A 109-amino-acid C-terminal fragment of Alzheimer's-disease amyloid precursor protein contains a sequence, -RHDS-, that promotes cell adhesion

Jorge GHISO,* Agueda ROSTAGNO,* Joseph E. GARDELLA,† Linda LIEM,* Peter D. GOREVIC†‡ and Blas FRANGIONE*

*Department of Pathology, New York University Medical Center, 550 First Avenue TH 427, New York, NY 10016, and Departments of †Pathology and ‡Medicine, State University of New York, Stony Brook, NY 11794, U.S.A.

Amyloid β ($A\beta$), the major constituent of the fibrils composing senile plaques and vascular amyloid deposits in Alzheimer's disease (AD) and related disorders, is a 39–42-residue self-aggregating degradation peptide of a larger multidomain membrane glycoprotein designated amyloid precursor protein (APP). An array of biological functions has been assigned to different APP domains, including growth regulation, neurotoxicity, inhibitory activity of serine proteinases and promotion of cell–cell and cell–matrix interactions. $A\beta$ is generated through an as-yet-unknown catabolic pathway that by-passes or inhibits the cleavage of APP within the $A\beta$ sequence. We have identified a 16 kDa intermediate APP C-terminal fragment containing $A\beta$ in leptomeningeal vessels of aged normal individuals and AD patients by means of its immunoreactivity with a panel of four different anti-(APP C-terminal) antibodies, indicating a different pathway of APP processing. Previous studies have indicated that the APP C-terminal domain is the most likely to be involved in cell–matrix interactions. A 109-amino-acid construct C109 with a sequence analogous to the C-terminal of APP (positions 587–695 of APP₆₉₅), similar in length and immunoreactivity to the 16 kDa fragment, was found to promote cell adhesion. By use of synthetic peptides, this activity was initially located to the extracellular 28 residues of $A\beta$. Inhibition studies demonstrated that the sequence RHDS (amino acids 5–8 of $A\beta$, corresponding to residues 601–604 of APP₆₉₅) was responsible for the adhesion-promoting activity. The interaction is dependent on bivalent cations and can be blocked either by the tetrapeptides RHDS and RGDS or by an anti-(β 1 integrin) antibody. Thus, through integrin-like surface receptors, APP or its derivative proteolytic fragments containing the sequence RHDS may modulate cell–cell or cell–matrix interactions.

INTRODUCTION

Alzheimer's disease (AD) is the most common form of human amyloidosis and the major cause of dementia. The amyloid fibrils deposited in neuritic plaque cores and cerebral blood vessels are mainly composed of polymeric forms of a unique peptide designated β -protein [1] or $A\beta$ [2]. $A\beta$ is an internal degradation product (39–42 residues [3,4]) of a larger precursor (Amyloid precursor protein, APP) whose predicted structure correlates with that of an integral transmembrane protein [5]. Major APP isoforms (APP₆₉₅, APP₇₁₄, APP₇₅₁ and APP₇₇₀) are derived by alternative splicing of a single gene located on chromosome 21 (for a review, see [6]). The two largest isoforms contain a 56-amino-acid domain, similar to the Kunitz family of proteinase inhibitors (KPI), inserted at codon 289. After synthesis, APP undergoes post-translational modifications that include the removal of the signal sequence, *N*- and *O*-glycosylation, phosphorylation and tyrosine sulphation [7–12].

The origin of $A\beta$ still remains poorly understood. It is clear that it arises by proteolysis of APP and not by aberrant splicing, since an intron interrupts the DNA sequence encoding $A\beta$ [13]. Studies performed with mammalian [14–17] and insect cells overexpressing APP isoforms [17a] indicate that a main APP cleavage occurs at the C-terminal side of Lys¹⁶ of the $A\beta$ sequence (corresponding to position 612 of APP₆₉₅). As a result, a large *N*-terminal segment is released, while a polypeptide of 83 amino acids that lacks the intact $A\beta$ is left behind and presumably

further processed. Therefore, when APP is targeted to this pathway, amyloidogenesis is prevented.

The structural multidomain organization of APP suggests that it may have different biological functions. Secreted forms of APP comprising almost the entire extracellular domain and containing the KPI domain have been identified as protease nexin II, a serine-proteinase inhibitor [18,19]. The presence of APP in platelet α -granules, as well as its identity with the Factor XIa inhibitor, indicate a function in wound healing and homeostasis [20–24]. APP has been related to growth regulation [25–28], neurite outgrowth [29], neurotoxicity [30] and cell–cell and cell–matrix interactions [9,31–37]. Recently it has been identified as the core protein of a chondroitin sulphate proteoglycan secreted by glial-cell line C6 [38]. Intact APP promotes cell–substratum adhesion of both neuronal and non-neuronal cells. Although the interaction was not studied in detail, the site responsible for the cell-adhesion-promoting activity appears to be located within the first 16 residues of $A\beta$, since an antibody directed against fragment 1–16 of $A\beta$ was able to inhibit cell–substratum adhesion [37].

We report here that (i) a 16 kDa fragment containing the $A\beta$ and the C-terminus of the APP (representing a second normal APP processing pathway) is present in leptomeningeal vessels obtained from aged normal and AD brains; (ii) a 109-residue construct similar to the 16 kDa fragment, as well as a synthetic peptide identical with the first 28 *N*-terminal residues of $A\beta$, both promote cell adhesion through an integrin-like receptor. The site

Abbreviations used: APP, amyloid precursor protein (the nomenclature committee has recommended that APP in future be called β PP); $A\beta$, amyloid β ; AD, Alzheimer's disease; KPI, Kunitz proteinase inhibitor; Fn, fibronectin; KLH, keyhole-limpet haemocyanin; NTA, nitrilotriacetate; PTH, phenylthiohydantoin; PBS, phosphate-buffered saline.

of the interaction was localized to the sequence RHDS (positions 5–8 of A β or 601–604 of APP₆₉₅), closely related to the conserved tetrapeptide RGDS, a widely distributed recognition site in proteins that mediate cell–matrix interactions.

