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ANTISERA TO AN AMINO-TERMINAL PEPTIDE DETECT THE AMYLOID PROTEIN PRECURSOR OF ALZHEIMER'S DISEASE AND RECOGNIZE SENILE PLAQUES

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SUMMARY: The cerebral amyloid deposited in Alzheimer's disease (AD) contains a 4.2 kDa ß amyloid polypeptide (ßAP) that is derived from a larger ß amyloid protein precursor (ßAPP). Three ßAPP mRNAs encoding proteins of 695, 751, and 770 amino acids have previously been identified. In each of these, there is a single membrane-spanning domain close to the carboxyl-terminus of the ßAPP, and the 42 amino acid ßAP sequence extends from within the membrane-spanning domain into the large extracellular region of the ßAPP. We raised rabbit antisera to a peptide corresponding to amino acids 45-62 near the amino-terminus of the ßAPP. We show that these antisera detect the ßAPP by demonstrating that they (i) label a set of ~120 kDa membrane-associated proteins in human brain previously detected by antisera to the carboxyl-terminus of ßAPP and (ii) label a set of ~120 kDa membrane-associated proteins that are selectively overexpressed in cells transfected with a full length ßAPP expression construct. The $ßAPP_{45-62}$ antisera specifically stain senile plaques in AD brains. This finding, along with the previous demonstration that antisera to the carboxyl-terminus of the ßAPP label senile plaques, indicates that both near amino-terminal and carboxyl-terminal domains of the ßAPP are present in senile plaques and suggests that proteolytic processing of the full length ßAPP molecule into insoluble amyloid fibrils occurs in a highly localized fashion at the sites of amyloid deposition in AD brains.

In the brains of patients with Alzheimer's disease (AD), proteinaceous amyloid fibrils are deposited in senile plaque cores and blood vessel walls. A 4.2 kDa polypeptide, referred to as A4 or the beta amyloid protein (ßAP), has been isolated from the amyloid in vessels (1) and plaque cores (2,3). Using oligonucleotides based on the ßAP sequence, several groups (4-7) have isolated cDNAs that encode ßAP as part of a 695 amino acid ß amyloid protein precursor (ßAPP). Two additional ßAPP mRNAs encoding proteins of 751 and 770 amino acids have subsequently been identified (8-10). Based on the sequence of $ßAPP_{695}$ cDNA, Kang *et al.* (4) have developed a model which predicts that, in each of the three ßAPPs,

ABBREVIATIONS: AD, Alzheimer's disease. **ßAP**, beta amyloid polypeptide islolated from the amyloid in AD brain. **ßAPP**, beta amyloid protein precursor. **$ßAPP_{45-62}$**, synthetic peptide corresponding to amino acids 45-62 in the ßAPP. **C_1**, synthetic peptide corresponding to the 20 amino acids at the carboxyl-terminus of the ßAPP. **SP28**, synthetic peptide corresponding to amino acids 1-28 in the ßAP.

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there is a single membrane-spanning domain close to the carboxyl-terminus and that the 42 amino acid βAP sequence extends from within the membrane-spanning domain into the putative extracellular region of the βAPP. Thus, two cleavages, one at the amino-terminus and one at the carboxyl-terminus of the βAP, appear to be required to release the βAP from the βAPP.

As part of an effort to evaluate the cellular location and post-translational processing of the βAPP, antisera were prepared to a peptide corresponding to amino acids 45-62 of the βAPP (βAPP_{45-62}). While these antisera were being characterized, (i) two reports were published showing that an antiserum to a cytoplasmic domain ($\beta APP_{666-676}$ in βAPP_{695}) labels a 92 kDa protein in human muscle (11) and mouse muscle and brain (12); (ii) Selkoe *et al.* reported that antisera to the carboxyl-terminus of the predicted βAPP (anti-C_1) specifically detect a set of ~110-135 kDa membrane-associated proteins in brain, a variety of peripheral tissues, and cultured cells (13,14); and (iii) Tabaton *et al.* (15) reported that an antiserum to synthetic βAP specifically detects a 130 kDa protein in human brain and fibroblasts. Moreover, Selkoe *et al.* (14) showed that the set of ~120 kDa proteins labeled by anti-C_1 represent the βAPP by transfecting cells with a full-length βAPP expression construct and showing marked augmentation of specifically immunolabeled proteins in the appropriate size range. In this report, we describe the initial results of a collaboration designed to compare βAPP_{45-62} antisera with anti-C_1.

