Apolipoprotein E forms stable complexes with recombinant Alzheimer's disease β-amyloid precursor protein

Cristina HAAS*‡, Pilar CAZORLA*‡, Carlos DE MIGUEL†, Fernando VALDIVIESO* and Jesús VÁZQUEZ*§

*Centro de Biología Molecular 'Severo Ochoa', Universidad Autónoma de Madrid, 28049 Madrid, Spain, and †Departamento de Bioquímica, Universidad de Navarra, 31080 Pamplona, Spain

Apolipoprotein E (apoE), a protein genetically linked to the incidence of Alzheimer's disease, forms SDS-stable complexes *in vitro* with β-amyloid peptide (Aβ), the primary component of senile plaques. In the present study, we investigated whether apoE was able to bind full-length Aβ precursor protein (APP). Using a maltose-binding-protein–APP fusion protein and human very-low-density lipoprotein (VLDL), we detected an interaction of apoE with APP that was inhibited by Aβ or anti-apoE antibody. Saturation-binding experiments indicated a single binding equilibrium with an apparent 1:1 stoichiometry and a dissociation constant of 15 nM. An interaction was also observed using apoE from cerebrospinal fluid or delipidated VLDL, as well as recombinant apoE. APP·apoE complexes were SDS-stable, and their formation was not inhibited by reducing conditions; however, they were dissociated by SDS under reducing conditions. ApoE·APP complexes formed high-molecular-mass aggregates, and competition experiments suggested that amino acids 14–23 of Aβ are responsible for complex-formation. Finally, no differences were found when studying the interaction of APP with apoE3 or apoE4. Taken together, our results demonstrate that apoE may form stable complexes with the Aβ moiety of APP with characteristics similar to those of complexes formed with isolated Aβ, and suggest the intriguing possibility that apoE–APP interactions may be pathologically relevant *in vivo*.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder the central pathological event of which is the deposition of β-amyloid peptide (Aβ) as amyloid fibrils within senile plaques and cerebral blood vessels [1]. Aβ is generated from the proteolysis of a larger precursor protein (APP). The first APP metabolic pathway to be described involves cleavage at position 16 of the Aβ sequence by a still unidentified protease, denominated α-secretase, which obviously precludes Aβ formation [2–4]. Although aggregated Aβ is one of the main components, several other proteins are also associated with extracellular senile plaques, including SP 40,40 [5], APP itself, α_1-antichymotrypsin, complement factors, immunoglobulins, amyloid P, glycosaminoglycans and, most notably, apolipoprotein E (apoE) [6]. Recently the product of the S182 gene, which is linked to most early-onset familial AD cases, has also been found associated with senile plaques [7].

ApoE is a secretory protein of 34 kDa that is involved in plasma cholesterol transport and clearance. This protein has been shown to be the main apolipoprotein synthesized in the brain, is present in the cerebrospinal fluid as a component of lipoproteins and lipid complexes [8–10], and is implicated in neuronal regeneration. The synthesis of apoE markedly increases after neuronal injury in both the peripheral [11] and central [12] nervous systems. In addition, there is an isoform-specific effect of apoE on the outgrowth and sprouting of cultured dorsal root ganglia neurons [13,14] and a murine neuroblastoma cell line [15], which appears to be associated with microtubule depolarization [14]. Recent genetic evidence suggests a link between apoE and AD pathogenesis. The three major protein isoforms of apoE (apoE2, E3 and E4) are the products of three alleles (ε2, ε3 and ε4 respectively) at a single gene locus on the proximal long arm of chromosome 19q13.2 [16]. The frequency of the ε4 allele is significantly higher in sporadic [17–19] and familial [20,21] late-onset AD than in the general population. It has also been observed that brain tissue from AD patients carrying the ε4 allele contains more amyloid than does brain tissue from AD patients with other apoE genotypes [18,22].

Several studies have demonstrated that synthetic Aβ binds *in vitro* to apoE from cell culture medium [23,24] and cerebrospinal fluid [5,21,25], as well as to purified apoE [25,26]. The complexes between apoE and Aβ are stable to boiling in SDS, implying strong intermolecular interactions, and it has also been shown that apoE enhances polymerization of Aβ *in vitro* [27–29], thereby supporting the idea that apoE may act as a 'pathological chaperone', facilitating Aβ fibrillogenesis and deposition [30]. Recently, evidence has been presented that amyloid-associated apoE from AD brains has formed complexes with polymers of Aβ [31]. All of these observations support the idea that the interaction with Aβ may underlie the physiological mechanism by which apoE contributes to AD pathology.

Given the occurrence of tight binding between apoE and Aβ, and since Aβ is formed by proteolytic processing of APP, we have been intrigued by the possibility that at least part of apoE may directly bind to APP through the Aβ moiety before the precursor is proteolytically processed, i.e. before Aβ is actually produced. In order to explore this hypothesis, we investigated whether apoE from human very-low-density lipoprotein (VLDL) and from other sources is able to interact with full-length recombinant APP. The results presented here clearly demonstrate that apoE binds APP, forming SDS-stable complexes resembling

Abbreviations used: AD, Alzheimer's disease; Aβ, β-amyloid peptide; APP, Aβ precursor protein; apoE, apolipoprotein E; VLDL, very-low-density lipoprotein; MBP, maltose-binding protein; APP_{695}, APP_{751} and APP_{770}, APP isoforms containing 695, 751 and 770 amino acids respectively.

‡ These authors contributed equally to this work.

§ To whom correspondence should be addressed.

those produced with Aβ, thus opening up the possibility that a direct interaction between two of the proteins involved in AD, i.e. apoE and APP, may be physiologically relevant.