Part of this paper was presented at the 21st Annual Meeting of the Society for Neuroscience, New Orleans, November 10–15, 1991.

MATERIALS AND METHODS

Proteins

Plasma fibronectin (Fn) was purchased from the New York Blood Center and further purified by gelatin-affinity chromatography as described in [39]. Bound Fn was eluted with 4 M-urea in 0.1 M-Tris/HCl, pH 7.4, dialysed against 0.1 M-Tris/HCl, pH 7.4, and stored at -70°C . Keyhole-limpet haemocyanin (KLH), egg lysozyme and BSA were from Sigma.

C109 is a recombinant protein which is identical with the C-terminal 109 residues of all three major APP translation products (APP₆₉₅, APP₇₅₁ and APP₇₇₀). An added C-terminal histidine hexamer enables the chromatographic purification of the expressed protein on nickel-ion-binding resin. Oligonucleotides A β -1759 (CCGGATCCATATGAAGACGGAGGAGATCTCTGAA) and A β 2065 (TTGGATCCGGTGACCGTTCTGCATCTGCTCAAAGAA) were synthesized to PCR-amplify a 355-bp segment out of a plasmid containing the APP₇₅₁ cDNA sequence. A β 1759 is the upstream primer and encodes an endonuclease-*NdeI* site in the 5' 13 bases; bases 14–34 are identical with bases 2051–2071 of the reported APP₇₅₁ cDNA sequence. A β 2065 is the downstream primer and encodes a *BstEII* site in the 5' 15 bases; bases 16–36 are complementary to bases 2377–2357 of the reported APP₇₅₁ cDNA sequence. Amplification was accomplished by using a GeneAmp Kit (Perkin-Elmer/Cetus), the manufacturer's specifications being followed. The 355-bp amplification product was purified on a preparative 4% (w/v)-agarose gel and extracted with a Gene Clean Kit (Bio 101). The purified C109 cDNA was sequentially cut by restriction endonucleases *NdeI* and *BstEII*, in order to make an in-frame insertion into the T7 expression vector pT7Ad23K13 (kindly provided by Dr. John Dunn, Brookhaven National Laboratory, Upton, NY, U.S.A.). Transformation of DH5 α *Escherichia coli*, mini-prep isolation of supercoiled DNA and transformation of BL-21 *E. coli* were as described in [40]. Screening of successful BL-21 transformants was performed by means of PCR detection of the 355-bp insert. C109 expression was induced in cultures in mid-exponential phase of growth by the addition of isopropyl thio- β -D-galactoside to a final concentration of 0.4 mM.

C109 purification was carried out by means of immobilized-metal-ion affinity chromatography, utilizing the metal-chelating adsorbent nickel trinitriloacetate (NTA)-agarose (Qiagen, Chatsworth, CA, U.S.A.). Following the manufacturer's specifications, induced *E. coli* were solubilized in 6 M-guanidinium chloride/0.1 M-NaH₂PO₄/0.01 M-Tris, pH 8.0, and loaded on to a 2 ml column of NTA resin equilibrated with the same buffer. The column was washed sequentially with 10 column vol. of 8 M-urea/0.1 M-NaH₂PO₄/0.01 M-Tris, pH 8.0, and 10 vol. of 8 M-urea/0.1 M-NaH₂PO₄/0.01 M-Tris, pH 6.3. Bound material was eluted with 2 vol. of 8 M-urea/0.1 M-NaH₂PO₄/0.01 M-Tris, pH 4.5. Purified C109 was concentrated by freeze-drying after exhaustive dialysis against distilled water; it was stored at -70°C as a 0.325 mg/ml solution in water, pH 4.1.

Antibodies

Anti-SP41 is a polyclonal antiserum raised in rabbits against a synthetic peptide similar to residues 584–624 of APP₆₉₅ [41]; anti-SP28 is a polyclonal antibody obtained in rabbits using as

immunogen a synthetic peptide similar to amino acids 597–624 of APP₆₉₅ [41]; anti-SP18 is a polyclonal antiserum raised in rabbits against a synthetic peptide similar to positions 45–62 of APP₆₉₅ [7]; 6E10 kindly provided by K. S. Kim is a monoclonal antibody specific for the first 17 residues of A β (positions 597–613 of APP₆₉₅) as previously described [42]; F12 is a monoclonal antibody that specifically recognizes the intracellular C-terminal 20 amino acids of APP (positions 676–695 of APP₆₉₅) [43]; monoclonal antibody anti-FnR5, specific for the β 1-subunit of the integrin receptors, was purchased from Pierce.

Synthetic peptides

Tetrapeptide RGDS was purchased from Sigma. Synthetic peptides RHDS, DAEFRHDSGYEVHHQKLFFAEDVGS-NK (SP28, positions 597–624 of APP₆₉₅), MNVQNGKWSD-PSGKTC (SP18, amino acids 45–62 of APP₆₉₅), NWCKRG-RKQ (SP9, residues 96–104 of APP₆₉₅), and LCNNIHQWCG-SNSNRYERC (SP19) were 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 peptides were purified via h.p.l.c. using a μ -Bondapak C₁₈ column (0.78 cm $\times$ 30 cm) (Waters) and a linear gradient of 0–66% 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. Sequences were corroborated via amino acid sequence, and concentrations were calculated by means of amino acid composition.

