Contribution of Cysteine 158, the Glycosylation Site Theonine 194, the Amino- and Carboxy-Terminal Domains of Apolipoprotein E in the Binding to Amyloid Peptide β (1−40)†

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ABSTRACT: Recent studies have shown that at physiological conditions (pH 7.6, 37 °C), the reactivity of recombinant apoE isoforms secreted by mammalian cells toward amyloid peptide β (Aβ40) follows the order apoE2 > apoE3 > apoE4 for the apoE monomer and apoE2 > apoE3 for apoE dimer that is formed via that intramolecular disulfide bridges. Different Aβ binding properties have been reported for the plasma-derived apoE and commercially available apoE preparations that differ from the native apoE forms in the degree of their O-glycosylation. To define structural elements of apoE involved in the interaction with Aβ, we have introduced point mutations as well as amino- and carboxy-terminal deletions in the apoE structure. The mutant apoE forms were expressed transiently using the Semliki Forest Virus system, and the culture medium was utilized to study the reactivity of the mutated proteins with Aβ 40. This analysis showed that a mutation in the O-glycosylation site of apoE2 (Thr194-Ala) did not affect the SDS-stable binding of apoE to Aβ. In contrast, introduction of cysteine at position 158 of apoE4 (Arg112, Cys158) increased the SDS-stable binding of apoE to Aβ to the levels similar to those observed in apoE2. Similar analysis showed that apoE truncated at residues 259, 249, 239, and 229 retains the SDS-stable binding to Aβ40, whereas apoE truncated at residues 185 and 165 does not bind to Aβ. The deletion of aminoterminal residues 2−19 reduced the SDS-stable binding of apoE2 to Aβ and deletion of residues 2−81 abolished binding to Aβ. It is also noteworthy that the (Δ2−81) apoE mutant exists predominantly as a dimer, suggesting that removal of residues 2−81 promoted dimerization of apoE. These findings suggest that the amino- and carboxy-terminal residues of apoE are required for SDS-stable binding of apoE to Aβ and that the presence of at least one cysteine contributes to the efficient Aβ binding.

Formation of amyloid plaques in the brain is a major histopathological hallmarks of Alzheimer's Disease (AD)[1] (*1*). Proteolysis of membrane-associated amyloid precursor protein (APP) generates the 39−42 aa long amyloid β peptides which polymerize into amyloid fibers and aggregate to form the plaques (*2*). It was demonstrated in human patients and mouse models that increased production of Aβ, particularly the Aβ1−42/43 form, can mediate its aggregation in the brain, leading to formation of the extracellular amyloid plaque (*3*−*6*). ApoE was recognized as one of the proteins associated with the amyloid plaque. Ensuing population studies established that one of the common isoforms of apolipoprotein E, the apoE4, is associated with higher incidence and earlier age of onset of late onset familial AD, as compared to apoE3 and apoE2 (*7*−*9*).

Apolipoprotein E is a ubiquitous 34.2 KDa protein of lipoprotein transport system which is synthesized by the liver and the majority of the peripheral tissues (*10*) including astrocytes (*11*−*19*), smooth muscle cells, and epithelial cells (*20*, *21*). Newly synthesized apoE is modified by O-glycosylation at Thr-194 (*22*, *23*). In the peripheral and the central nervous system, the secretion of apoE by nonneuronal cells increases dramatically following neuronal injury, and it has been suggested that apoE participates in the growth and repair of the nervous system (*24*, *25*). (There are three apoE ϵ2, ϵ3, and ϵ4 alleles which give rise to three homozygous E2, E3, and E4, and three heterozygous E4/3, E3/2, and E4/2 apoE phenotypes (*26*, *27*). They differ in arginine-cysteine substitution at positions 112 and 158 (*28*).

We and others have shown that the interaction of apoE isoforms produced by mammalian cells with Aβ follows the order apoE2 > apoE3 ≫ apoE4 (*29*−*32*). ApoE2 and apoE3, which bind strongly, protect and apoE4, which binds weakly, predisposes to AD, suggesting that strong binding of nascent apoE to Aβ may correlate inversely with the risk of developing AD (*7*−*9*).