EXPERIMENTAL

Materials

Recombinant apoE3 and apoE4 were purchased from Calbiochem. Peptide p186 [Aβ-(1–29)] was synthesized by Professor Manuel Patarroyo (Instituto de Inmunología, Hospital de San Juan, Bogotá, Colombia). Peptides p4 [Aβ-(12–21)], p5 [Aβ-(14–23)], p6 [Aβ-(16–25)] and p7 [Aβ-(18–27)] were synthesized by Professor Juan Pablo Albar (Centro Nacional de Biotecnología, Madrid, Spain). The purity of the peptides (> 90 %) was checked by HPLC analysis. Goat anti-apoE antibodies were from Calbiochem, and rabbit anti-[maltose-binding protein (MBP)] antibodies were from New England Biolabs. Synthetic Aβ-(1–40) was purchased from Sigma.

Preparation of human and delipidated VLDL

Human VLDLs were prepared according to the simple discontinuous density-gradient procedure described by Redgrave et al. [32], with some modifications. This procedure uses high-speed rotors in a single centrifugation step to separate the individual plasma lipoprotein species in relatively small samples. Briefly, samples of human plasma from fasted individuals with the $\epsilon 3/\epsilon 3$ genotype (unless otherwise indicated) were adjusted to $d = 1.21$ with KBr, and 920 μl aliquots were pipetted into 3 ml polycarbonate TL100.3 centrifuge tubes (13 mm × 51 mm) (Beckman). A discontinuous gradient was formed by layering 690 μl of salt solution of $d = 1.063$ above the plasma, followed by 690 μl of salt solution of $d = 1.019$ and 575 μl of salt solution of $d = 1.006$. The tubes were centrifuged for 4 h at 334000 ***g*** in a TL-100 centrifuge (Beckman), and bands I–IV [32] were separated. The distribution of lipoproteins was checked by immunoblot analysis of each of the fractions using antibodies specific for apoA-I, apoA-II, apoC-II, apoB and apoE (Calbiochem). The fraction corresponding to band I contained VLDL, as described [32], and was used for interaction experiments after extensive dialysis against 0.01 % EDTA, pH 7.4. The apoE content in VLDL preparations was measured by SDS/PAGE followed by densitometry. VLDLs were delipidated by chloroform/methanol extraction and redissolved as described in [33].

Production of recombinant MBP–APP

Expression of the three APP isoforms APP_{695}, APP_{751} and APP_{770} (containing 695, 751 and 770 amino acids respectively) was accomplished using the $pMal_c$ plasmid system (New England Biolabs). Clones containing the cDNA sequences of the three isoforms (pSP65-695, pSP65-751 and pSP65-770 respectively; donated by Professor K. Beyreuther, Center for Molecular Biology, University of Heidelberg, Heidelberg, Germany) were used to construct plasmids $pMal_c$-695, $pMal_c$-751 and $pMal_c$-770, which code for the three fusion proteins (MBP–APP) composed of MBP fused to APP_{695}, APP_{751} or APP_{770} respectively, starting from amino acid Val^{20} in all cases. To obtain APP cDNAs, pSP65-APP vectors were digested with *Ban*I, their recessed 3′ termini were filled in with Klenow fragment, and they were finally subjected to *Hin*dIII digestion. The $pMal_c$ (pIH821) vector was digested with the restriction enzymes *Stu*I and *Hin*dIII.

The ligation mixtures of the APP cDNAs and the digested $pMal_c$ vector were used to transform competent *Escherichia coli* TG1 cells. The constructs were checked by automated DNA sequencing in a Pharmacia-LKB A. L. F. DNA sequencer. Cells containing plasmids that coded for fusion proteins were grown and induced with isopropyl β-D-thiogalactosidase. Bacterial cells were sedimented by low-speed centrifugation and the pellet was suspended in lysis buffer (30 mM NaCl, 10 mM β-mercaptoethanol, 10 mM sodium phosphate, pH 7.2, 0.25 % Tween 20), subjected to a freeze–thaw cycle, sonicated and centrifuged at 9000 ***g*** for 30 min. The supernatant was adjusted to 0.5 M NaCl and passed five times through an amylose resin column (New England Biolabs) pre-equilibrated with column buffer (0.5 M NaCl, 10 mM sodium phosphate, pH 7.2, 1 mM sodium azide) containing 0.25 % Tween 20. The column was washed with 15 vol. of 0.25 % Tween 20 in column buffer and with 30 vol. of column buffer. Bound MBP–APP was then eluted with column buffer containing 10 mM maltose.

Detection of MBP–APP · apoE complexes

To study the interaction of apoE with MBP–APP, 1 ml aliquots of washed amylose resin bound to MBP–APP were packed on to 2 ml minicolumns (Pierce). Aliquots of 1 ml of freshly prepared VLDL diluted 10-fold (3 μg of apoE), recombinant apoE (1 μg) or human cerebrospinal fluid diluted 2-fold in column buffer containing 0.25 % Tween 20 were added to the columns and incubated batchwise with gently agitation overnight, unless otherwise stated. In the inhibition experiments, VLDLs were preincubated with 20 μg of synthetic peptide or with 0.1 vol. of anti-apoE antibody before dilution. The columns were washed with 20 vol. of column buffer and eluted with 3 ml of 10 mM maltose in column buffer. The eluted fractions were then concentrated using a 3-kDa cut-off YM3 Diaflo membrane (Amicon), resuspended in sample buffer and subjected to SDS/PAGE (under reducing conditions unless otherwise stated) using 7.5 % acrylamide. Proteins were then electrotransferred on to nitrocellulose sheets (0.45 μm pore size; Bio-Rad) in 39 mM glycine, 0.0375 % SDS, 48 mM Tris/HCl, pH 9.0, and 20 % (v/v) methanol for 3 h at a current intensity of 1.3 mA/cm², according to the semi-dry standard procedure [34], and unreactive binding sites were blocked by overnight incubation at 4 °C in a buffer containing 3 % BSA and 0.2 % Tween 20. The blots were incubated for 2 h at room temperature with goat anti-(human apoE) antibodies (Calbiochem) diluted 1:7500. After washing, the sheets were incubated for 90 min with biotin-conjugated rabbit anti-goat IgG antibodies (Sigma) diluted 1:50000, followed by streptavidin-conjugated horseradish peroxidase (Boehringer-Mannheim). Blots were then developed using the luminol/H_2O_2 method (ECL Western Blotting Reagent; Amersham) according to the manufacturer's instructions, and the plaques were subjected to densitometric analysis in a 300A Computing Densitometer (Molecular Dynamics). The amounts of MBP–APP eluted were also quantified either by direct Coomassie Blue staining or by immunoblotting using an anti-APP antibody as described previously [35], and were found to be essentially constant throughout each of the experiments.