SP28 was coupled to KLH using the homobifunctional imido-ester dimethyl pimelimidate (Pierce) according to the manufacturer's instructions.

Amino acid and sequence analyses

Purified peptides were hydrolysed under reduced pressure in 0.2 ml of 6 M-HCl for 20 h at 110°C , and their amino acid compositions were determined with a Waters Pico-Tag amino acid analyser. Automatic Edman degradation analyses were carried out on a 477A protein sequencer, and the resulting phenylthiohydantoin (PTH) derivatives were identified using an on-line 120A PTH analyser (Applied Biosystems, Foster City, CA, U.S.A.).

Electron microscopy

Specimens were placed on Formvar-coated nickel grids, negatively stained with 2% uranyl acetate, and examined in an electron microscope.

Source of tissue

Brain tissue from six AD cases (aged 86, 66, 87, 80, 53 and 68 years; delay *post mortem*: 12, 10, 8, 10, 12 and 24 h respectively) and five aged non-demented individuals (aged 62, 85, 83, 79 and 78 years; delay *post mortem*: 9, 10, 10, 6 and 8 h respectively) were obtained, with consent of the next-of-kin, from Columbia-Presbyterian Medical Center, Neuropathology Department, New York, NY, U.S.A.

Vessel-wall homogenates

The following protocol was performed at 4°C . Leptomeninges were stripped and washed extensively with pre-cooled PBS (10 mM-phosphate/150 mM-NaCl, pH 7.5), containing proteinase inhibitors (10 μg of aprotinin/ml, 5 μg of soybean trypsin inhibitor/ml, 10 μg of pepstatin A/ml, 1 μg of tosyl-lysylchloromethane ('TLCK')/ml, 2 mM-phenylmethanesulphonyl fluoride, 2 mM-EDTA and 2 mM-EGTA) until all blood was removed. The material was homogenized in the same buffer using a 7 ml Ten-Broeck tissue grinder, centrifuged at 16000 *g* for 10 min and the supernatants collected (PBS fractions). Pellets were re-

suspended in PBS/proteinase inhibitors containing 2% Triton X-100, frozen and thawed three times, followed by sonication for 10 s in a W-380 sonicator (Heat Systems-Ultrasonics, Farmingdale, NY, U.S.A.). After centrifugation under conditions identical with those described above, supernatants were collected (Triton fractions). The remaining insoluble pellets were re-suspended in 10 mM-Tris, pH 8.8, containing 2% SDS and boiled for 5 min (SDS fractions).

Immunoblotting

The different fractions described above were electrophoresed on SDS/17%-polyacrylamide minigels and transferred on to 0.45 μ m-pore-size nitrocellulose membranes (Bio-Rad, Richmond, CA, U.S.A.) using 3-cyclohexylamino-1-propane-sulphonic acid buffer, pH 11, containing 10% (v/v) methanol. Membranes were blocked with 5% non-fat dry milk in TBST (20 mM-Tris/HCl/150 mM-NaCl/0.05% Tween-20, pH 7.4) and incubated overnight at 4 °C with the following antibodies diluted in TBST: polyclonal anti-SP28 (1:500), polyclonal anti-SP41 (1:700), polyclonal anti-SP18 (1:1000), monoclonal F12 (1:1000) and monoclonal 6E10 (1:1000). Alkaline-phosphatase-labelled goat anti-rabbit IgG 1:3000 (Promega) or alkaline-phosphatase-labelled goat anti-mouse IgG 1:3000 (Promega) were used as a second antibody. Immunoblots were developed using 5-bromo-4-chloroindol-3-yl phosphate and Nitroblue Tetrazolium (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.).

Cells and cell cultures

The human monocytoid cell line U937 (A.T.C.C., Rockville, MD, U.S.A.) was maintained in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal-bovine serum (Gibco), 100 units of penicillin (base)/ml and 100 μ g of streptomycin (base)/ml. For cell-adhesion assays, cells were grown for 3 days at 37 °C in a humidified 5% CO_2 atmosphere, washed three times with RPMI 1640 and resuspended in attachment medium [20 mM-Tris/HCl/150 mM-NaCl, pH 7.4 (TBS), containing 2 mM- CaCl_2 /2 mM- MgCl_2 /1% BSA and 5 mM-glucose]. Cell viability was assessed by Trypan Blue dye exclusion at the time of harvest.

Cell-attachment assay

Flat-bottom multi-well plates Falcon 3915 (Becton-Dickinson Labware, Lincoln Park, NJ, U.S.A.) were coated overnight at 4 °C with 100 μ l of fibronectin (Fn), SP28, SP28-KLH, C109, unrelated peptides, BSA, KLH or lysozyme, at various concentrations (0.1–50 μ g/ml). The plates were washed three times with TBS and incubated for 1 h at 37 °C with 100 μ l of 1% BSA in TBS in order to block non-specific binding sites. The plates were rinsed with TBS, and 100 μ l of a cell suspension containing 10^6 cells of attachment medium/ml was added to each well, followed by 1 h incubation at 37 °C in a 5% CO_2 atmosphere. Non-adherent cells were removed by three gentle washes with attachment medium; attached cells were fixed for 15 min in 3% paraformaldehyde, pH 7.2, and stained with 0.5% Crystal Violet in 20% (v/v) methanol for 5 min [44]. Specifically stained material was eluted with 0.1 M-sodium citrate, pH 4.2, and quantified in a Microplate reader MR600 (Dynatech) at 540 nm.