Complexing of apoE with Aβ may affect either the polymerization of Aβ to form amyloid fibers or the subsequent clearance of the apoE−Aβ complex. In this study, we

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[1] Abbreviations: AD, Alzheimer's disease; Aβ, amyloid peptide β; APP, amyloid precursor protein; apoE, apolipoprotein E; BHK, baby hamster kidney; LRP, lipoprotein receptor related protein; LDL, low-density lipoprotein; SFV, Semliki Forest Virus; VLDL, very low-density lipoprotein.

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Table 1: Oligonucleotides Used for Mutagenesis and Mutants Created

oligonucleotide name	oligonucleotides	mutations	designation of mutants
			C-terminal truncated mutants
E3260RXS	5′ ATT CCA GGC CTG ACT CAA GAG CTG 3′	ApoE2 Arg (CGC) 260 → stop (TGA)	E2−259
E3260RXA	5′ CAG CTC TTG AGT CAG GCC TGG AAT 3′		
E3250IXS	5′ GCC CAG CAG TGA CGC CTG CAG GCC 3′	ApoE2 Ile (ATA) 250 → stop (TAA)	E2−249
E3250IXA	5′ CTG CAG GCG TCA CTG CTG GGC CTG 3′		
E3240 RXS	5′ GCG GAG GTG TGA GCC AAG CTG GAG 3′	ApoE2 Arg (CGC) 240 → stop (TGA)	E2−239
E3240RXA	5′ CAG CTT GGC TCA CAC CTC CGC CAC 3′		
E3230DXS	5′ GAC CGC CTG TAG GAG GTG AAG GAG 3′	ApoE2 Asp (GAC)230 → stop (TGA)	E2−229
E3230DXA	5′ CCT TCA CCT CCT ACA GGC GGT CGC 3′		
E3186EXS	5′ CCC CTG GTG TAA CAG GGC CGC GTG 3′	ApoE2 Glu (GAA)186 → stop (TGA)	E2−185
E3186EXA	5′ GCG GCC CTG TTA CAC CAG GGG CCC 3′		
E3166AXS	5′ CAG GCC GGG TGA CGC GAG GGC 3′	ApoE2 Ala (GCC)166 → stop (TGA)	E2−165
E3166AXA	5′ GCC CTC GCG TCA CCC GGC CTG 3′		
			N-terminal truncated mutants
E20 NXS	5′ TTC CTG GCA GGA TGC CAG GCC AAG ↓ TGG CAG AGC GGC CAG CGC TGG GAA 3′	ApoE2 Lys (AAG)1-Δ(2−19)-Trp (TGG) 20	E2Δ2−19
20 NXA	5′ TTC CCA GCG CTG GCC GCT CTG CCA ↓ CTT GGC CTG GCA TCC TGC CAG GAA 3′	ApoE2 Lys (AAG) 1-Δ(2−81)-Leu (CTG) 82	E2Δ2−81
E82NXS	5′ TTC CTG GCA GGA TGC CAG GCC AAG ↓ 5′ CTG ACC CCG GTG GCG GAG GAG ACG 3′		
E82 NXA	5′ CGT CTC CTC CGC CAC CGG GGT CAG ↓ CTT GGC CTG GCA TCC TGC CAG GAA		
E84 NXS	5′ GTG CGG GCC GCC GCC GTG GGC TCC CTG 3′	ApoE2 Thr (ACT)194 → Ala (GCC)	E2 Thr194 → Ala
E194NXA	5′ CAG GGT GCC CAC GGC GGC GGC CCG CAC 3′		
E158NXS	5′ CCT GCA GAAGTGCCTGGCAG3′		
E158**NXA**	5′ CTGCCAGGCACTTCTGCAGG 3′	ApoE4 Arg158 (CGC) → Cys (AGC)	E4 Arg158 → Cys

have investigated the effects of specific point mutations and apoE deletions on the formation of SDS stable apoE−Aβ complexes. Our findings indicate that both amino- and carboxy-terminal regions of apoE and the Cys residues contribute to the formation of SDS stable apoE−Aβ complexes whereas the O-linked carbohydrate moiety does not affect apoE−Aβ interactions.