Analysis of MBP–APP · apoE complexes by sucrose-density-gradient centrifugation

Maltose eluates obtained after incubation of columns containing bound MBP–APP with recombinant apoE were concentrated to 100 μl by ultrafiltration through a 3-kDa cut-off membrane (YM3). The concentrates and 100 μl aliquots of recombinant apoE (10 μg/ml) were layered on to parallel 5–20 % (w/v) sucrose-density gradients prepared in 3 ml polycarbonate tubes, and centrifuged in a Beckman TL100.3 rotor at 4 °C at the velocity and for the times indicated in the legend to Figure 3.

Fractions of 250 μl were collected from top to bottom and directly dot-blotted on to a nitrocellulose membrane. The blots were then developed by immunostaining using anti-apoE antibody followed by densitometric analysis, as described above. Sedimentation coefficients were estimated using the method of McEwen [36], assuming a particle density of 1.3 g/ml.

Saturation-binding analysis of the interaction of apoE with MBP–APP

Columns containing bound MBP–APP were incubated with various amounts of either recombinant apoE3 or recombinant apoE4 and washed, and bound proteins were eluted as described above. Amounts of apoE in the eluates were quantified by spotting the samples on to nitrocellulose sheets using a conventional dot-blot apparatus and subjecting the sheets to immunodetection using an anti-apoE antibody, under the same conditions as employed for Western blot analysis (see above). Saturation-binding data were fitted directly to a model that assumes the existence of a single population of binding sites, by a conventional non-linear iterative least-squares algorithm. In order to better compare data obtained from independent experiments, bound apoE was typically expressed as the fraction of occupied binding sites ($B_r = B/B_{max}$, where B is the concentration of bound apoE and B_{max} is the asymptotic value of B derived from curve fitting). Since B was never greater than 2% of the total concentration of apoE applied, Scatchard analysis were routinely performed by plotting $B_r/[apoE]_t$ against B_r, where $[apoE]_t$ is the total amount of apoE. The dissociation constant of the complex was also estimated from the regression lines. Dissociation constants obtained by the two methods were not significantly different from each other.

N-terminal and internal protein sequencing

Fractions containing either intact MBP–APP or MBP–APP subjected to controlled digestion with chymotrypsin (Sigma) were subjected to SDS/PAGE and then electrotransferred on to Immobilon membranes (Millipore). The bands of interest were then visualized by Ponceau Red staining, excised and sequenced directly using an Applied Biosystems 473A pulsed-liquid phase protein sequencer, using the manufacturer's protocols.

RESULTS

Expression of recombinant MBP–APP fusion proteins

We engineered constructs that allowed us to produce in *E. coli* the three major isoforms of APP (containing 695, 751 and 770 amino acids) as proteins fused to MBP, using the $pMal_c$ plasmid. This system produced recombinant MBP–APP fusion proteins with high yields (10–20 mg of protein/litre of cell culture). The MBP–APP fusion proteins were rapidly and efficiently affinity-purified in an single step through an amylose resin, which specifically binds MBP-containing proteins. SDS/PAGE analysis of the maltose eluate corresponding to the MBP–APP_{695} isoform revealed the presence of a unique band of approx. 157 kDa (Figure 1A). The 157 kDa band was unambiguously identified as the MBP–APP_{695} fusion protein on the basis of N-terminal and internal protein sequencing, and immunoblot analysis using anti-MBP and anti-APP antibodies (results not shown). Similar results were obtained for the MBP–APP_{751} and MBP–APP_{770} fusion products. In some instances, minor contaminating bands were observed below the MBP–APP band; these bands presumably correspond to C-terminally truncated MBP–APP proteins, since they were detected by anti-MBP antibody.

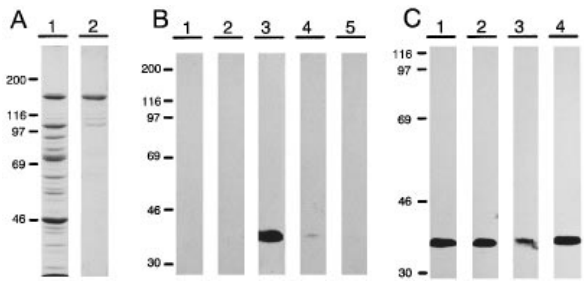

Figure 1 Binding of various preparations of apoE to columns containing bound MBP–APP

(**A**) Coomassie Blue-stained gel of fractions from a column containing bound MBP–APP incubated with VLDL. Lane 1, crude bacterial extracts containing recombinant MBP–APP; lane 2, maltose eluate. (**B**) Anti-apoE staining of immunoblots from maltose eluates after incubation of various columns with VLDL. Lane 1, column with resin alone; lane 2, column with bound MBP; lane 3, column with bound MBP–APP; lane 4, as for 3, except that VLDL was preincubated with Aβ-(1–40)-peptide; lane 5, as for 3, except that VLDL was preincubated with anti-apoE antibody. (**C**) Anti-apoE staining of immunoblots from maltose eluates after incubation of columns containing bound MBP–APP with various apoE sources. Lane 1, VLDL; lane 2, delipidated VLDL; lane 3, cerebrospinal fluid; lane 4, recombinant apoE. Positions of molecular mass markers (kDa) are shown on the left of each gel. For further details, see the Experimental section.

