Epitope map of two polyclonal antibodies that recognize amyloid lesions in patients with Alzheimer's disease

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Two synthetic peptides with sequences identical with those of fragments of the extracellular domain of the Alzheimer's-disease amyloid precursor protein (APP) were used to raise antibodies. SP28 comprises positions 597–624 of the APP₆₉₅ isoform, whereas SP41 extends towards the *N*-terminus (amino acids 584–624) and contains the entire SP28 peptide. Using e.l.i.s.a. and inhibition experiments we identified the two β -turn-containing segments 602–607 and 617–624 as the epitopes recognized by anti-SP41 and anti-SP28 respectively. Both antibodies immunolabelled amyloid lesions in brains from Alzheimer's-disease patients and patients with related disorders, whereas they were unreactive in control brains. However, when probed on immunoblots, anti-SP28 failed to detect full-length APP from baculovirus-infected Sf9 cells, and anti-SP41 reacted weakly compared with other anti-APP antisera. The data suggest that these antibodies are directed to conformational epitopes not existent in the native molecules but present after alternative APP processing.

INTRODUCTION

Amyloid-fibril deposition restricted to the central nervous system has been described in a variety of diseases of different etiology, including hereditary forms [1]. Among them, β -amyloid diseases (β AD) [2] share a unique peptide (designated β -protein [3], A4 [4], or more recently $A\beta$ [5]) deposited as cerebrovascular amyloid in medium-sized arteries and arterioles and/or as amyloid cores in neuritic plaques. Fibrils composed of aggregated forms of the $A\beta$ peptide are the main component of the lesions found in Alzheimer's disease (AD) [3,4], Down's syndrome (DS) [4,6], hereditary cerebral haemorrhage with amyloidosis of Dutch origin (HCHWA-D) [7], sporadic cerebral amyloid angiopathy (CAA) [8], and asymptomatic age-related amyloidosis [9]. $A\beta$ is an internal aberrant degradation product of a larger molecule (molecular mass 105–135 kDa), named β -amyloid precursor protein (APP), whose predicted structure correlates with that of a transmembrane cell-surface receptor [10]. The APP primary structure discloses a multidomain-like organization comprising an *N*-terminal extracellular region, a transmembrane hydrophobic segment and a short *C*-terminal intracellular domain.

Several forms of APP mRNA encoding 695, 714, 751 and 770 amino acids [10–17], and a shorter form devoid of the transmembrane domain [18], are derived by alternative splicing [14] of a single gene located on chromosome 21 [10–13]. Since an intron interrupts the DNA sequence at position 613 (APP₆₉₅ numbering) [19], it is believed that $A\beta$ arises by proteolysis of its precursor and not by aberrant splicing. The mRNA forms 714, 751 and 770 were shown to contain an extra domain encoding 19, 56 and 75 residues respectively, interpolated at position 289 of the APP and entirely encoded by separate exons. The extra domains found in APP₇₅₁ and APP₇₇₀ have sequence similarities with the Kunitz family of proteinase inhibitors [14–16].

Soluble APP derivatives have been detected in serum, brain homogenates, cerebrospinal fluid and tissue-culture medium [20–24] as a complex group of 105–135 kDa glycoproteins. The primary structure and biological properties of the soluble APP₇₅₁

mRNA transcript are analogous to those of a cell-secreted proteinase inhibitor nexin II [25,26]. The *N*-terminus starts at position 18 of the deduced sequence, whereas the *C*-terminus is 10 kDa shorter than the membrane-bound APP [10,20,21]. Studies performed on human embryonic kidney 293 cells transfected with cDNA constructs encoding full-length APP₇₅₁ and APP₆₉₅ indicate that APP processing occurs either at residue 15 (Gln) or 16 (Lys) of the $A\beta$ sequence [27], although an alternative pathway was recently identified in leptomeningeal vessel wall (J. Ghiso & B. Frangione, unpublished work).

Synthetic peptides similar to different segments of $A\beta$ have been used to raise antibodies, to perform conformational structural studies and to demonstrate that amyloid fibrils can be spontaneously formed *in vitro* [28–31]. In this regard: (i) The first 11 residues of $A\beta$ appear not to be essential for fibril formation; (ii) the segment 12–28 possesses cross- β -pleated sheet conformation and can successfully adopt a fibrillar configuration; and (iii) the 11–14 *C*-terminal stretch seems to be related to the insolubility of $A\beta$ rather than to the fibril formation [31]. Antibodies generated against synthetic peptides with sequences identical with those of $A\beta$ are currently useful tools for diagnostic purposes.