MATERIALS AND METHODS

The synthetic peptide used in this study corresponds to amino acids 45-62 (βAPP_{45-62}) of the 695-residue protein predicted from the full length cDNA isolated by Kang *et al.* (4). The βAPP_{45-62} peptide was prepared by Peninsula Laboratories and generously provided by P. Gambetti and G. Perry. The data in this report are based on antisera from two of the three rabbits that we immunized with βAPP_{45-62} peptide linked to keyhole limpet hemocyanin. These two antisera labeled the same proteins on immunoblots but with different intensities. Antiserum to SP28, a synthetic peptide homologous to the first 28 amino acids of the βAP [amino acids 597-624 of the Kang sequence (4)], was generously provided by B. Frangione. The antiserum raised to a synthetic peptide corresponding to 676-695 (C_1) of the Kang sequence has been described previously (14).

Immunoblots and immunocytochemistry were performed using frozen tissue from histologically confirmed AD (age = 72 years, post-mortem interval = 8.5 hours) and control (age = 89 years, post-mortem interval = 3 hours) brains obtained at autopsy. Selkoe *et al.* (14) previously described the human 293 embryonic kidney cells employed here. Transcription in these cells, which express recombinant βAPP_{695} cDNA, is driven by the human cytomegalovirus immediate early gene enhancer/promoter unit. The construct also contains the SV40 t-splice and late polyadenylation signal.

Membrane-associated proteins were prepared as described by Selkoe *et al.* (14). Tissue was homogenized in 2 volumes of TS buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 2 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 μg/ml pepstatin, and 1 μg/ml TLCK, pH 7.6), and membrane-associated proteins were extracted from the 100,000 g pellet with TS containing 2% Triton X-100. Immunoblot analysis was performed on nitrocellulose blots of 7% SDS-polyacrylamide gels using 5% defatted milk to block non-specific binding of primary antibody and goat anti-rabbit IgG coupled to alkaline phosphatase (Promega) for immunodetection. Immunocytochemistry employed the Vectastain-Elite ABC kit (Vector laboratories) and was carried out conventionally on cryostat sections from tissue blocks that had been rapidly frozen in isopentane cooled with liquid nitrogen. Cryostat sections were fixed 10 min in 10% phosphate-buffered formalin (Fisher) prior to immunostaining.

Specificity of immunochemical reactions was confirmed by peptide absorption. Fifty μg of unconjugated peptide were incubated at 4°C overnight with 1 μl of antiserum diluted (1:100 or 1:300) in Tris buffered saline containing either (i) 0.05% Tween 20 and 1 mg/ml bovine serum albumin for immunoblots or (ii) 1% normal goat serum for immunocytochemistry. After centrifugation at 16,000 x g for 5-10 min, the supernatant was applied to blots or tissue sections.

RESULTS AND DISCUSSION

Fig. 1A shows immunoblots of membrane-associated proteins from human cerebral cortex stained with antisera to C_1 and to βAPP_{45-62}. As previously reported, anti-C_1 labels a set of ~110-135 kDa proteins in human brain (lane 2) that are not detected after absorption with C_1 (14). Anti-βAPP_{45-62} labels a set of proteins that comigrates with the set of ~120 kDa proteins detected by anti-C_1 as well as a ~55 kDa protein (lane 1). Anti-βAPP_{45-62} does not, however, label the ~11 kDa protein previously reported to react with anti-C_1 (14), as expected from its carboxyl-terminal location (data not shown). Absorption with