Interaction of apoE with MBP–APP

In order to investigate whether apoE was able to interact with APP, aliquots of diluted VLDL were incubated in mini-columns containing resin-bound MBP–APP. After the addition of maltose, the presence of apoE in the eluates was tested by immunoblotting with an anti-apoE antibody. As shown in Figure 1(B), whereas no signal was detected in the eluate from columns containing resin alone or MBP-bound resin, an intense band corresponding to apoE was found in the eluate from the column containing MBP–APP_{695}-bound resin. This result indicated that the APP_{695} moiety was responsible for the retention of apoE in the column. Similar results were obtained when MBP–APP_{751} or MBP–APP_{770} fusion proteins were used (not shown).

In order to investigate further the specificity of this interaction, the VLDL preparation was incubated with either synthetic Aβ-(1–40) or an anti-apoE antibody, before being added to the columns. These treatments almost completely abolished the detection of apoE in the eluate (Figure 1B), indicating that apoE retention by the column was not due to a minor interacting component of VLDL different from apoE, and that the Aβ moiety of APP was probably responsible for the interaction observed.

To investigate whether the presence of lipids in the lipoprotein complexes is required for the APP–apoE interaction, VLDLs were subjected to delipidation by chloroform/methanol extraction. This treatment did not inhibit binding to the column (Figure 1C). Consistent with this, both a preparation of recombinant apoE and apoE from a sample of human cerebrospinal fluid were also found to interact with the column (Figure 1C). These results indicate that the presence of lipids is not essential for the interaction, and supports the notion that apoE is binding APP directly.

Stability of apoE·APP complexes

We then investigated the stability of apoE·APP complexes under reducing conditions and to boiling in SDS. We knew from the previous experiments that the apoE·APP complexes were not resistant to boiling in SDS under reducing conditions, since the

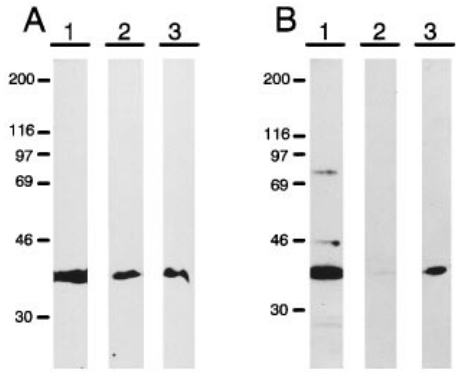

Figure 2 Evidence that MBP–APP·apoE complexes can be formed under reducing conditions and are SDS-stable under non-reducing conditions

Aliquots of VLDL preparations (lanes 1) and of maltose eluates after incubation of columns containing bound MBP–APP with VLDL in either the absence (lanes 2) or the presence (lanes 3) of 0.4% β-mercaptoethanol were subjected to SDS/PAGE under either reducing (**A**) or non-reducing (**B**) conditions, and subjected to immunoblotting using an anti-apoE antibody. Positions of molecular mass markers (kDa) are shown on the left of each gel.

maltose eluates yielded a single apoE band with an apparent molecular mass of 34 kDa, indistinguishable from that of native apoE (Figure 2A, lanes 1 and 2). However, a 34 kDa apoE band was also detected in the maltose eluate after incubation of VLDL with a column containing bound MBP–APP in the presence of β-mercaptoethanol (Figure 2A, lane 3). This observation clearly demonstrated that the reducing agent did not inhibit the interaction of apoE with APP.

ApoE from our preparation of human VLDL migrated on non-reducing SDS/PAGE as a single band of 34 kDa, sometimes accompanied by a band of about 80 kDa, probably corresponding to the apoE3 dimer (Figure 2B, lane 1). However, when an aliquot of the maltose eluate was subjected to immunoblotting after non-reducing SDS/PAGE, no traces of apoE were detected (Figure 2B, lane 2), indicating that the apoE present cannot enter the gel under these conditions, presumably because it is forming a high-molecular-mass complex with APP (see below). Consistently, the apoE band was detected after non-reducing SDS/PAGE when a reducing agent was present in the incubation medium (Figure 2B, lane 3), thus demonstrating that the high-molecular-mass complex can be dissociated by the combined effects of SDS and reducing agent.

Analysis of apoE and MBP–APP·apoE complexes by sucrose-density-gradient centrifugation

Since our efforts to detect high-molecular-mass complexes containing apoE by immunoblotting after non-reducing SDS/PAGE were unsuccessful, we sedimented MBP–APP·apoE complexes and isolated apoE through linear 5–20% sucrose gradients to obtain a comparative estimate of their molecular sizes. As shown in Figure 3, the apoE-containing complexes showed a considerably faster migration than did intact apoE, a result indicative of their greater molecular size. From data in Figures 3(B)–3(F), the sedimentation coefficient of apoE alone was estimated as