We present here immunohistological studies and the epitope map of two polyclonal antibodies, anti-SP41 (raised against the synthetic peptide SP41; positions 584–624 of APP₆₉₅) and anti-SP28 (made against the synthetic peptide SP28; residues 597–624 of APP₆₉₅). The data indicate that anti-SP28 recognizes the β -turn located at the *C*-terminus of SP28 (between residues 617 and 624 of APP₆₉₅), whereas anti-SP41 reacts with the hydrophilic stretch, 602–612, of APP₆₉₅ (also containing a β -turn). Both antibodies detect neuritic plaques, vascular amyloid and 'pre-amyloid deposits' in β AD brain tissues.

MATERIALS AND METHODS

Source of proteins and peptides

Samples of cerebrospinal fluid (CSF) from AD and control

Abbreviations used: β AD, β -amyloid disease(s); AD, Alzheimer's disease; DS, Down's syndrome; HCHWA-D, hereditary cerebral haemorrhage with amyloidosis of Dutch origin; CAA, (sporadic) cerebral amyloid angiopathy; APP, β -amyloid precursor protein; CSF, cerebrospinal fluid; PTH, phenylthiohydantoin.

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Immunohistochemistry

Sections of the hippocampus taken from formalin-fixed brains of five cases with AD, two cases with DS, two cases with CAA and two normal controls were studied. Deparaffinated paraffin sections were first incubated with TBS containing 5% (w/v) non-fat dried milk, 3% (w/v) BSA and 0.05% (v/v) Tween-20. To enhance amyloid staining, all sections were pretreated with 98% (v/v) formic acid for 10 min. Endogenous peroxidase activity was quenched by washing the sections in methanolic 0.3% (v/v) H_2O_2 for 20 min. Horseradish peroxidase activity was revealed with 3,3'-diaminobenzidine. Preimmune serum and TBS were used instead of primary antibody as negative controls. The specificity of the immunoreaction with anti-SP28 and anti-SP41 was confirmed by immunoabsorption with SP28 as described above.

RESULTS

Synthetic peptide SP28, injected unconjugated into a rabbit, induced specific antibody synthesis that was detectable 20 days after the first stimulation, reaching a maximum titre plateau of

Fig. 1. Binding of anti-SP28 (a) and anti-SP41 (b) to a panel of synthetic peptides (SP41, SP28, SP17, SP14, SP8 and SP10) and unrelated sequences KSNFNGAILSSC and LCNNHQWCGSNSNRYERC (UR), and (c) binding of anti-SP28 and anti-SP41 to the SP28 tryptic-generated peptides

Each data point represents the mean $\pm$ 2 S.D. for three independent duplicate experiments.

Fig. 2. H.p.l.c. purification of SP28 tryptic peptides

SP28 was digested with tosyl-lysylchloromethane-treated trypsin for 1 h at 37 °C at an enzyme/substrate ratio of 1:100. Peptides were separated in a μ -Bondapak C_{18} column using a 60 min linear gradient of 0–66% acetonitrile in 0.1% trifluoroacetic acid (flow rate: 2 ml/min); [B], concentration of buffer B (66% acetonitrile in 0.1% trifluoroacetic acid); ----, buffer B gradient.

1:20000 3 months later. When tested in an e.l.i.s.a., anti-SP28 recognized not only SP41 and SP28, but also their derivatives, SP17, SP14 and, to a slightly lesser extent, SP8. SP10, as well as unrelated peptides used as negative controls, were not recognized by the antibody (Fig. 1a).

The antibody induced by SP41 (also injected in an unconjugated form into a rabbit) was detected 4 weeks after the first stimulation, and the maximum titre (1:8000) was reached after 4 months. When tested in e.l.i.s.a. experiments, anti-SP41 was able to recognize SP41 and SP28 specifically, whereas only background levels were obtained with the rest of the peptides tested (Fig. 1b).

To characterize further the location of the epitopes, proteolytic fragments of SP28 were generated by trypsin digestion. Since SP28 contains one arginine and two lysine residues (one C-terminal), the three expected tryptic fragments, named SP(t)5, SP(t)11 and SP(t)12, were separated by h.p.l.c., as indicated in Fig. 2. Three major peaks, eluted at 35, 42 and 60% buffer B (see Fig. 2) were obtained. Identification by amino acid analysis as well as by amino acid sequence revealed that peptide SP(t)11 was eluted first, followed by SP(t)5; SP(t)12 was the most strongly retained by the column. As a control, undigested SP28, separated under identical conditions, eluted at 55% buffer B.