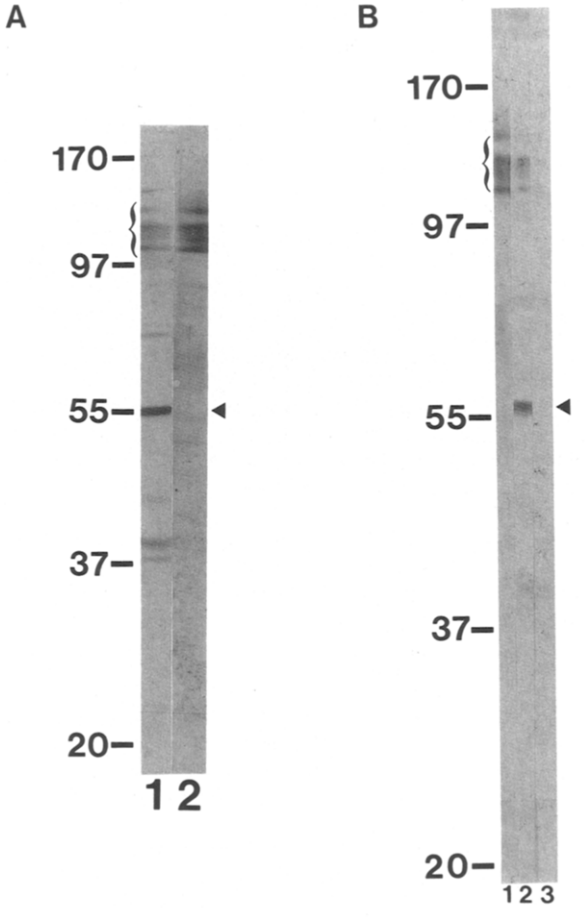

Fig. 1. Immunoblots of membrane-associated proteins in human cerebral cortex. **A.** Comparison of anti-C_1 and anti-βAPP_{45-62} staining. Lane 1, anti-βAPP_{45-62} (1:100); lane 2, anti-C_1 (1:150). The ~55 kDa protein detected by anti-βAPP_{45-62} in lane 1 may represent a fragment of the βAPP containing the amino-terminal region. **B.** Staining of membrane-associated human cerebral cortical proteins with anti-βAPP_{45-62} before and after absorption with βAPP_{45-62}. Lane 1 is stained with anti-C_1 (1:150) to aid in visualizing the proteins in lane 2, which are stained with anti-βAPP_{45-62} (1:100). Lane 3 is stained with anti-βAPP_{45-62} (1:100) that was absorbed with the βAPP_{45-62} peptide. Gels are 7% SDS-polyacrylamide; all lanes loaded with 70 μg protein. Molecular weight markers (bars) are 170, 97, 55, 37, and 20 kDa. The proteins identified in these blots (brackets, arrows) are those that have been shown by peptide absorption to be specifically labeled (Fig. 1B). The proteins that continue to be labeled after peptide absorption are presumably unrelated to the βAPP. The four membrane-associated proteins (brackets) detected by both anti-C_1 and anti-βAPP_{45-62} have calculated molecular weights of 131, 121, 115, and 111 kDa that are essentially identical to those previously reported by Selkoe *et al.* (14). The prominent membrane associated protein labeled by anti-APP_{45-62} (arrow) and not detected by anti-C_1 has a calculated molecular weight of 54 kDa.

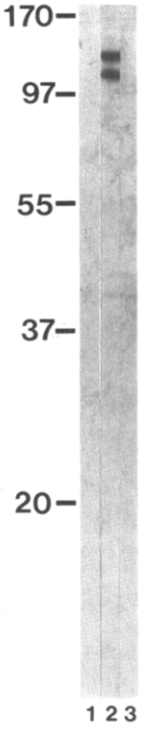

Fig. 2. Membrane-associated proteins labeled by anti-ßAPP$_{45-62}$ in human 293 embryonic kidney cells transfected with a full length ßAPP$_{695}$ expression construct. Lane 1 shows protein from control 293 cells stained with anti-ßAPP$_{45-62}$ (1:300), lane 2 shows protein from transfected 293 cells stained with anti-ßAPP$_{45-62}$ (1:300), and lane 3 shows protein from transfected cells stained with anti-ßAPP$_{45-62}$ (1:300) after absorption with ßAPP$_{45-62}$. Gels are 7% SDS-polyacrylamide; all lanes loaded with 20 μg protein. Molecular weight markers (bars) as in Fig. 1. The two intensely labeled proteins in lane 2 have calculated molecular weights of 130 and 117 kDa.