EXPERIMENTAL PROCEDURES

Materials

Bactotryptone and bacto yeast extract were purchased from Difco. Acrylamide, sodium dodecyl sulfate (SDS), urea, and Tris were from International Biotechnologies, Inc. In vitro transcription kit was from Stratagene. Aprotinin, pepstatin A, and leupeptin were from American Bioanalytical Corp. Centricon-30 or Centriprep-30 cartridges were from Amicon, Inc. Maxisorb multi-well plates were from Nunc (Denmark). Goat polyclonal anti-human apoE antibody was from Calbiochem Corp. Synthetic β-amyloid peptide (1−40) was from Bachem Bioscience Inc. (lot 506063) and QCB Inc. (lot 03013612). Immobilon-P membranes were from Millipore Corp. Rabbit anti-goat IgG antibody conjugated to horseradish peroxidase, rabbit polyclonal anti-human Aβ (1−40) antiserum, goat anti-rabbit IgG antibody conjugated to horseradish peroxidase, and 3′,3′-diaminobenzidine were from Sigma. Enhanced chemiluminescence (ECL) kit was from Amersham. Biomax film was from Kodak. pSFV-1 was a gift courtesy of Dr. Peter Liljeström, Karolinska Institute, Sweden. Mouse anti-human apoE 6H7 monoclonal antibody was a gift courtesy of Dr. Yves Marcel, University of Ottawa, Heart Institute, Canada.

Methods

Generation of the Expression Vectors Carrying Mutated ApoE cDNA Derivatives. Generation of apoE2, apoE3, and apoE4 constructs and expression plasmids have been described previously (*29*). The pUC-E2 plasmid was amplified and mutagenized by PCR using a set of external 5′ and 3′ amplification primers and a set of mutagenic primers as shown in Table 1 (*29*). The first reaction utilizes the 5′ external primer and the antisense mutagenic primer covering codon that was mutagenized. The second utilizes the 3′ external primer and the sense mutagenic primer covering the same codon. The codons altered by mutagenesis were Thr-194 ACT → Ala (GCT), Arg-158 CGC → Cys (AGC), Arg-260 or −240 (CGC) → stop (TGA), Ile-250 (ATA) → stop (TAA), Asp-230 GAG → stop (TAA), Glu-186 GAA → stop (TAA), Ala-166 (TCC) → stop (TAA).

An aliquot of 4% of the volume of each PCR reaction was mixed, and the sample was amplified with the 5′ and 3′ external primers. The mutagenic primers were designed to create the aminoterminal deletions extending 24 nucleotides upstream and 24 nucleotides downstream of the codon which specify the first and the last amino acids of the deletion (Table 1). The amplified fragment containing the desired mutation was then excised with *Bst*EII/*Nar*I digestion and was used to replace the corresponding region in the pUC-E2 plasmid, giving rise to a new mutant pUC-E2 plasmid. The cDNAs of the mutant pUC-E plasmid derivatives were excised by *Bam*HI and *Bgl*II digestion and cloned into the *Bam*HI site of the SFV vector.

Expression of ApoE cDNAs Using the SFV-1 Expression System. BHK-21 cells were grown at 36.5 °C in 5% CO_2 in complete Glasgow medium containing 5% fetal calf serum, 10% tryptose phosphate broth and 1× antibiotic/antimycotic solution. Approximately 10^7 cells were electroporated with the appropriate apoE mRNA produced by in vitro transcription of the mutant pSFV-E plasmids as described (*29*). Serum-free condition medium was harvested from the electroporated BHK-21 cells and utilized for analysis of the mutant proteins and their interaction with Aβ. Labeling of

electroporated cells with [^{35}S]methionine and SDS−PAGE of the mutant proteins was performed as described (*29*).

Preparation of Conditioned Medium and Quantitation of ApoE. Mock and apoE-conditioned media were centrifuged for 10 min at 2000*g* and 20 min at 12000*g* and then concentrated at 4 °C using Centricon-30 or Centriprep-30 cartridges. The concentrated media were aliquoted and stored in liquid nitrogen. Aliquots were thawed in a waterbath at 37 °C immediately before use. The concentration of apoE was determined by ELISA as described (Aleshkov, 1997).

Preparation of β-Amyloid Peptide (1−40). The peptide was dissolved in degassed HPLC grade water at 1 mM, aliquoted, and stored in liquid nitrogen. Aliquots were thawed in a waterbath at 37 °C, centrifuged for 5 min at 13000*g* and immediately added to reaction mixtures. All aliquots were frozen and thawed only once and the rest of the material was discarded.