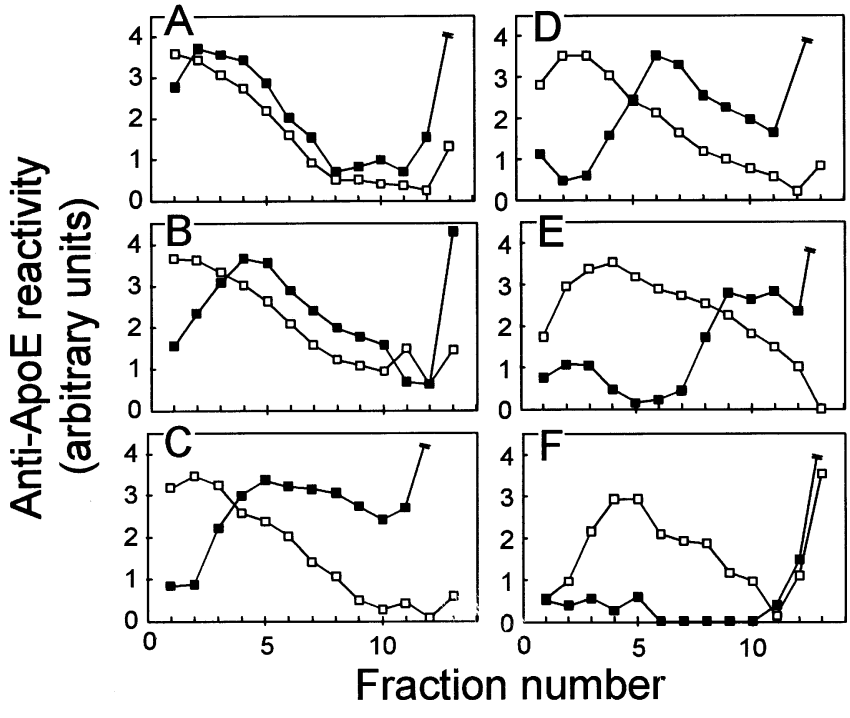

Figure 3 Sucrose-density-gradient centrifugation of apoE and MBP–APP·apoE complexes

Recombinant apoE (□) or maltose eluates from a column containing bound MBP–APP incubated with apoE (■) were layered on to 5–20% (w/v) sucrose-density gradients and subjected to centrifugation; fractions were collected from top (left) to bottom (right) and subjected to immunoblot analysis using anti-apoE antibody. Centrifugations were performed at 83500 ***g*** for 0.5 (**A**), 1 (**B**), 2 (**C**) and 3 h (**D**), or at 372000 ***g*** for 1 (**E**) and 2 h (**F**).

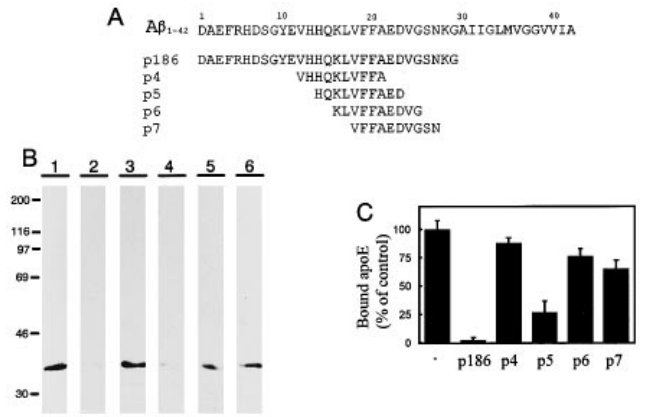

Figure 4 Identification of the region of the APP molecule implicated in apoE binding

(**A**) Scheme showing the composition of peptides used in these experiments. (**B**) Anti-apoE staining of immunoblots from maltose eluates after incubation of columns containing bound MBP–APP with VLDL preincubated in the presence of no peptide (lane 1), p186 (lane 2), p4 (lane 3), p5 (lane 4), p6 (lane 5) or p7 (lane 6). Positions of molecular mass markers (kDa) are shown on the left. (**C**) Densitometric analysis of immunoblots similar to that presented in (**B**). Data are expressed as means $\pm$ S.D. of two independent determinations.

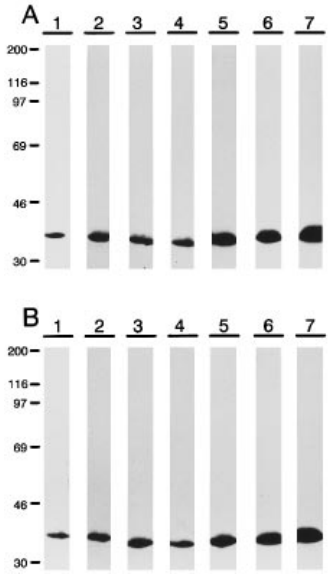

Figure 5 Time course of the interaction of MBP–APP with VLDL isolated from the plasma of subjects with the ε3/ε3 and ε4/ε4 genotypes

Maltose eluates after incubation of columns containing bound MBP–APP with VLDL from ϵ3/ϵ3 (**A**) or ϵ4/ϵ4 (**B**) homozygotes for 0.5 (lanes 1), 1 (lanes 2), 2 (lanes 3), 3 (lanes 4), 5 (lanes 5), 7 (lanes 6), or 24 (lanes 7) h were subjected to immunoblotting using an anti-apoE antibody. Positions of molecular mass markers (kDa) are shown on the left of each gel.

11 ± 1 S (mean $\pm$ S.E.M. of five determinations), corresponding to a particle of approx. 200–300 kDa. In clear contrast, the estimated sedimentation coefficient of apoE-containing complexes was 37 ± 4 S, which corresponds to a particle of considerable higher molecular mass (approx. 1000 kDa). This result supports our notion that apoE is eluted from columns tightly bound to MBP–APP, and provides an explanation as to why the undissociated complexes cannot be detected in standard SDS/PAGE gels.

Identification of the region of APP involved in the interaction with apoE

In order to study the region of the Aβ moiety within the APP molecule that is responsible for apoE binding, a number of peptides overlapping the region comprising residues 1–28 of Aβ (Figure 4A) were tested for their ability to inhibit the binding of apoE to APP. As shown in Figures 4(B) and 4(C), preincubation of VLDL with peptide p186, corresponding to Aβ-(1–29), completely abolished the retention of apoE by the MBP–APP-bound resin. Peptides p4–p7, from the region comprising residues 12–27 of Aβ, also partially inhibited binding, with peptide p5, corresponding to Aβ-(14–23), having the greatest effect. These results suggest that the amino acids of APP and of Aβ responsible for the interaction with apoE are most probably the same, further supporting the idea that apoE·Aβ and apoE·APP complexes are stabilized by similar binding mechanisms.