ßAPP$_{45-62}$ abolishes the immunolabeling of both the 120 kDa complex and the ~55 kDa protein (Fig. 1B, compare lanes 2 and 3). Anti-ßAPP$_{45-62}$ detects similar ~120 kDa and ~55 kDa membrane-associated proteins in rat brain and in human nucleus basalis of Meynert, hippocampus, and cerebellum (data not shown). Preliminary comparison of AD and control brains revealed no obvious qualitative differences in the proteins labeled by anti-ßAPP$_{45-62}$.

Fig. 2 shows immunoblots of membrane-associated proteins in control human 293 embryonic kidney cells and in 293 cells transfected with a full length ßAPP$_{695}$ expression construct. Anti-ßAPP$_{45-62}$ stains two proteins in the 110-135 kDa range that are intensely labeled in the transfected cells (lane 2) and not detected in control cells (lane 1). As shown in lane 3, the immunostaining of these proteins in the transfected cells is completely abolished by absorption with ßAPP$_{45-62}$.

The data in Figs. 1 and 2 demonstrate that, in brain and cDNA-transfected cells, anti-ßAPP$_{45-62}$ labels the set of ~110-135 kDa membrane-associated proteins previously identified as ßAPP using anti-C_1. Selkoe *et al.* (14) observed that anti-C_1 stains senile plaques in the hippocampus of patients with AD. Fig. 3 shows that anti-ßAPP$_{45-62}$ also stains senile plaques (A) and demonstrates that this staining is removed by absorption with ßAPP$_{45-62}$ (B). For comparison, plaques stained with anti-C_1 (C) and anti-SP28 (D) are