Binding of Recombinant ApoE Isoforms with Amyloid Peptide β (Aβ). For a typical binding reaction, a 30 μL aliquot of conditioned medium containing 30 μg of apoE/mL was mixed with 1 μL of 1 M Tris HCl buffer, pH 7.4, and 3.4 μL of stock 1 mM Aβ solution and incubated at 37 °C for 2 h. The final concentration of apoE isoforms was 27 μg/mL and of Aβ 100 μM. Reactions were stopped by addition of 5× Laemmli buffer without β-mercaptoethanol and the concentration of SDS in the mixture was adjusted to 2%. The samples were boiled for 5 min and loaded on 7.50 or 10% polyacrylamide gels containing 2% SDS, electrophoresed, and transferred to Immobilon-P membranes. The membranes were blocked for 1 h in 10% nonfat dried milk-PBS-0.1% and Tween-20 and probed successively with rabbit polyclonal anti-human Aβ (*1−40*) antiserum or goat polyclonal anti-human apoE antibody, followed by secondary antibody conjugated to horseradish peroxidase. 3′,3′-Diaminobenzidine or enhanced chemiluminescence (ECL) reagent were used as substrate of the horseradish peroxidase to detect the protein bands. In the latter case, the protein bands were detected by exposing the blots to Biomax film and quantified by densitometry. Each analysis was performed in triplicate in independent experiments.

RESULTS

Previous studies have shown that apoE4, which has Arg residues at positions 112 and 158, has reduced ability to form SDS-stable complexes with Aβ as compared to apoE2, which contains Cys residues at these positions (*29*). To assess the importance of Cys in the formation of apoE−Aβ complexes, the Arg158 of apoE4 was mutated into Cys. ApoE2, apoE4, and the apoE4 (Arg158−Cys) were expressed using the Semliki Forest Virus expression system and equal quantities of apoE (0.7 μg) were used for Aβ binding assays in solution at pH 7.4 and 37 °C. The apoE Aβ and apoE−Aβ complexes were resolved in SDS polyacrylamide, blotted into nitrocellulose membrane, and detected by immunostaining. This analysis showed that substitution of Arg158 of apoE4 by Cys restored binding of Aβ to this mutant apoE4 (Arg158 → Cys) form to levels similar to those of apoE2. Figure 1, panels A and B, shows that when equal apoE concentrations were used, formation of apoE−Aβ complex was much greater for apoE2 than for apoE4 (Figure 1, panels A and B). In contrast, when the Arg158 of apoE4 was substituted by Cys, the amount of apoE−Aβ complex formed with the WT apoE2, assessed by scanning densitometry, was similar to that formed with the mutant apoE4 (Arg158 → Cys) (Figure 1, panels B and C). It has been shown previously that apoE3, which has Cys and Arg residues at positions 112 and 158, respectively, has much greater ability to form stable apoE−Aβ complexes as compared to apoE4 (*29*). The findings suggest that the cysteine residues at positions 158 and 112 are important determinants in the formation of stable apoE−Aβ complexes. Analysis of the flotation properties of apoE secreted by BHK cells indicated that approximately 45% of apoE is distributed in lipoprotein particles which float in the HDL region in $d = 1.08-1.20$ g/mL, 40% in lipid-poor particles which float in the $d = 1.22-1.28$ g/mL region, and 15% in a lipid-free fraction $d > 1.33$ g/mL (data not shown). Other studies showed that delipidation of apoE attenuated the differences between apoE3 and apoE4 to form SDS stable apoE−Aβ complexes (*32*).

We and others have shown previously that the isoform-specific differences of apoE−Aβ complex formation are lost when apoE is purified from plasma or when commercially available apoE preparations are used (*29*−*32*). A major difference between plasma and newly synthesized apoE is the extent of modification of apoE. Newly synthesized apoE is mostly modified with O-linked carbohydrate chains containing sialic acid whereas most of the plasma apoE exists in the nonmodified form (*22*, *27*, *33*). To assess the importance of apoE modification, we mutated Ala194, which is the O-glycosylation site of apoE, into Ala. ApoE-Aβ binding assays showed that the nonglycosylated mutant apoE2 (Thr194 → Ala) forms SDS stable apoE−Aβ complexes with similar efficiency to the original apoE2 form as assessed by scanning densitometry (Figure 1, panels E and F). The findings indicate that the O-linked carbohydrate chains do not affect the formation of SDS stable apoE−Aβ complexes.

