

## RESEARCH COMMUNICATION

# The cerebrospinal-fluid soluble form of Alzheimer's amyloid beta is complexed to SP-40,40 (apolipoprotein J), an inhibitor of the complement membrane-attack complex

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The amyloid fibrils deposited in Alzheimer's neuritic plaque cores and cerebral blood vessels are mainly composed of aggregated forms of a unique peptide, 39–42 amino acids long, named amyloid beta ( $A\beta$ ). A similar, although soluble,  $A\beta$  ('s $A\beta$ ') has been identified in cerebrospinal fluid, plasma and cell supernatants, indicating that it is normally produced by proteolytic processing of its precursor protein, amyloid precursor protein (APP). Using direct binding experiments we have isolated and

characterized an 80 kDa circulating protein that specifically interacts with a synthetic peptide identical with  $A\beta$ . The protein was unmistakably identified as SP-40,40 or ApoJ, a cytolytic inhibitor and lipid carrier, by means of amino acid sequence and immunoreactivity with specific antibodies. Immunoprecipitation with anti-SP-40,40 retrieved soluble  $A\beta$  from cerebrospinal fluid, indicating that the interaction occurs *in vivo*.

## INTRODUCTION

Amyloid beta ( $A\beta$ ) is the major constituent of the fibrils composing senile plaques and vascular deposits in Alzheimer's disease (AD) and related disorders. It is an internal degradation product (39–42 residues) of a larger precursor (amyloid precursor protein, APP) generated through still-unclear proteolytic mechanisms (for a review, see [1]). So far, two main APP-processing pathways have been described. Pathway I involves a proteolytic cleavage inside the  $A\beta$  sequence that prevents amyloid formation [2–5]. Pathway II, in contrast, generates N-terminal fragments of APP lacking  $A\beta$  [6] and C-terminal peptides containing intact  $A\beta$ . These fragments have been described in membrane-associated fractions isolated from cerebral cortex, leptomeningeal vessels, cerebral microvessels, baculovirus-infected Sf9 cells overexpressing APP and cell lines stably transfected with full-length APP [7–14]. Very recently, soluble  $A\beta$ -like peptides (s $A\beta$ ) were identified in media from cell cultures of untransfected and APP-transfected cells as well as in cerebrospinal fluid (CSF) and plasma obtained from normal and AD patients [15–17]. Amino-acid-sequence analysis indicated that s $A\beta$  is similar to the amyloid protein extracted from cerebrovascular lesions.

Synthetic peptides identical with  $A\beta$  have been used to study different physicochemical properties of  $A\beta$ , among them cell adhesion and fibril formation [8,18–21].  $A\beta$  is able to promote cell adhesion by an interaction of the sequence RHDS (one-letter amino acid code; positions 5–8) with an integrin-like receptor [8]. Synthetic peptides identical with residues 1–40 of  $A\beta$  and their shorter derivatives SP28 ( $A\beta_{1-28}$ ) and SP12 ( $A\beta_{17-28}$ ) spontaneously form fibrils *in vitro* [18–20]. Moreover, the presence of the Dutch mutation at codon 618 of APP<sub>695</sub> [22] (corresponding

to residue 22 of  $A\beta$ ) accelerates the rate of fibril formation [21] and transfected cells expressing the Swedish mutations (codons 595 and 596 of APP<sub>695</sub>, associated with Familial AD of early onset in two families [23]), produce higher quantities of s $A\beta$  [24]. These findings indicate that the primary structure of  $A\beta$  and its concentration may play a pivotal role in amyloid formation. However, the question of why s $A\beta$  does not spontaneously form fibrils in biological fluids has not been addressed. It is possible that, under normal conditions, its concentration is too low for it to polymerize; alternatively, specific factors may maintain  $A\beta$  in solution.

We hypothesized that s $A\beta$  binds to carrier proteins and that this interaction may maintain  $A\beta$  in soluble form, preventing its aggregation and therefore suppressing its fibril-forming propensity. We have tested the first part of this hypothesis using (i) synthetic peptides similar to  $A\beta$  immobilized on affinity matrices in direct binding experiments with plasma and CSF and (ii) immunoprecipitation of s $A\beta$  from CSF with a selected set of antibodies. A specific ionic interaction was detected between the above-mentioned synthetic peptide and an 80 kDa soluble plasma protein. Immunoblot and amino-acid-sequence analysis unequivocally identified the bound protein as SP-40,40 or ApoJ, an inhibitor of the cytolytic activity of the complement complex C5b-9.