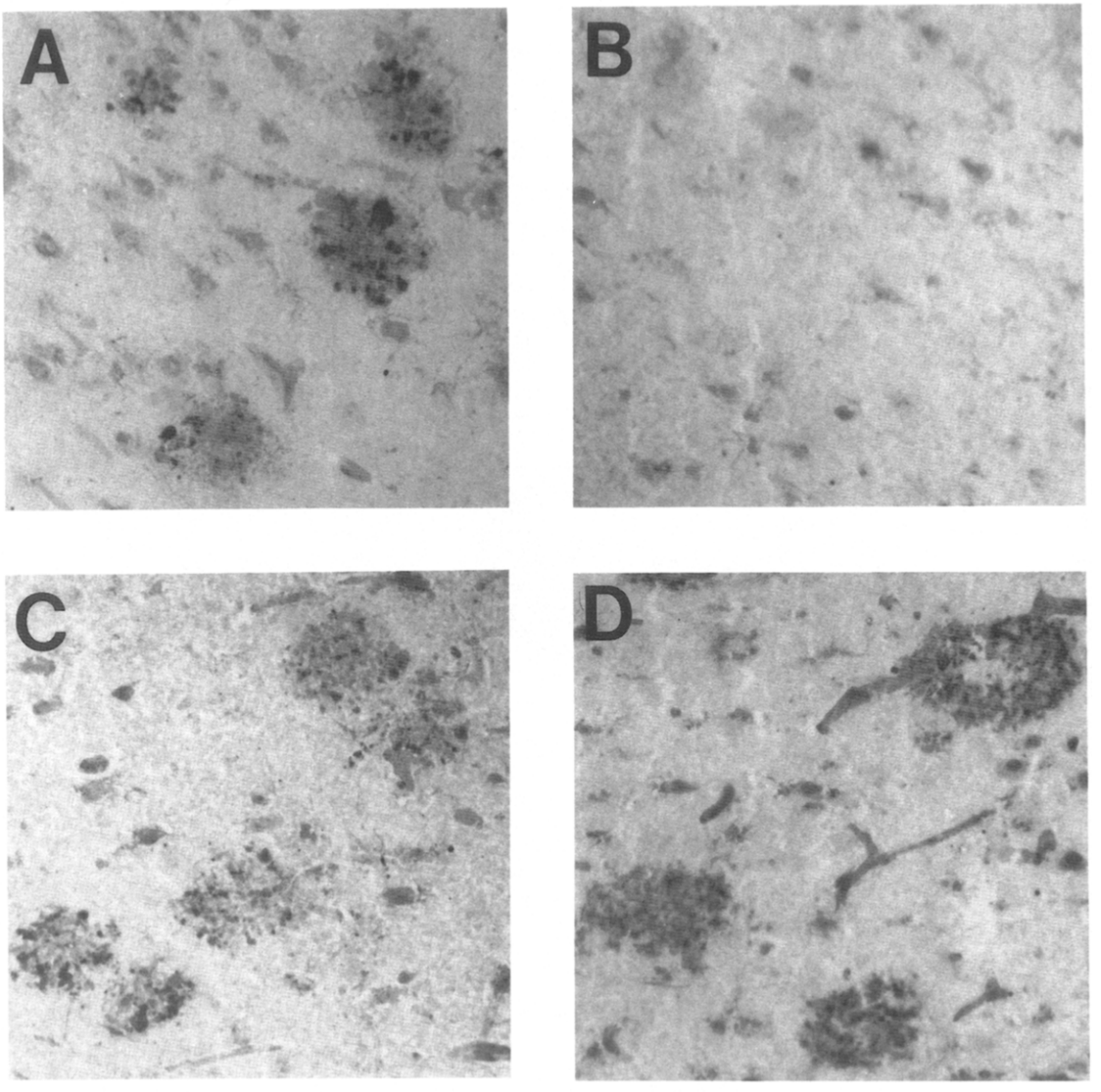

Fig. 3. Immunocytochemistry of various antibodies on cryostat sections of temporal cortex from a patient with Alzheimer's disease. **A.** Anti-ßAPP$_{45-62}$ (1:300). **B.** Anti-ßAPP$_{45-62}$ (1:300) after absorption with ßAPP$_{45-62}$. **C.** Anti-C_1 (1:150). **D.** Anti-SP28, an antiserum corresponding to amino acids 1-28 in the ßAP (1:150). Magnification 200x.

also shown. Overall, both anti-C_1 and anti-ßAPP$_{45-62}$ appear to stain fewer plaques than anti-SP28 (although this is not apparent in Fig. 3).

The fact that antiserum to C_1 labels senile plaques in AD brain indicates that the carboxyl-terminal portion of the ßAPP (which is located approximately 60 amino acids distal to the amyloidogenic ßAP domain) is present in the immediate vicinity of the amyloid fibrils within plaques (14). Our demonstration here that antiserum to ßAPP$_{45-62}$ stains plaques indicates that the near amino-terminal portion of the ßAPP is also located with the ßAP fibrils in senile plaques and suggests that proteolytic processing of the entire ßAPP molecule into insoluble fibrils occurs, at least in part, in a highly localized fashion directly at the sites of amyloid deposition in AD brains.

In a study that will be reported separately, we have found that, in addition to the ~120 kDa membrane-associated proteins, our antisera to βAPP_{45-62} detect a set of large, soluble proteins in brain and βAPP_{695}-transfected cells that are not detected by anti-C_1. For this reason, the immunostaining of senile plaques with anti-βAPP_{45-62} reported here could be due not only to membrane-associated forms of the βAPP detected by anti-C_1 but also to soluble forms of the βAPP not detected by antisera to the carboxyl-terminus.

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