Inhibition of cell adhesion

By synthetic peptides. To test the inhibition of cell attachment to either Fn-, SP28- or C109-coated plates by synthetic peptides RGDS, RHDS, SP28 and SP28-KLH, equal volumes of U937 cells (10^6 /ml) in attachment medium and of the corresponding peptides (final concn. 50 μ g/ml to 5 mg/ml) were incubated for 1 h at 37 °C in a 5% CO_2 atmosphere. Aliquots (100 μ l) of each mixture were transferred to wells coated either with 250 ng of Fn

or 500 ng of SP28 or C109, and incubated for 1 h in humidified 5% CO_2 atmosphere. Non-adherent cells were removed and attached cells were fixed, stained and evaluated as described above. As negative controls, U937 cells were incubated either with synthetic peptides SP18, SP19 or SP9, which do not possess the sequence RHDS in their primary structure, or BSA and lysozyme, before addition to substratum-coated wells. The cells that attached to Fn-, SP28-, or C109- coated wells, under identical assay conditions and in the absence of inhibitors, were considered to be the controls at 100% binding. Results were expressed as percentage of cells attached compared with the control.

By anti-(β 1 integrin) antibody. The inhibition of cell attachment to either SP28- or C109-coated surfaces was assessed as follows. A 100 μ l portion of U937 cells (10^6 /ml) in attachment medium were preincubated for 1 h at 37 °C in a 5% CO_2 atmosphere with different quantities (0.01–5 μ g) of anti-(β 1 integrin) antibody (mAb a-FnR5). Each mixture was transferred into SP28- or C109-coated wells (500 ng/well) and incubated for another 1 h under the same conditions as those described above. After removal of non-adherent cells, the cells attached were determined as described above. Values obtained with cells that were preincubated with unrelated antibodies were considered to be the control at 100% binding. The results were expressed as percentage of cell adhesion compared with controls.

RESULTS AND DISCUSSION

Four different antibodies (Table 1) immunolabelled a 16 kDa fragment on immunoblot experiments performed with SDS-soluble fractions obtained from leptomeninges of AD and aged-control subjects (Fig. 1): polyclonal antisera anti-SP41 (lane B) and anti-SP28 (lane D), and monoclonal antibodies 6E10 (lane F) and F12 (lane H). Anti-SP18, directed towards the N-terminus of APP, did not react with the 16 kDa component (lane A). Absorption of the antibodies with the corresponding synthetic peptides completely abolished the observed immunoreactivity (lanes C, E, G and I). Anti-SP28 also immunolabelled monomers of A β (lane D, *), reactivity that disappeared after absorption with SP28 (lane E). The staining observed at high molecular mass was interpreted as non-specific, since it was present in almost every lane regardless of the antibody used and in very few cases disappeared after absorption.

Anti-SP41 as well as anti-SP28 recognizes two different antigenic determinants both located in the extracellular portion of the amyloid A β (residues 6–11 and 21–28 of A β , respectively) [41]. Both antibodies detect neuritic plaques, vascular amyloid and 'preamyloid' deposits. Anti-SP28 does not recognize full-length APP, but it is capable of immunolabelling amyloid fibrils in electron-microscopic studies [45], which indicates that the

Table 1. Immunoreactivity of a panel of anti-APP antibodies probed against full-length APP and its derivative fragments

Antibody	Full-length APP*	Soluble APP†	16 kDa fragment‡	C109	A β ‡
Anti-SP18	+	+	—	—	—
Anti-SP41	±	±	+	+	+
Anti-SP28	—	—	+	+	+
mAb 6E10	+	+	+	+	+
mAb F12	+	—	+	+	—

* From baculovirus-infected Sf9 cells.

† From cerebrospinal fluid.

‡ From vascular wall.

Fig. 1. Immunoblot analysis

SDS-soluble fractions extracted from leptomeningeal vessels of AD patients were probed against anti-APP antibodies; anti-SP18 (lane A), anti-SP41 (lane B), anti-SP28 (lane D), mAb 6E10 (lane F) and mAb F12 (lane H). The arrowhead indicates the 16 kDa immunoreactive fragment; * indicates the A β monomer. Antibodies preabsorbed with the corresponding synthetic peptides are shown in lanes C, E, G and I. Molecular-mass (*M*) markers: 92 kDa, phosphorylase *b*; 67 kDa, BSA; 46 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 20 kDa, soybean trypsin inhibitor; 14 kDa, α -lactalbumin.

Fig. 2. Electron microscopy of C109 construct

The electronmicrograph shows C109 negatively stained with uranyl acetate. The bar indicates 100 nm (magnification $\times 150\,000$).

antibody recognizes a spatial structure present in the fibrils and not in the parent molecule. Monoclonal 6E10 recognizes amyloid deposits in AD patients [42], but also reacts with both soluble and full-length APP on Western-blot analysis [41]. Monoclonal F12 [43] recognizes full-length APP on immunoblots [41]. The naturally occurring 16 kDa fragment displayed a unique immunoreactivity, since it was immunolabelled by two antibodies directed against conformational epitopes present in A β ; therefore it adopts similar tertiary structure and might represent a potentially amyloidogenic fragment. This is in agreement with recent reports that describe the presence of membrane-associated low-molecular-mass components (14–22 kDa) containing A β in cerebral cortex, cell lines, cerebral microvessels and baculovirus-infected Sf9 cells overexpressing APP [46–51]. The variation in molecular mass may be due to technical differences or, more likely, may reflect dissimilar types of processing in different cells. Although lysosomal involvement was proposed [50], it is not clear how the 16 kDa fragment originates, how it may produce A β and where the additional processing takes place (e.g. lysosomal vesicles, plasma membrane or extracellular compartment). Conformational modifications due to free radicals [52], genetic alterations [53] and/or other unknown factors may render APP fragment(s) resistant to the normal catabolic pathway. Cell impairment to complete degradation of the amyloidogenic fragment will result in accumulation of self-aggregating peptides and, eventually, amyloid-fibril formation. In this regard it is noteworthy that a 19 kDa C-terminal APP fragment was reported in brain tissue from aged individuals [46]. However, the possibility that A β originates from intact APP or other still unknown APP fragments cannot be ruled out.