## MATERIALS AND METHODS

## Synthetic peptides and antibodies

Synthetic peptide DAEFRHDSGYEVHHQKLVFFAEDVGS-NKGAIIGLMVGGVV ( $A\beta_{1-40}$ , positions 597–636 of APP<sub>695</sub>) was synthesized at the Center for the Analysis and Synthesis of Macromolecules (State University of New York, Stony Brook,

NY, U.S.A.) by solid-phase techniques. Crude peptide was dissolved in 0.1% trifluoroacetic acid/30% acetonitrile and purified via h.p.l.c. using a 20  $\mu$ m Aquapore C8 (250 mm  $\times$  10 mm) column (Brownlee) and a linear gradient of 30–100% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 2.0 ml/min. The column effluent was monitored by absorbance at 214 nm. Peptides DAEFRHDSGYEVHHQKLVF-FAEDVGSNK (SP28, positions 597–624 of APP<sub>695</sub>) [18] and LGNNIHQWCGSNSNRYERC (SP19, amino acids 196–215 of human plasma gelsolin) [25] were available in the laboratory. Sequences were corroborated via amino-acid-sequence analysis. BSA and keyhole-limpet haemocyanin (KLH) were from Sigma.

Peptide A $\beta$ <sub>1-40</sub> was used to obtain anti-A $\beta$ <sub>1-40</sub> polyclonal antibodies. Rabbits were immunized every 15 days with 200  $\mu$ g of uncoupled peptide dissolved in 0.15 M NaCl mixed with incomplete Freund's adjuvant in a 1:1 ratio. The animals were bled according to the evolution of the antibody titre (evaluated by e.l.i.s.a.) and boosted every 20 days with 50  $\mu$ g of A $\beta$ <sub>1-40</sub> in 0.15 M NaCl. Monoclonal and polyclonal anti-[human apolipoprotein E (ApoE)] antibodies were from Chemicon International, Temecula, CA, U.S.A.; monoclonal antibodies IF12 (anti-SP-40,40  $\beta$ -chain) and IVF4 (anti-SP-40,40  $\alpha$ -chain) were previously characterized by us (N. H. C.-M. and M. T.); monoclonal antibody 4G8 was obtained from K. S. Kim [26].

#### Affinity chromatography

Either purified peptides A $\beta$ <sub>1-40</sub>, SP28 and SP19 or control proteins BSA and KLH were coupled to CNBr-activated Sepharose 4B (Pharmacia) at a concentration of 5 mg/ml of beads, according to the manufacturer's instructions. Briefly, peptides or proteins were solubilized in 0.1 M NaHCO<sub>3</sub>, pH 8.3, except for A $\beta$ <sub>1-40</sub>, which was solubilized in the same buffer containing 30% acetonitrile. In all cases, solubilized peptides/proteins were allowed to react with the matrix support for 16 h at 4 °C. After blocking the remaining unchanged groups with 0.1 M Tris/HCl, pH 8.3, the resulting affinity matrices were washed with 0.5 M ammonium acetate, pH 3.5, and 1 M acetic acid, pH 2.5, and finally equilibrated with 20 mM Tris/HCl, pH 7.2, containing 150 mM NaCl (TBS). Plain Sepharose was prepared by blocking all the available sites with 0.1 M Tris/HCl, pH 8.3.

Of the above-described affinity matrices equilibrated with TBS, 2 ml were allowed to interact in batch with either 10 ml of human plasma or 30 ml of CSF for 3 h at 37 °C, under continuous rotation. Unbound material was removed by extensive washing of the matrix with PBS, and the bound fraction was eluted using 1 M acetic acid, pH 2.5. Samples were dialysed against distilled deionized water and freeze-dried. In order to detect any remaining bound protein, 100  $\mu$ l of beads were mixed with 50  $\mu$ l of sample buffer (2% SDS in 0.1 M Tris/HCl buffer, pH 6.8), containing 100 mM dithiothreitol (DDT), and boiled for 5 min before electrophoresis.

#### Amino acid sequence

Automatic Edman degradation analyses were carried out on a 477A protein sequencer, and the resulting phenylthiohydantoin derivatives identified using an on-line 120A phenylthiohydantoin-derivative analyser (Applied Biosystems, Foster City, CA, U.S.A.). When necessary, samples were electrophoresed on an SDS/10%-polyacrylamide [27] Minigel and transferred on to poly(vinylidene difluoride) (PVDF) membranes (Immobilon; Millipore) using 3-cyclohexylamino-1-propanesulphonic acid,

pH 11, containing 10% (v/v) methanol. Membranes were stained with Coomassie Blue and the protein bands excised and sequenced.