E.l.i.s.a. plates were coated with the tryptic peptides generated from SP28 and allowed to react with both anti-SP28 and anti-SP41. As Fig. 1(c) shows, peptides SP(t)5 and SP(t)11 failed to bind anti-SP28, and the reaction with SP(t)12 mimicked that of intact SP28. On the other hand, anti-SP41 was able to recognize only SP(t)11 and reacted with neither SP(t)5 nor SP(t)12.

Inhibition experiments with the synthetic peptides in solution were carried out to confirm the direct binding assays. As Fig. 3(a) shows, the reaction SP28–anti-SP28 can be inhibited by SP28 itself and by SP(t)12. SP(t)5 and SP(t)11 failed to inhibit the antigen–antibody reaction, and only values comparable with

Fig. 3. Inhibition of SP28-anti-SP28 (a) and SP28-anti-SP41 (b) interactions by SP28 and its tryptic peptides

Key to symbols: ●, SP28; open symbols, SP28-tryptic peptides [△, SP(t)5; ▽, SP(t)11; □, SP(t)12]. Data represent the means for two independent duplicate experiments. The S.D. value never exceeded $\pm 8\%$.

Fig. 4. Immunoreactivity of anti-SP28 and anti-SP41 on immunoblot analysis

(a) Full-length APP₇₅₁ from baculovirus-infected Sf9 cells; (b) soluble nexin II from baculovirus-infected Sf9 cells culture media; (c) soluble forms of APP from CSF. Lane a, anti-SP41 (1:700); lane b, anti-SP18 (1:1000); lane c, monoclonal antibody F12 (1:1000); lane d, monoclonal antibody 6E10 (1:1000); lane e: anti-SP28 (1:500). Note that, in (a), lane e was divided into two halves: the left half was allowed to immunoreact with anti-SP28 and the other half was stained with Fast Green.

control unrelated peptides were obtained. When identical dilutions of antibody were allowed to react with various concentrations of either SP(t)12 or SP28 before being added to SP28-coated wells, almost the same inhibition was obtained. On the other hand, the reaction SP41-anti-SP41 was inhibited by SP(t)11 and SP28, but not by SP(t)5 or SP(t)12 (Fig. 3b).

In immunoblot experiments, anti-SP28 was unable to recognize soluble forms of APP, either in CSF or in culture media (Figs. 4b and 4c, lane e). When it was probed against 20 μ g of full-length APP₇₅₁, a faint immunoreactivity was observed (Fig. 4a, lane e)

Fig. 5. Immunohistochemical staining

(a) Immunoreactivity of vessel wall in a case of sporadic congophilic angiopathy with anti-SP28 antibodies (magnification $\times 260$); (b) same vessel as in (a) immunoreacted with anti-SP41 ($\times 260$); (c) classical plaque with amyloid core (arrows) and 'preamyloid' or diffuse plaque (open arrowhead) allowed to immunoreact with anti-SP28 from an AD patient ($\times 130$); (d) sequential section of same area as in (c) allowed to immunoreact with anti-SP41 ($\times 130$); (e) AD brain tissue from the same area as (c) and (d), allowed immunoreact with anti-SP41 after absorption with SP28. No immunoreactivity is present ($\times 65$); (f) 'preamyloid' or diffuse plaque showing immunoreactivity with anti-SP41; dystrophic neurons are indicated by arrowheads in the vicinity of the plaque ($\times 260$).

compared with the strong signal generated by anti-SP18 or monoclonal antibodies F12 and 6E10 (Fig. 4a, lanes b-d). Tested in the same system, anti-SP41 also failed to recognize soluble forms of APP in CSF or culture media (Figs. 4b and 4c, lane a), but reactivity was observed when probed against full-length APP (Fig. 4a, lane a), although with less intensity than anti-SP18 or monoclonal antibodies F12 or 6E10. To confirm these findings further, soluble forms of APP were probed with both

Fig. 6. Amino acid sequence of the fragment 584–624 of APP₆₉₅.

The underlined italics represent the first 28 residues of A β . (a) Hydropathic index of the segment 584–624, according to [42]. (b) Secondary-structure prediction calculated according to [36]; —, α -helix; ---, β -strand; ■, β -turn. (c) and (d). Topographical location of segment 584–624 (c) within APP₆₉₅ (d). The box indicates A β . Arrowheads indicate the location of the transmembrane domain.

antibodies in direct e.l.i.s.a. experiments. Anti-SP28 failed to recognize them, corroborating the results obtained on immunoblot; however, anti-SP41 reacted with soluble APP forms, suggesting that the protein attached in the solid phase adopts different spatial conformation.