The role of APP in cell-cell or cell-extracellular-matrix interaction was previously suggested [9,31–37]. An antibody directed to the first 16 residues of A β successfully inhibited PC12 cell adhesion to plastic surfaces [37], implying that the site of the interaction may be located in that 16-amino-acid stretch. C109, a highly insoluble construct analogous to the C-terminal residues 587–695 of APP₆₉₅, that immunochemically behaves like the 16 kDa fragment described above (see Table 1) and spontaneously polymerizes forming twisted fibrils (Fig. 2), was used to test for potential cell adhesion of U937 cells. Positive and

Fig. 3. Cell-adhesion-promoting activity of C109

U937 cells were plated ($10^6/\text{ml}$ in attachment media) on wells coated with various concentrations (0.1–50 $\mu\text{g/ml}$) of C109 (Δ), Fn ($\blacktriangle$) BSA ($\blacklozenge$) or lysozyme ($\diamond$). After 1 h incubation at 37 $^{\circ}\text{C}$ in a 5% CO_2 atmosphere, attached cells were quantified as described in the text. Each point represents the mean for three independent experiments. The vertical bars indicate ± 2 S.D.

negative controls for cell attachment were carried out using Fn and BSA or lysozyme-coated substrata respectively. As shown in Fig. 3, C109 promotes adhesion of U937 cells in a dose-dependent manner, yielding maximum adhesion at a concentration of 50 $\mu\text{g/ml}$. The sigmoid curve obtained, as well as the maximum values of cells adhered, are similar to those obtained for the adhesion of U937 cells to Fn.

Synthetic peptide SP28 (positions 1–28 of A β), coupled to

Fig. 4. Cell-adhesion-promoting activity of SP28

U937 cells were plated (10^6 /ml in attachment media) on wells coated with variable concentrations (0.1–100 μ g) of SP28–KLH ($\blacktriangle$), KLH ($\blacklozenge$) or unrelated peptides SP18 and SP9 ($\triangle$). After 1 h incubation at 37 °C in a 5%–CO₂ atmosphere, cell adhesion was evaluated as described in the text. Each point represents the mean for three independent experiments. The vertical bars indicate ± 2 s.d.

Fig. 5. Inhibition of cell adhesion to Fn-coated wells by (a) RHDS and (b) SP28

U937 cells (10^6 /ml of attachment media) were preincubated for 1 h at 37 °C in a 5%–CO₂ atmosphere with various concentrations (50–1000 μ g/ml) of synthetic peptides RHDS ($\blacktriangledown$), RGDS ($\blacklozenge$), SP28 ($\blacktriangle$), SP28–KLH ($\bullet$), SP19 ($\triangle$) or SP18 ($\diamond$), as well as control proteins BSA, KLH or lysozyme ($\circ$), before being transferred to Fn-coated wells (250 ng/well). After 1 h at 37 °C in a 5%–CO₂ atmosphere, cell adhesion was evaluated as described in the text. Each point represents the mean for three independent experiments. The vertical bars indicate ± 2 s.d.

KLH to avoid amyloid-fibril formation [54–56], was also able to mediate cell adhesion of U937 cells specifically. As shown in Fig. 4, a dose-response behaviour was obtained using increasing concentrations of SP28–KLH to coat the microtitre plates. Negative controls, in which unrelated peptides SP18 and SP9 as well as KLH and BSA were used to coat the wells, never induced more than 5% of the total cell adhesion.

C109 and SP28 both contain the tetrapeptide RHDS (positions

Fig. 6. RHDS inhibition of cell adhesion to (a) SP28- and (b) C109-coated plates

U937 cells (10^6 /ml of attachment media) were preincubated for 1 h at 37 °C in a 5%–CO₂ atmosphere with various concentrations (50–5000 μ g/ml) of synthetic peptides RHDS ($\blacktriangledown$), RGDS ($\blacklozenge$), as well as control unrelated peptides SP9, SP19 or SP18 ($\triangle$) before being transferred to SP28- (a) or C109- (b) coated wells (500 ng/well). After 1 h at 37 °C in a 5%–CO₂ atmosphere, cell adhesion was evaluated as described in the text. Each point represents the mean for three independent experiments. The vertical bars indicate ± 2 s.d.

601–604 of β PP₆₉₅); it is located in a predicted β -turn, presumably exposed on the surface of the molecule [41]. The sequence RHDS is similar to the tetrapeptide RGDS, the recognition signal for many integrins [57], a widely distributed family of heterodimeric membrane proteins that mediate cell–cell and cell–extracellular-matrix interactions [58,59]. RGDS-mediated cell adhesion was competitively inhibited by the synthetic peptide RHDS in solution and by larger molecules that contain the sequence RHDS. As shown in Fig. 5(a), RHDS was able to mimic the peptide RGDS in its ability to inhibit the adhesion of U937 cells to Fn-coated surfaces. Both peptides at concentrations of 500 μ g/ml reduced the cell attachment by 50% or more when compared with the controls performed in the absence of inhibitors. It is possible that the replacement of histidine for glycine may render a fully active cell-adhesion peptide only under certain conditions, i.e. at a pH closer to histidine's isoelectric point, since the interaction appears to be pH-dependent (results not shown).