#### Immunoblot analysis

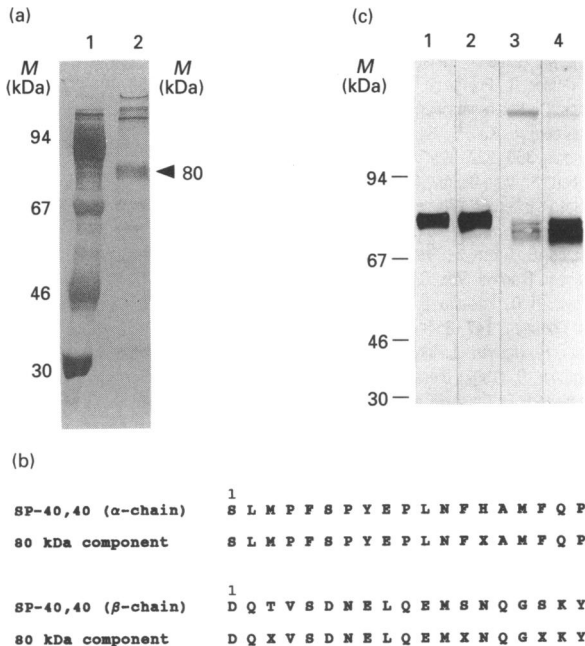
A 10–20  $\mu$ g sample of plasma or CSF fractions that bound to the affinity matrices were electrophoresed on a SDS/10%-polyacrylamide Minigels and transferred on to 0.45  $\mu$ m-pore-size nitrocellulose membranes (Bio-Rad), using the same conditions as those described above. Membranes were blocked with 5% non-fat dry milk in TBST [20 mM Tris/HCl (pH 7.2)/150 mM NaCl/0.05% Tween-20] and incubated overnight at 4 °C with monoclonal IVF4 (anti-SP-40,40  $\alpha$ -chain), 1:200, and monoclonal IF12 (anti-SP-40,40  $\beta$ -chain), 1:500. After extensive washing with TBST, the membranes were incubated with goat F(ab')<sub>2</sub> anti-mouse immunoglobulins (alkaline phosphatase-labelled; 1:3000; Tago). Immunoblots were developed using 5-bromo-4-chloro-indol-3-yl phosphate and Nitroblue Tetrazolium (Kirkegaard and Perry, Gaithersburg, MD, U.S.A.).

#### Immunoprecipitation

For immunoprecipitation of sA $\beta$ , 3 ml of CSF were mixed with 3 ml of 2  $\times$  RIPA [150 mM NaCl/50 mM Tris/HCl (pH 8.0)/1% Triton X-100/0.5% cholic acid/0.1% SDS/5 mM EDTA]/LPT [1  $\mu$ g/ml leupeptin/0.1  $\mu$ g/ml pepstatin/1 mM phenylmethanesulphonyl fluoride/1  $\mu$ g/ml tosyl-lysylchloromethane ('TLCK')] buffer; either anti-SP-40,40  $\beta$ -chain or anti-(lipoprotein E) antibodies (20  $\mu$ l each) and RIPA/LPT-washed Protein A-Sepharose (50  $\mu$ l) were added. The mixture was rocked at 4 °C for 24 h. After washing twice with RIPA/LPT and once with TBS, the immunoprecipitated protein was separated by Tris/Tricine/SDS/10/16.5%-polyacrylamide gel electrophoresis and transferred to Immobilon P (Millipore) for 2 h at 100 V using 25 mM Tris/HCl/192 mM glycine, pH 8.3, containing 20% methanol. Membranes were blocked with 5% non-fat dry milk and labelled with monoclonal antibody 4G8 (1:400) overnight at 4 °C. Fluorograms were then prepared with an ECL Western Blotting Kit (Amersham), according to the manufacturer's specifications. For control purposes, 5  $\mu$ g of A $\beta$ <sub>1-40</sub> were added to 3 ml of CSF and immunoprecipitated with anti-A $\beta$ <sub>1-40</sub>.