Interaction of APP with apoE isoforms

The differential binding to APP of isoforms apoE3 and apoE4 was investigated. We studied the time course of interaction of VLDL isolated from the plasma of subjects with the ϵ3/ϵ3 and ϵ4/ϵ4 genotypes. As shown in Figure 5(A), the extent of the interaction gradually increased along the time course considered, with maximal binding being reached after about 24 h. This profile is similar to that reported by other authors for the apoE·Aβ interaction [23,24,26]. However, under our experimental conditions, no significant differences could be observed in the interactions of VLDLs from individuals with ϵ3/ϵ3 and ϵ4/ϵ4 genotypes, either in the time-course profiles or in maximal binding (Figures 5A and 5B).

Saturation-binding analysis of the MBP–APP·apoE interaction

In order to perform a quantitative characterization of the interaction of apoE with MBP–APP, and since only a single band was detected in the column eluates by the anti-apoE antibody after Western blot analysis, a direct immunoblot protocol was developed for determining apoE concentrations. As shown in Figure 6, when MBP–APP-containing columns were incubated with increasing concentrations of either apoE3 or apoE4, apoE was retained by the columns in a concentration-dependent fashion. A good fit of the saturation-binding data was obtained using a model which assumes the existence of a single population of binding sites (Figure 6, solid curves). The saturation curves obtained using apoE3 and apoE4 were qualitatively indistinguishable, and the calculated dissociation constants (15 ± 3 nM and 18 ± 3 nM respectively, expressed as means $\pm$ S.D.) did not differ significantly. Similar conclusions were obtained after performing a Scatchard analysis of the binding data (Figure 6, insets).

The maximal amount of apoE bound under the experimental conditions employed was calculated as 1.3 ± 0.3 pmol for both apoE3 and apoE4. Since the amount of MBP–APP present in column eluates was 1.9 pmol, and given the possibility that some proportion of column-bound MBP–APP molecules may remain inaccessible or acquire a conformation unfavourable for apoE binding, these data support a 1:1 stoichiometry of binding of APP to apoE.

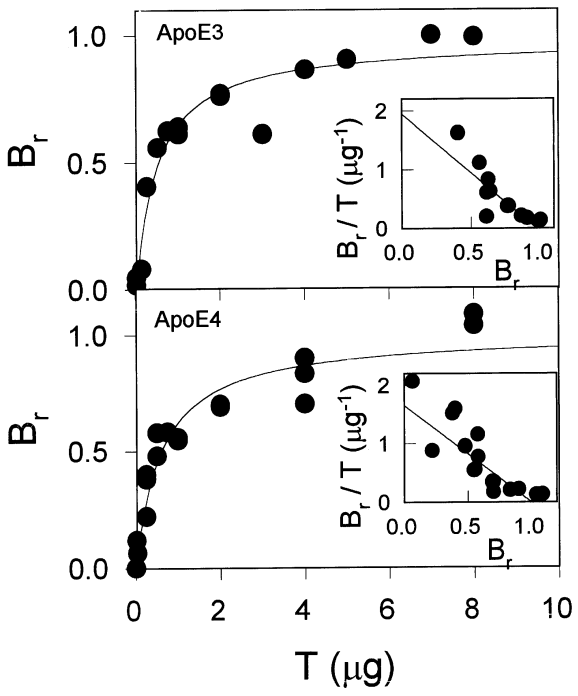

Figure 6 Saturation-binding analysis of the binding of apoE isoforms to MBP–APP

Columns containing bound MBP–APP were incubated with increasing amounts (T) of either apoE3 (upper panel) or apoE4 (lower panel), and apoE in maltose eluates from the columns was quantified by dot-blot analysis as described in the Experimental section. Bound apoE is expressed as the fraction of occupied binding sites (B_r). Insets: Scatchard plots modified as described in the Experimental section. Continuous lines are drawn assuming a single class of binding sites, using the best-fit parameters obtained in each case. Data are representative of three independent experiments.

DISCUSSION

The results presented here clearly demonstrate that apoE interacts with full-length recombinant APP. The experimental approach employed in this work was based on the reversible binding of the MBP–APP fusion protein to columns containing amylose resin. The mild conditions used for MBP–APP elution also allowed us to preserve the integrity of the apoE-containing complexes, thus allowing the study of their stability to boiling in SDS and the presence of reducing agents, as well as the estimation of their molecular size.

The competition experiments indicate that the region of APP implicated in apoE binding is most probably located between amino acids 14 and 23 of Aβ. This finding is in very good agreement with the results of Strittmatter et al. [21], who reported amino acids 12–28 as being the probable region of apoE binding to isolated Aβ. Interestingly, residues 16–20 of Aβ have been reported to serve as a binding sequence during Aβ polymerization and fibril formation [37]. Therefore the same region of Aβ appears to be responsible for the interaction of apoE with both Aβ and APP, and for Aβ–Aβ interactions. Our results also indicate that APP binding is apparently not affected by the lipidic environment of apoE or by the source of lipoprotein, in agreement with data obtained with Aβ by several groups [5,21,23–26,33]. This similarity between the binding of both Aβ and APP to apoE suggests that we are detecting the same kind of molecular interaction (see also below).

We found consistently that apoE formed complexes with MBP–APP that were resistant to boiling in SDS in the absence of reducing agents, behaviour which mimics that of complexes formed between apoE and Aβ [21,23,24,26,32]. This indicates that the existence of strong intermolecular interactions between apoE and the Aβ moiety of APP are also possible. In addition, we found that MBP–APP·apoE complexes appear to form high-molecular-mass aggregates. In this regard, it should be noted that the formation of high-molecular-mass complexes between apoE and Aβ *in vitro* has been described [31].

