associated with both neurons (20–22) and astrocytes (23). A distinct difference between A4 and P2 antigens was the presence of the latter in CA. Perhaps germane to the present investigations was the observation that, in AD, CA were found within synaptic processes in Alzheimer striatum and caused distortion of synaptic vesicles (24). It has been known for some time that CA contain a glycogen-like substance (25) and recent studies identified a glycosaminoglycan as a major constituent (26). It was recently determined that proteoglycan secreted from PC12 cells shares similarities with the ECD of APP (27). Thus, there is a striking parallel between these data and our own observation that an ECD site is associated with PAS-positive CA.

Conclusions. The APP extracytoplasmic region occurs in control and AD brains in association with CA and certain extracellular sites that are devoid of A4. Thus, the ECD may undergo processing mechanisms that are unique to this domain. Concomitantly, the data also support the association

of ECD antigen with a number of AD senile plaques. Thus, the possibility exists that more than one processing mechanism is involved in the metabolism of different APP subdomains and that at least one mechanism is unique to the AD brain and involves both A4 as well as non-A4 sites.

The authors thank T. Wheelock for technical advice on staining protocols. This work was supported by funds from the following sources: National Institutes of Health Grant AG02126; Alzheimer's Disease Research, a program of the American Health Assistance Foundation, the Axelrod Family Fund, the Katherine Smith Bolt Fund, the Familial Alzheimer's Disease Research Foundation, and the Sandoz Foundation for Gerontological Research. The Massachusetts Alzheimer's Disease Research Center is supported by National Institutes of Health Grant AG05134 and the McLean Hospital Brain Tissue Resource Center is supported by Grant MH/NS31862. Sequence searches carried out by means of BIONET are supported by National Institutes of Health, Division of Research Resources, RR01865.

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