The larger synthetic peptide, SP28, either alone or coupled to the carrier molecule KLH, was used to inhibit cell attachment to Fn-coated surfaces. As indicated in Fig. 5(b), the number of cells adhering to the plate decreased when the cells were preincubated with increasing concentrations of either SP28 or SP28–KLH. The number of cells attached to Fn for the various concentrations of SP28–KLH tested matched the values obtained for RGDS-mediated inhibition of cell adhesion. Total suppression of the cell adhesion was observed when uncoupled SP28 was used as inhibitor. In this case, electron-microscopic examination revealed that formation of fibrils indeed occurred during the incubation period (results not shown). In order to rule out the possibility

Fig. 7. Inhibition of cell adhesion by anti-(β_1 integrin) antibodies

U937 cells (10^6 /ml of attachment media) were preincubated for 1 h at 37 °C in a 5% CO_2 atmosphere with various amounts (0.01–5 μg) of anti-(β_1 integrin) antibody (mAb a-FnR5) before being transferred to SP28- (▲) or C109- (◆) coated wells (500 ng/well). After 1 h at 37 °C in a 5% CO_2 atmosphere, cell adhesion was evaluated as described in the text. Unrelated antibodies were used as negative controls for inhibition (△). Each point represents the mean for three independent experiments. The vertical bars indicate ± 2 s.d.

that the fibrils might physically interfere with the cell adhesion (i.e. increasing the viscosity of the attachment medium), the unrelated peptide SP19 derived from a novel amyloid molecule, gelsolin [60], which also forms fibrils *in vitro* (B. Frangione, unpublished work), was used as control. Cells preincubated with the same concentrations of either unrelated peptides SP9 and SP18, or unrelated proteins such as BSA, KLH and lysozyme, never exhibited less than 90% cell attachment. Similar values were obtained with SP19, although fibrils were formed.

Corroborating the specificity of the interaction, the cell adhesion mediated by SP28–KLH was abolished by preincubation of the cells with synthetic peptides RGDS or RHDS, while control peptides SP9, SP19 or SP18 at identical concentrations failed to inhibit cell adhesion (Fig. 6a). Similar inhibition was obtained when the same synthetic peptides were probed to block the adhesion of U937 cells to C109-coated plates. As indicated in Fig. 6(b), a concentration of 500 μg of RHDS or RGDS/ml reduced the number of cells attached by 80%, while unrelated peptides SP9, SP19 or SP18 did not affect the adhesion.

Addition of 10 mM-EDTA to the attachment media totally suppressed the adhesion of the cells to either SP28–KLH- or C109-coated wells, indicating that the interaction is $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent, a property shared by all the integrins [59].

Preincubation of U937 cells with anti-(β_1 integrin) antibody (mAb a-FnR5) blocked their attachment to either SP28–KLH- or C109-coated plates. As indicated in Fig. 7, preincubation of the cells with increasing concentrations of the mAb decreased cell attachment to either SP28–KLH- or C109-coated wells, while unrelated antibodies did not affect the interaction. A 100% inhibition of cell attachment was achieved when the cells were preincubated with 2 μg of anti-(β_1 integrin) antibodies. In the brain, β_1 integrins are strongly expressed by capillary endothelial cells, where the basement-membrane proteins laminin and collagen type VI are their main ligands [61]. It is noteworthy that preincubation of U937 cells with RHDS or RGDS completely inhibited cell adhesion either to C109- or SP28–KLH-coated wells, while the same peptides yielded only a maximum of 60% inhibition of cell attachment to Fn-coated wells. U937 cells express two receptors for Fn: (a) integrin $\alpha_5\beta_1$, RGDS-dependent [62] and (b) integrin $\alpha_4\beta_1$, which recognizes a structure unrelated to RGDS in the Fn molecule [63]. Thus complete inhibition of cell attachment to Fn cannot be achieved by blocking only one of the Fn receptors.

Our data strongly suggest that, through the interaction of the tetrapeptide RHDS with an integrin-like receptor, APP and/or its derivatives may function as cell-adhesion proteins (preliminary data also indicate that nexin II, the soluble form of APP, can promote cell attachment to some extent). The biological implications of this interaction, as well as its potential role in amyloidogenesis, remain to be determined.

Note added in proof (received 13 October 1992)

Recent reports indicate the existence of soluble forms of A β in cerebrospinal fluid, plasma and cell-culture-medium supernatants [64–66], supporting the idea that A β -like peptides are produced by normal proteolytic processing.

This research was supported by National Institutes of Health Grants AG 08721, AG 05891, and AG 10491, and by the Anna Schermerhorn Fund.