The formation of MBP–APP·apoE complexes was not inhibited by the presence of reducing agents in the incubation medium. This behaviour is similar to that of apoE·Aβ complexes, as reported by Näslund et al. [31], but apparently contradicts the results of other authors, who observed that inclusion of reducing agents abolished complex-formation [5,28]. The reason for these discrepancies is still unclear, although they have been attributed to differences in the protocols used for complex-solubilization prior to SDS/PAGE [31]. In the case of apoE·APP complexes, our column approach allowed us to determine directly that, although such complexes can be formed in the presence of reducing agents, they are only stable to boiling in SDS in the absence of reducing agents. Therefore, although the complexes are SDS-resistant, they can be disrupted by the combined effects of boiling in SDS and reducing conditions. Although direct comparisons should be viewed carefully owing to the different experimental approaches and the nature of the reactants, this explanation could be tentatively applied to the contradictory results obtained with apoE·Aβ complexes. In the work of Strittmatter et al. [26], the reducing agent was present in the medium that was mixed with SDS/PAGE sample buffer; in this case, if apoE·Aβ complexes were actually formed, they could have been dissociated by the synergistic effects of reducing agent and SDS. On the other hand, in the work of Näslund et al. [31], apoE·Aβ complexes formed in the presence of dithiothreitol were pelleted, resuspended in hexafluoro-2-propanol and dried, a process that may have rendered the reducing agent unable to contribute to complex-dissociation in the presence of SDS. Nevertheless, more detailed work needs to be done to determine the molecular mechanisms that underlie the effects of reducing agents on the stability of apoE-containing complexes.

The time-course profile of the apoE–APP interaction was also essentially similar to that reported for the apoE–Aβ interaction [23,24,26]. However, we did not observe any difference between the behaviour of apoE3 and apoE4 with respect to APP binding. Similarly, no significant differences in either the dissociation constants or the binding stoichiometries of the two apoE isoforms were evident. These observations contrast with previous reports [23,24,26], in which isoform-specific differences in Aβ binding were described. The reason for this discrepancy is currently unknown, but it may be related to the source of apoE employed in this and previous studies, as has already been suggested [23,24]. Alternatively, it may be a consequence of the different experimental protocols or binding conditions employed in the present study. Finally, it is also possible that the differences between apoE3 and apoE4 may become negligible when binding is to APP in comparison with Aβ, due to reasons of steric hindrance or specific spatial arrangements within the APP molecule of amino acids involved in the interaction.

Our data support the existence of a single binding equilibrium with a 1:1 stoichiometry and an apparent dissociation constant of around 15 nM. These results agree very well with a recent report [38] in which a dissociation constant of 20 nM was described for the interaction of Aβ with recombinant apoE and no differences were found between apoE3 and apoE4. Interestingly, the high affinity of the apoE–APP interaction is

typical of that of a ligand–receptor complex, thus supporting the notion that such an interaction may play a physiological role, and opening up the possibility that membrane-bound APP acts as an apoE receptor.

In conclusion, our data clearly support the notion that apoE may form stable complexes with the Aβ moiety of full-length APP with similar characteristics to those of complexes formed with isolated Aβ. The physiological relevance of the APP–apoE interaction is at present unknown. The apoE·APP complexes produced *in vitro* appear to be stabilized by strong intermolecular interactions, and it is important to note that, besides Aβ and apoE, full-length APP has been detected immunochemically in AD amyloid [6]. Therefore the role of apoE as a pathological chaperone facilitating Aβ fibrillogenesis and deposition may be extended to contribute to the association of APP with amyloid plaques.

Finally, given that the region of the APP molecule that appears to mediate apoE binding (amino acids 14–23 of Aβ) overlaps the site of action of α-secretase, the intriguing possibility is raised that bound apoE exerts a protective effect on the APP molecule that switches the proteolytic metabolism of APP towards the amyloidogenic pathway. Given the strong molecular forces apparently involved, apoE should remain tightly associated with the Aβ moiety throughout the proteolytic cascade of APP degradation, and a mechanism may be conceived by which at least some of the nascent Aβ molecules are already bound to apoE. Although the proportion of apoE-bound Aβ molecules may be very low, it may be sufficient to act as nucleus of fibrillogenesis and aggregation of Aβ into amyloid. The interesting possibility that apoE is involved in the modulation of the proteolytic processing of APP awaits more detailed investigation.

We thank Professor Manuel Patarroyo and Professor Juan Pablo Albar for peptide synthesis, Professor Konrad Beyreuther for APP-containing cDNA clones, and Ms. Esther Romero for excellent technical assistance. This work was supported by Dirección General de Investigación Científica y Técnica (DGICYT; Grant #PB93-0182), Boehringer Ingelheim España and an institutional grant from Fundación Ramón Areces to CBMSO. C.H. and P.C. are recipients of fellowships from the Comunidad Autónoma de Madrid.