#### RESULTS AND DISCUSSION

In order to identify potential physiological ligands for sA $\beta$ , a synthetic peptide, A $\beta$ <sub>1-40</sub>, was immobilized on to an affinity matrix and allowed to interact with either plasma or CSF obtained from normal donors. A $\beta$ <sub>1-40</sub>-Sepharose was able to retain a small amount of the total protein applied. From 10 ml of plasma, 260  $\mu$ g were recovered in the bound fraction, representing approx. 0.04% of the total protein applied, whereas 24  $\mu$ g were obtained from 30 ml of CSF (approx. 0.26% of the total protein offered). SDS/PAGE analysis of the unreduced bound fractions (Figure 1a) indicated the presence of a main protein with an apparent molecular mass of 80 kDa. When the same samples were reduced with DTT, only one component of 40 kDa was detected (result not shown). The native 80 kDa protein was subjected to automatic Edman degradation analysis after electrotransfer to a PVDF membrane, yielding 19 steps of two different equimolar sequences (SLMPFSPYEPLNFXAMFQP and DQXVSDNELQEMXNQGXKY). An homology search using the PIR database (National Biomedical Research Foundation, Georgetown, VA, U.S.A.) identified both sequences as the N-terminal  $\alpha$ - and  $\beta$ -chains of the protein SP-40,40 (also known as

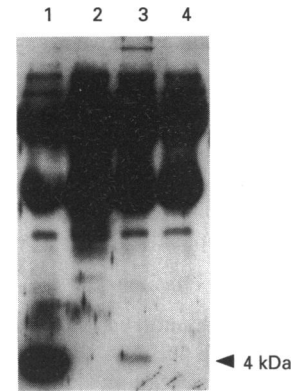


**Figure 1** (a) Binding of SP-40,40 to immobilized  $A\beta_{1-40}$ , (b) N-terminal sequence of the 80 kDa component and (c) Immunoblot identification of SP-40,40 in bound plasma (lanes 1 and 2) and CSF (lanes 3 and 4) fractions

(a) The affinity matrices described in the Materials and methods section were allowed to interact with either human plasma or CSF. Unbound material was washed off the matrix and the bound fraction was eluted with 1 M acetic acid, pH 2.5. Bound fractions were separated on 10% SDS-PAGE, transferred on to a PVDF membrane and stained with Coomassie Blue. Lane 2 shows the plasma-bound fraction containing a major component (arrowed) of 80 kDa. Molecular-mass (*M*) markers are shown in lane 1. (b) The 80 kDa bound component described in (a) was excised from the PVDF membrane and N-terminally sequenced. (c) Either monoclonal IVF4 {anti-[SP-40,40 (α-chain)] (1:200)} (lanes 1 and 3) or monoclonal IF12 {anti-[SP-40,40 (β-chain)] (1:500)} (lanes 2 and 4) were used in the immunoblot analysis.

ApoJ or human plasma clusterin) (Figure 1b). The results were corroborated via immunoblot analysis using two monoclonal antibodies against SP-40,40 α- and β-chains. As shown in Figure 1(c), the 80 kDa component either from plasma or CSF was immunoreactive with both antibodies. When the shorter peptide SP28 (analogous to the first 28 residues of  $A\beta_{1-40}$ ) coupled to a solid matrix was used for binding experiments, SP-40,40 was also recovered in the bound fraction after acid elution (results not shown), suggesting that the fragment 29–40 of  $A\beta$  is not critical for the interaction to occur. The specificity of the binding was corroborated by using plain Sepharose, the fibrillogenic synthetic peptide SP19 coupled to the solid matrix and the unrelated proteins BSA or KLH, also coupled to Sepharose. In all these cases, SP-40,40 was not retained by the control affinity matrices.

In order to identify any remaining bound material that was not removed by acid elution,  $A\beta_{1-40}$ -beads were boiled in 2% SDS/100 mM DTT and the released material analysed via SDS/PAGE and immunoblot. Although no SP-40,40 was found, a 35 kDa component was eluted by SDS and further identified as ApoE. We and others [27a,28] have obtained similar results using  $A\beta$  peptides immobilized on Immobilon AV (Millipore) affinity membranes. The fact that ApoE was not present in the acid-eluted fractions where SP-40,40 was detected indicates that the interaction of each of these proteins with  $A\beta$  has different physicochemical requirements.



**Figure 2** Immunoprecipitation of s $A\beta$  in CSF

Analysis was performed as described in the Materials and methods section. A 5  $\mu$ g portion of exogenous synthetic  $A\beta_{1-40}$  precipitated with anti- $A\beta_{1-40}$  antiserum was used as a positive control (lane 1). Soluble  $A\beta$  was only precipitated by anti-[SP-40,40 (β-chain)] (lane 3) and not by monoclonal or polyclonal anti-ApoE antibodies (lanes 2 and 4 respectively). High-molecular-mass signals are due to the reaction of the monoclonal antibody (heavy and light chains) used to precipitate SP-40,40 and the anti-mouse serum employed as second antibody.