In another set of experiments, we examined the contribution of the amino- and carboxy-terminal domains of apoE in the formation of SDS stable apoE−Aβ complexes. Analysis of the synthesis and secretion of the truncated apoE forms showed that apoE forms carrying deletions of amino-terminal amino acids extending to amino acid 81 as well as apoE forms carrying carboxy-terminal deletions extending to amino acids 259, 249, 239, 229, 185, and 165 are secreted efficiently into the culture medium (Figure 2, panels B and D). It is interesting that the amino-terminal deletion mutant E2 (Δ2−81) exists as an SDS stable dimer, suggesting that deletion of residues 2−81 promotes dimerization of the protein. The Aβ binding assay showed that the E2 (Δ2−81) as well as the E2−185 and E2−165 mutant do not form SDS-stable apoE−Aβ complexes, whereas the apoE−Aβ complex formation was reduced to approximately 50% in the other mutants (Figure 2, panels A and C). Figure 2C shows also the presence of higher Mr Aβ immunoreactive complexes in the lanes that contain the truncated apoE forms. These bands do not correspond to the apoE immunoreactive bands observed in panel D and may represent complexes with other proteins. Scanning densitometry showed that the higher M_r bands represent 30−40% of the specific bands of M_r 32−40 KDa. The average intensity of the specific complex formed with the truncated apoE2 forms extending to residues 259, 249, 239, and 220 is 22% of the intensity