REFERENCES

- Glenner, G. G. & Wong, C. S. (1984) *Biochem. Biophys. Res. Commun.* **120**, 885–890.
- Husby, G., Azaki, S., Benditt, E. P., Benson, M. D., Cohen, A. S., Frangione, B., Glenner, G. G., Natvig, J. B. & Westermark, P. (1991) in *Amyloid and Amyloidosis* (Natvig, J. B., Forre, O., Husby, G., Husebekk, A., Skogen, B., Sletten, K. & Westermark, P., eds.), pp. 7–11, Kluwer Academic Publishers, Dordrecht.
- Prelli, F., Castaño, E. M., Glenner, G. G. & Frangione, B. (1988) *J. Neurochem.* **51**, 648–651.
- Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L. & Beyreuther, K. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4245–4249.
- Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., Multhaup, G., Beyreuther, K. & Müller-Hill, B. (1987) *Nature (London)* **325**, 733–736.
- Selkoe, D. J. (1991) *Neuron* **6**, 487–498.
- Ghiso, J., Tagliavini, F., Timmers, W. F. & Frangione, B. (1989) *Biochem. Biophys. Res. Commun.* **163**, 430–437.
- Gandy, S. E., Czernik, A. J. & Greengard, P. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6218–6221.
- Dyrks, T., Wiedemann, A., Multhaup, G., Salbaum, J. M., Lemaire, H. G., Kang, J., Müller-Hill, B., Masters, C. L. & Beyreuther, K. (1988) *EMBO J.* **7**, 949–957.
- Weidemann, A., König, G., Bunke, D., Fisher, P., Salbaum, J. M., Masters, C. L. & Beyreuther, K. (1989) *Cell (Cambridge, Mass.)* **57**, 115–126.
- Oltersdorf, T., Ward, P. J., Henriksson, T., Beattie, E. C., Neve, R., Lieberburg, I. & Fritz, L. C. (1990) *J. Biol. Chem.* **265**, 4492–4497.
- Buxbaum, J. D., Gandy, S. E., Cicchetti, P., Ehrlich, M. E., Czernik, A. J., Fracasso, R. P., Ramabhadran, T. V., Unterbeck, A. J. & Greengard, P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6003–6006.
- Lemaire, H. G., Salbaum, J. M., Multhaup, G., Kanz, J., Bayney, R. M., Unterbeck, A., Beyreuther, K. & Müller-Hill, B. (1989) *Nucleic Acids Res.* **17**, 517–522.
- Sisodia, S. S., Koo, E. H., Beyreuther, K., Unterbeck, A. & Price, D. L. (1990) *Science* **248**, 492–495.
- Esch, F. S., Keim, P. S., Beattie, E. C., Blacker, R. W., Culwell, A. K., Oltersdorf, T., McClure, D. & Ward, P. J. (1990) *Science* **248**, 1122–1124.
- Wang, R., Meschia, J. F., Cotter, R. J. & Sisodia, S. S. (1991) *J. Biol. Chem.* **266**, 16960–16964.
- Anderson, J. P., Esch, F. S., Keim, P. S., Sambamurti, K., Lieberburg, I. & Robakis, N. K. (1991) *Neurosci. Lett.* **128**, 126–128.
- Ramabhadran, T. V., Gandy, S. E., Ghiso, J., Czernik, A. J., Ferris, D., Bhasin, R., Goldgaber, D., Frangione, B. & Greengard, P. (1992) *J. Biol. Chem.*, in the press.
- Oltersdorf, T., Fritz, L. C., Schenk, D. B., Lieberburg, I., Johnson-Wood, K. L., Beattie, E. C., Ward, P. J., Blacher, R. W., Dolvey, H. F. & Sinha, S. (1989) *Nature (London)* **341**, 144–147.
- Van Nostrand, W. E., Wagner, S. L., Suzuki, M., Choi, B. H., Farrow, J. S., Geddes, J. W., Cotman, C. W. & Cunningham, D. D. (1989) *Nature (London)* **341**, 546–549.
- Gardella, J. E., Ghiso, J., Gorgone, G. A., Marratta, D., Kaplan, A. P., Frangione, B. & Gorevic, P. (1990) *Biochem. Biophys. Res. Commun.* **173**, 1292–1298.