REFERENCES

1 Selkoe, D. J. (1991) Neuron **6**, 487–498
2 Sisodia, S. S., Koo, E. H., Beyreuther, K., Unterbeck, A. and Price, D. L. (1990) Science **248**, 492–495
3 Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D. and Ward, P. J. (1990) Science **248**, 1122–1124
4 Wang, R., Meschia, J. F., Cotter, R. J. and Sisodia, S. S. (1991) J. Biol. Chem. **266**, 16960–16964
5 Ghiso, J., Matsubara, E., Koudinov, A., Choi-Miura, N. H., Tomita, M., Wisniewski, T. and Frangione, B. (1993) Biochem. J. **293**, 27–30
6 Beyreuther, K. and Masters, C. L. (1991) Brain Pathol. **1**, 241–251
7 Wisniewski, T., Palha, J. A., Ghiso, J. and Frangione, B. (1995) Lancet **346**, 1366
8 Boyles, J. K, Pitas, R. E., Wilson, E., Mahley, R. W. and Taylor, J. M. (1985) J. Clin. Invest. **76**, 1501–1513
9 Pitas, R. E., Boyles, J. K, Lee, S. H., Foss, D. and Mahley, R. W. (1987) Biochim. Biophys. Acta **917**, 148–161
10 Pitas, R. E., Boyles, J. K., Lee, S. H., Hui, D. Y. and Weisgraber, K. H. (1987) J. Biol. Chem. **262**, 14352–14360
11 Boyles, J.K, Zoellner, C. D., Anderson, L. J., Kosik, L. M., Pitas, R. E., Weisgraber, K. H., Hui, D. Y., Mahley, R. W., Gebicke-Haerter, P. J., Ignatius, M. J. and Shooter, E. M. (1989) J. Clin. Invest. **83**, 1015–1031
12 Snipes, G. J., McGuire, C. B., Norden, J. J. and Freeman, J. A. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1130–1134
13 Handelmann, G. E., Boyles, J. K., Weisgraber, K. H., Mahley, R. W. and Pitas, R. E. (1992) J. Lipid Res. **33**, 1677–1688
14 Nathan, B. P., Bellosta, S., Sanan, D. A., Weisgraber, K. H., Mahley, R. W. and Pitas, R. E. (1994) Science **264**, 850–852
15 Nathan, B. P., Chang, K. C., Bellosta, S., Brisch, E., Ge, N., Mahley, R. W. and Pitas, R. E. (1995) J. Biol. Chem. **270**, 19791–19799
16 Mahley, R. W. (1988) Science **240**, 622–628
17 Mayeux, R., Stern, Y., Ottman, R., Tatemichi, T. K., Tang, M. X., Maestre, G., Ngai, C., Tycko, B. and Ginsberg, H. (1993) Ann. Neurol. **34**, 752–754
18 Rebeck, G. W., Reiter, J. S., Strickland, D. K. and Hyman, B. T. (1993) Neuron **11**, 575–580
19 Saunders, A. M., Strittmatter, W. J., Schmechel, D., St George-Hyslop, P. H., Pericak-Vance, M. A., Joo, S. H., Rosi, B. L., Gusella, J. F., Crapper-MacLachlan, D. R., Alberts, M. J., et al. (1993) Neurology **43**, 1467–1472
20 Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., Roses, A. D., Haines, J. L. and Pericak-Vance, M. A. (1993) Science **261**, 921–923
21 Strittmatter, W. J., Saunders, A. M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G. S. and Roses, A. D. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 1977–1981
22 Schmechel, D. E., Saunders, A. M., Strittmatter, W. J., Crain, B. J., Hulette, C. M., Joo, S. H., Pericak-Vance, M. A., Goldgaber, D. and Roses, A. D. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 9649–9653
23 LaDu, M. J., Falduto, M. T., Manelli, A. M., Reardon, C. A., Getz, G. S. and Frail, D. E. (1994) J. Biol. Chem. **269**, 23403–23406
24 LaDu, M. J., Pederson, T. M., Frail, D. E., Reardon, C. A., Getz, G. S. and Falduto, M. T. (1995) J. Biol. Chem. **270**, 9039–9042
25 Wisniewski, T., Golabek, A., Matsubara, E., Ghiso, J. and Frangione, B. (1993) Biochem. Biophys. Res. Commun. **392**, 359–365
26 Strittmatter, W. J., Weisgraber, K. H., Huang, D. Y., Dong, L.-M., Salvesen, G. S., Pericak-Vance, M., Schmechel, D., Saunders, A. M., Goldgaber, D. and Roses, A. D. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 8098–8102
27 Ma, J., Yee, A., Brewer, Jr., H. B., Das, S. and Potter, H. (1994) Nature (London) **372**, 92–94
28 Sanan, D. A., Weisgraber, K. H., Russel, S. J., Mahley, R. W., Huang, D., Saunders, A., Schmechel, D., Wisniewski, T., Frangione, B., Roses, A. D. and Strittmatter, W. J. (1994) J. Clin. Invest. **94**, 860–869
29 Wisniewski, T., Castaño, E. M., Golabek, A., Vogel, T. and Frangione, B. (1994) Am. J. Pathol. **145**, 1030–1035
30 Wisniewski, T. and Frangione, B. (1992) Neurosci. Lett. **135**, 235–238
31 Näslund, J., Thyberg, J., Tjernberg, L. O., Wernstedt, C., Karlström, A. R., Bogdanovic, N., Gandy, S. E., Lannfelt, L., Terenius, L. and Nordstedt, C. (1995) Neuron **15**, 219–228
32 Redgrave, T. G., Roberts, D. C. K. and West, C. E. (1975) Anal. Biochem. **65**, 42–49
33 Rall, Jr., S. C., Weisgraber, K. H. and Mahley, R. W. (1986) Methods Enzymol. **128**, 273–287
34 Towbin, H., Staehelin, T. and Gordon, J. (1970) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
35 Cazorla, P., Aldudo, J., Haas, C., Vázquez, J., Valdivieso, F. and Bullido, M. J. (1995) Biochim. Biophys. Acta **1270**, 149–156
36 McEwen, C. R. (1967) Anal. Biochem. **20**, 114–149
37 Tjernberg, L. O., Näslund, J., Lindqvist, F., Johansson, J., Karlström, A. R., Thyberg, J., Terenius, L. and Nordstedt, C. (1996) J. Biol. Chem. **271**, 8545–8548
38 Golabek, A. A., Soto, C., Vogel, T. and Wisniewski, T. (1996) J. Biol. Chem. **271**, 10602–10606

Received 27 September 1996/27 February 1997; accepted 11 March 1997