To test whether the interaction SP-40,40– $A\beta$  and/or ApoE– $A\beta$  occurs *in vivo*, aliquots of CSF were immunoprecipitated with anti-[SP-40,40 (β-chain)] and two different anti-ApoE antibodies. The resulting precipitates were tested for  $A\beta$  immunoreactivity with monoclonal antibody 4G8. As shown in Figure 2, s $A\beta$  co-precipitated with SP-40,40, indicating that SP-40,40 and s $A\beta$  are complexed in CSF. The electrophoretic mobility of the retrieved s $A\beta$  was identical with that of synthetic  $A\beta_{1-40}$  immunoprecipitated with anti- $A\beta_{1-40}$ .

SP-40,40 is a heterodimeric human serum protein [29] composed of two different 40 kDa subunits (termed α- and β-chains, of 222 and 205 amino acids respectively) covalently linked by five disulphide bonds [30]. The gene for SP-40,40 maps to chromosome 8 [31,32], and cDNA sequence studies indicated that the two chains are coded in a single open reading frame on the same mRNA molecule [33].

SP-40,40 functions as an inhibitor of the cytolytic terminal complement complex C5b-9 (also termed membrane-attack complex, MAC). It is present in the cytolytically inactive and water-soluble sC5b-9 complex, whereas it is absent in the membrane-bound C5b-9 complex, suggesting that SP-40,40 likely functions as an inhibitor similar to S-protein (vitronectin), possibly by binding to nascent metastable C5b-7 in conjunction with S-protein [34,35]. SP-40,40 is normally present in serum (35–105  $\mu$ g/ml) [28], although it is about four times more concentrated in seminal fluid [36], where it is known as clusterin, a major secreted product of Sertoli cells [37–39].

SP-40,40 (also named ApoJ) has been identified in human plasma associated with high-density lipoproteins [40], and its mRNA distribution indicates high levels of expression in the brain [41]. SP-40,40 has been quantified in CSF of normal individuals and AD patients [42]. The reported average concentration in normal donors was  $2.4 \pm 1.2$   $\mu$ g/ml, whereas it was slightly elevated ( $3.1 \pm 1.3$   $\mu$ g/ml) in AD patients, although no significant statistical differences were found. SP-40,40 has been immunohistochemically demonstrated in senile plaques of patients with AD [42,43]. Several proteins of the complement system have been also immunohistologically detected in senile plaques of AD brains, among them C1q, activation products of C3 and C4, MAC [44], as well as the inhibitor S-protein [45]. It

was recently demonstrated [44] that A $\beta$  deposits can bind C1q and activate the classical complement cascade without mediation of immunoglobulin. As a result, it was proposed that this mechanism may significantly contribute to the A $\beta$  neurotoxicity as well as to the neuronal and neuritic damage associated with AD. Our findings that SP-40,40 can specifically bind to A $\beta$  *in vitro* and *in vivo*, taken together with the immunohistochemical demonstration of SP-40,40 [42,43] and S-protein [45] in amyloid plaques, adds further complexity to the speculation that the complement system plays a role in the tissue damage associated with senile plaques.

Several proteins ( $\alpha_1$ -antichymotrypsin [46], P-component [47], proteoglycans [48] and ApoE [49]) have been also found accumulated in A $\beta$  amyloid deposits, leading to the concept of 'pathological chaperones' (unrelated proteins that mediate  $\beta$ -pleated amyloid formation [49]). ApoE can also be retrieved from CSF or plasma using either affinity chromatography with A $\beta_{1-40}$  or binding to A $\beta$  on membranes; however, the elution conditions are completely different from those required by SP-40,40 [27a].

It is noteworthy that both SP-40,40 (ApoJ) and ApoE have intriguing structural and functional similarities. Since both proteins can exist in plasma and compartments separated by a physiological barriers (i.e. blood/brain barrier), they can transport lipids, fatty acids and hydrophobic compounds between organs and facilitate lipid transport among cells within a particular organ [41]. Moreover, hybridization experiments *in situ* indicate that ApoJ is expressed by a variety of cells lining fluid compartments [50]; therefore ApoJ may play an important role in preserving cell membranes from hydrophobic elements. The interaction of A $\beta$  with apolipoproteins, which may be involved in maintaining the solubility of A $\beta$  in biological fluids, points to a potential mechanism of membrane protection and sA $\beta$  transport through the blood-brain barrier.

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