21. Van Nostrand, W. E., Schmaier, A. H., Farrow, J. S. & Cunningham, D. D. (1990) *Science* **248**, 745–748
22. Bush, A. I., Martins, R. N., Rumble, B., Moir, R., Fuller, S., Milward, E., Currie, J., Ames, D., Weidemann, A., Fischer, P., Multhaup, G., Beyreuther, K. & Masters, C. L. (1990) *J. Biol. Chem.* **265**, 15977–15983
23. Cole, G. M., Galasko, D., Shapiro, I. P. & Saitoh, T. (1990) *Biochem. Biophys. Res. Commun.* **170**, 288–295
24. Smith, R. P., Higuchi, D. A. & Broze, G. J. (1990) *Science* **248**, 1126–1128
25. Saitoh, T., Sundsmo, M., Roch, J. M., Kumura, N., Cole, G., Schubert, D., Oltersdorf, T. & Schenk, D. B. (1989) *Cell* (Cambridge, Mass.) **58**, 615–622
26. Schubert, D., Cole, G., Saitoh, T. & Oltersdorf, T. (1989) *Biochem. Biophys. Res. Commun.* **162**, 83–88
27. Whitson, J. S., Selkoe, D. J. & Cotman, C. W. (1989) *Science* **243**, 1488–1490
28. Bodmer, S., Podlisny, M. B., Selkoe, D. J., Heid, I. & Fontana, A. (1990) *Biochem. Biophys. Res. Commun.* **171**, 890–897
29. Whitson, J. S., Glabe, C. G., Shintani, E., Abcar, A. & Cotman, C. W. (1990) *Neurosci. Lett.* **110**, 319–324
30. Yankner, B. A., Dawes, L. R., Fischer, S., Villa-Komanoff, L., Oster-Granite, M. L. & Neve, R. L. (1989) *Science* **245**, 417–420
31. Schivers, B. D., Hiblich, C., Multhaup, G., Salbaum, M., Beyreuther, K. & Seeburg, P. H. (1988) *EMBO J.* **7**, 1365–1370
32. Octave, J.-N., de Sauvage, F. & Maloteaux, J.-M. (1989) *Brain Res.* **486**, 369–371
33. Schubert, D., Jin, L.-W., Saitoh, T. & Cole, G. (1989) *Neuron* **3**, 689–694
34. Ueda, K., Cole, G., Sundsmo, M., Katzman, R. & Saitoh, T. (1989) *Ann. Neurol.* **25**, 246–251
35. Klier, F. G., Cole, G., Stallcup, W. & Schubert, D. (1990) *Brain Res.* **515**, 336–342
36. Breen, K. C., Bruce, M. & Anderton, B. H. (1991) *J. Neurosci. Res.* **28**, 90–100
37. Chen, M. & Yankner, B. A. (1991) *Neurosci. Lett.* **125**, 223–226
38. Shioi, J., Anderson, J. P., Ripellino, J. A. & Robakis, N. K. (1992) *J. Biol. Chem.* **267**, 13819–13822
39. Engvall, E. & Ruoslahti, E. (1977) *Int. J. Cancer* **20**, 1–5
40. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, chapter 17, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
41. Ghiso, J., Wisniewski, T., Vidal, R., Rostagno, A. & Frangione, B. (1992) *Biochem. J.* **282**, 517–522
42. Kim, K., Wen, G. Y., Bancher, C., Chen, C. M., Sapienza, V. J., Hong, H. & Wisniewski, H. M. (1990) *Neurosci. Res. Commun.* **7**, 113–122
43. Gardella, J. E., Gorgone, G. A., Munoz, P. C., Ghiso, J., Frangione, B. & Gorevic, P. (1992) *Lab. Invest.* **67**, 303–313
44. Gillies, R. J., Didier, N. & Denton, M. (1986) *Anal. Biochem.* **159**, 109–113
45. Castaño, E., Ghiso, J., Prelli, F., Gorevic, P., Migheli, A. & Frangione, B. (1986) *Biochem. Biophys. Res. Commun.* **141**, 782–789
46. Nordstedt, C., Gandy, S. E., Alafuzoff, I., Caporaso, G. L., Iverfeldt, K., Grebb, J. A., Winblad, B. & Greengard, P. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8910–8914
47. Matsumoto, A. & Fujiwara, Y. (1991) *Biochem. Biophys. Res. Commun.* **175**, 361–365
48. Gandy, S. E., Bhasin, R., Ramabhadran, T. V., Koo, E. H., Price, D. L., Goldgaber, D. & Greengard, P. (1992) *J. Neurochem.* **58**, 383–386
49. Estus, E., Golde, T. E., Kunishita, T., Blades, D., Lowery, D., Eisen, M., Usiak, M., Qu, X., Tabira, T., Greemberg, B. D. & Younkin, S. G. (1992) *Science* **255**, 726–728
50. Golde, T. E., Estus, E., Younkin, L. H., Selkoe, D. J. & Younkin, S. G. (1992) *Science* **255**, 728–730
51. Tamaoka, A., Kalaria, R. N., Lieberburg, I. & Selkoe, D. J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1345–1349
52. Smith, C. D., Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Stadtman, E. R., Floyd, R. A. & Markesbery, W. R. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10540–10543
53. Castaño, E. M., Wisniewski, T. & Frangione, B. (1991) *Curr. Opin. Neurobiol.* **1**, 448–454
54. Kirschner, D. A., Abraham, C. & Selkoe, D. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 503–507
55. Gorevic, P. D., Castaño, E. M., Sarma, R. & Frangione, B. (1987) *Biochem. Biophys. Res. Commun.* **147**, 854–862
56. Wisniewski, T., Ghiso, J. & Frangione, B. (1991) *Biochem. Biophys. Res. Commun.* **179**, 1247–1254
57. Ruoslahti, E. & Pierschbacher, M. D. (1987) *Science* **238**, 491–497
58. Haynes, R. O. (1992) *Cell* (Cambridge, Mass.) **69**, 11–25
59. Humphries, M. J. (1990) *J. Cell Sci.* **97**, 585–592
60. Ghiso, J., Haltia, M., Prelli, F., Novello, J. & Frangione, B. (1990) *Biochem. J.* **272**, 827–830
61. McGeer, P. L., Akiyama, H. & Kawamata, T. (1991) in *Alzheimer's Disease: Basic Mechanisms, Diagnosis and Therapeutic Strategies* (Iqbal, K., McLachlan, R. R. C., Winblad, B. & Wisniewski, H. M., eds.), pp. 587–601, John Wiley and Sons, New York
62. Garcia-Pardo, A., Ferreira, O. C., Valinsky, J. & Bianco, C. (1989) *Exp. Cell Res.* **181**, 420–431
63. Ferreira, O. C., Garcia-Pardo, A. & Bianco, C. (1990) *J. Exp. Med.* **171**, 351–356
64. Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Selkoe, D., Lieberburg, I. & Schenk, D. (1992) *Nature* (London) **359**, 325–327
65. Haass, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B. L., Lieberburg, I., Koo, E. H., Schenk, D., Teplow, D. B. & Selkoe, D. (1992) *Nature* (London) **359**, 322–325
66. Shoji, M., Golde, T. E., Ghiso, J., Cheung, T. T., Estus, S., Shaffer, L. M., Cai, X.-D., McKay, D. M., Tintner, R., Frangione, B., Younkin, S. G. (1992) *Science* **258**, 126–129

Received 30 April 1992/30 June 1992; accepted 7 July 1992