Anti-SP28 and anti-SP41 antisera both reacted with neuritic plaques, 'preamyloid' lesions and vascular deposits in the cases studied (Figs. 5a–5e). Anti-SP28 was slightly more sensitive in detecting neuritic plaques. However, regardless of the concentration of the antibodies, on grid sections neuritic plaques often stained with different intensity. In addition, anti-SP41 occasionally stained neurons in the vicinity of amyloid deposits (Fig. 5f). Neither antibody reacted with sections of normal brain. As with the e.l.i.s.a. experiments, the immunoreactivity of anti-SP28 could be absorbed with SP28 itself and SP(t)12. Anti-SP41 could be absorbed with SP28 and SP(t)11.

DISCUSSION

A β peptides (39–42 residues) isolated from cerebral vessel walls or senile plaques are degradation products of APP [4,35]. Secondary-structure-prediction analysis [36] indicates that two β -turns (positions 7–11 and 24–28) are located immediately after an α -helix stretch (Fig. 6). The latter has recently been confirmed by n.m.r. studies [37]. These types of structures are usually exposed on the molecular surfaces, and they are generally constituents of structural epitopes.

Anti-SP28, raised against a 28-residue synthetic peptide (Table 1), recognizes an epitope located in the last eight residues of the original peptide (positions 617–624 of APP₆₉₅). This assumption is based on the reactivity of the antibody in direct e.l.i.s.a. experiments (Fig. 1) and on the ability of SP(t)12 (Fig. 2) to inhibit the reaction between the antibody and its SP28 native

antigen (Fig. 3). SP(t)12, one out of the three tryptic fragments generated from SP28, is located at the C-terminus of SP28 (amino acids 17–28 of A β) and contains a β -turn (Fig. 6).

To confirm these results, anti-SP28 was tested on immunoblots against full-length APP₇₅₁ obtained from baculovirus-infected Sf9 cells, soluble APP forms from CSF and baculovirus-infected Sf9-cell-culture media. As expected, anti-SP28 was not able to recognize the soluble APP forms because the main APP processing takes place at position 15/16 of A β [27], just before the epitope recognized by the antibody. When it was probed against APP₇₅₁ (up to 20 μ g), a faint positive reaction was obtained. The reactivity was weak compared with the signal rendered by other anti-APP antibodies, suggesting that the epitope is somehow hidden within the spatial conformation of the molecule.

Anti-SP41 raised in rabbits using a 41-residue synthetic peptide as immunogen (Table 1) detects a different determinant also contained in SP28. The antibody recognizes the peptides SP41 and SP28 in direct e.l.i.s.a. assays (Fig. 1), and the reaction can be prevented by preincubation with SP28 or SP(t)11, a tryptic fragment derived from SP28 (Figs. 2 and 3). Therefore the epitope is located within the segment A β 6–11 (corresponding to positions 602–607 of APP₆₉₅), which contains a β -turn (Fig. 6). When tested on immunoblots, anti-SP41 was able to detect full-length APP (although with less intensity than anti-SP18 and monoclonals F12 and 6E10). Surprisingly, anti-SP41 did not react with soluble APP forms on immunoblots, but was able to recognize them in e.l.i.s.a. experiments, probably owing to the APP molecule immobilized on the solid phase having a different conformation.

In addition to the above-mentioned reactivity, both anti-SP28 and anti-SP41 clearly detect a peptide of 16 kDa in SDS-soluble fractions from normal brain vessel homogenates that appears to

be the result of an alternative processing pathway for APP (J. Ghiso & B. Frangione, unpublished work).

Immunohistochemically, anti-SP28 has been found to label two types of structures in brains from AD, DS, CAA and HCHWA-D patients: (i) birefringent amyloid deposits in the neuropil and/or vessel walls [7–9,20,38]; and (ii) non-refracting structures in the neuropil [39,40] named 'preamyloid deposits'. These preamyloid lesions lack the optical and electron-microscopical properties of amyloid fibrils, and it is conceivable that they are composed of intermediate precursor fragments already conformationally modified, and/or unpolymerized forms of non-fibrillar A β monomers [41]. However, whether preamyloid deposits represent early stages of senile plaques remains to be determined [39]. Anti-SP28 recognizes both the fibrillar and the preamyloid lesions, but it is unable to identify any structure in normal brains, indicating that some conformational changes have indeed occurred after APP processing, and the segment 21–28 of the A β is somehow exposed.

When tested in brain tissue sections from AD, DS and CAA cases, anti-SP41 was also able to detect 'preamyloid' lesions, neuritic plaques and vascular amyloid. In addition, anti-SP41 occasionally stained neuronal cell bodies in the vicinity of senile plaques, indicating that these epitopes may be revealed in membrane-bound APP under certain pathological conditions. These results indicate that A β expresses unique epitopes recognized by antibodies after APP processing.

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