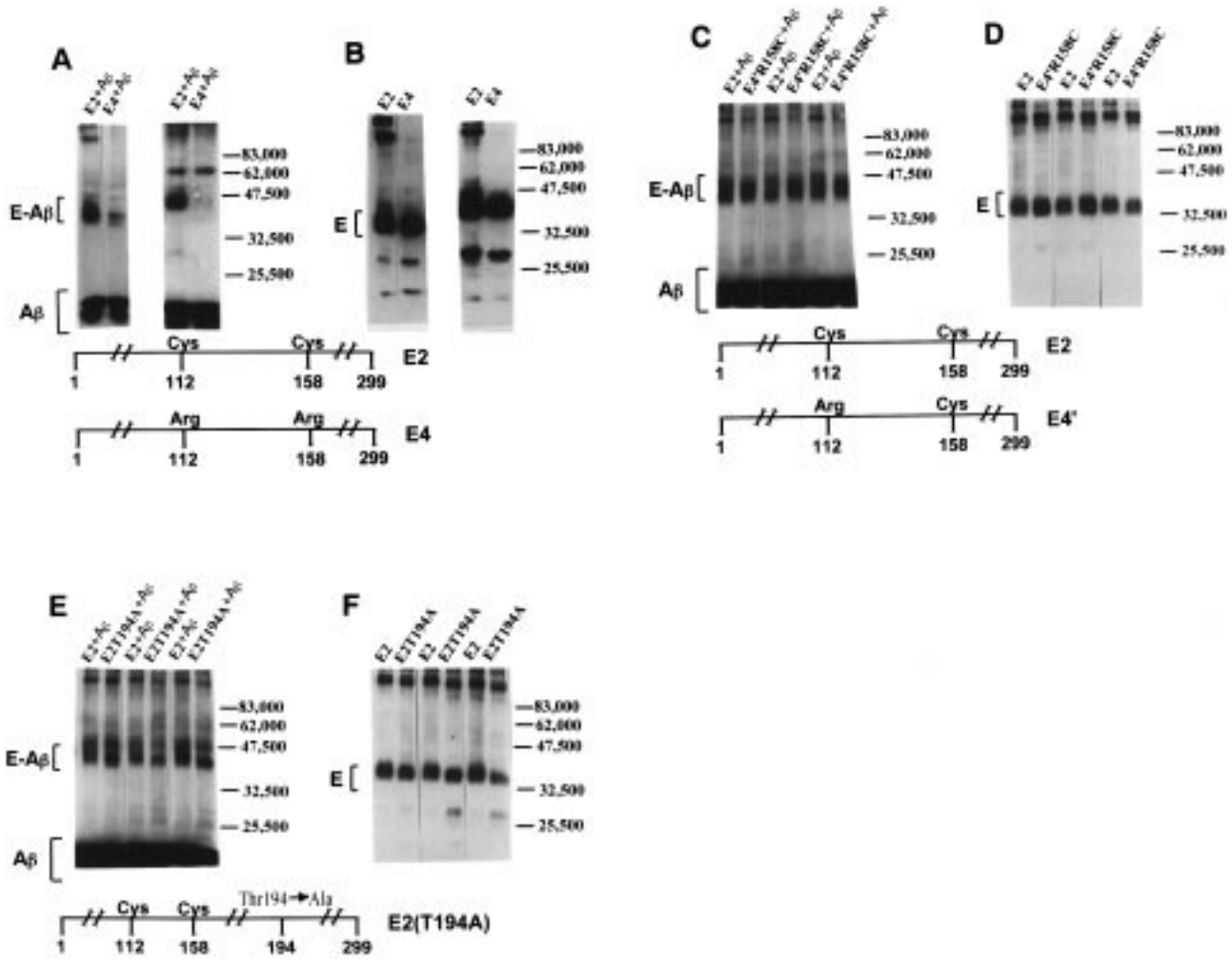

FIGURE 1: Western blotting of apoE, apoE variants and the apoE−Aβ complexes following interaction of different apoE forms with Aβ in solution and separation of the complex in 10% SDS gel as described (*29*). (A, B) Analysis of apoE2 and apoE4. (C, D) Analysis of apoE4 (R158 → C). (E, F) Analysis of apoE2 (T194A). The amount of apoE used was 0.7 μg. The residues at positions 112 and 158 of the apoE2 and apoE4 and the sites of the mutations in apoE are indicated at the bottom of the figure. ApoE was expressed in BHK-21 cells using the SFV expression system as described (*29*). In this figure as well as in Figure 2, the interaction of apoE with Aβ was performed at 37 °C, pH 7.4 for 2 h as explained in the Methods. The membranes were treated successively with anti-apoE or monoclonal Aβ, secondary antibody conjugated to horseradish peroxidase and a solution of enhanced chemiluminescence reagent for 1 min. The protein bands were detected by exposing the blots to Biomax film.

of the full-length apoE2 (range 12−28%). The findings suggest that amino as well as carboxy-terminal apoE residues contribute independently to the formation of SDS stable apoE−Aβ complexes.

DISCUSSION

Epidemiological and genetic data have established that the ϵ4 allele is a risk factor for familial late onset Alzheimer's disease (*7*−*9*). The frequency of the ϵ4 allele in the Caucasian population is approximately three times higher in patients with AD than non-AD subjects (0.42 vs 0.15), and there is a dosage effect of the ϵ4 allele on lifetime risk for AD and the age of onset of the disease (*8*, *9*). In vitro biochemical and functional assays also show isoform specific properties of apoE including (a) binding to Aβ (*29*−*32*, *34*, *35*); (b) binding to tau and MAP2C (*36*−*39*); (c) cholinergic deficit in the frontal cortex and the hippocampus (*40*, *41*); (d) effects on neuronal morphology and cytoskeletal structure in vitro (*42*−*44*); (e) effects on neuronal degeneration and dendritic remodeling in vivo (*45*); (f) inhibition of fusion of liposomes by apoE which is promoted by Aβ (*46*); (g) binding to soluble APP (sAPP), which has a protective effect for the hippocampal neurons (*47*) and inhibits activation of microglia (*48*). These different properties of apoE isoforms could be the result of either loss or gain of function as a result of the structural differences which characterize the three naturally occurring apoE isoforms.

On the basis of these observations, several hypotheses have been advanced that ascribe the roles of apoE isoforms in either the pathogenesis or protection from AD. (*37*−*41*, *45*, *49*−*51*).

Our previous work indicated that newly synthesized apoE2 and apoE3 bind more strongly to Aβ as compared to apoE4 in vitro. It is tempting to speculate that similar in vivo binding may remove Aβ from the extracellular space and thus prevent polymerization of Aβ and formation of amyloid fibers and plaques. The protective forms of apoE, apoE2, and apoE3, may inhibit fibrillogenesis by binding to amyloid protofibrils and preventing formation of amyloid fibrils (*52*). In this regard, it has been proposed that the face of the growing Aβ fibril displays hydrophobic surface to allow association with apoE helices and thus prevent fibril extension (*53*). The

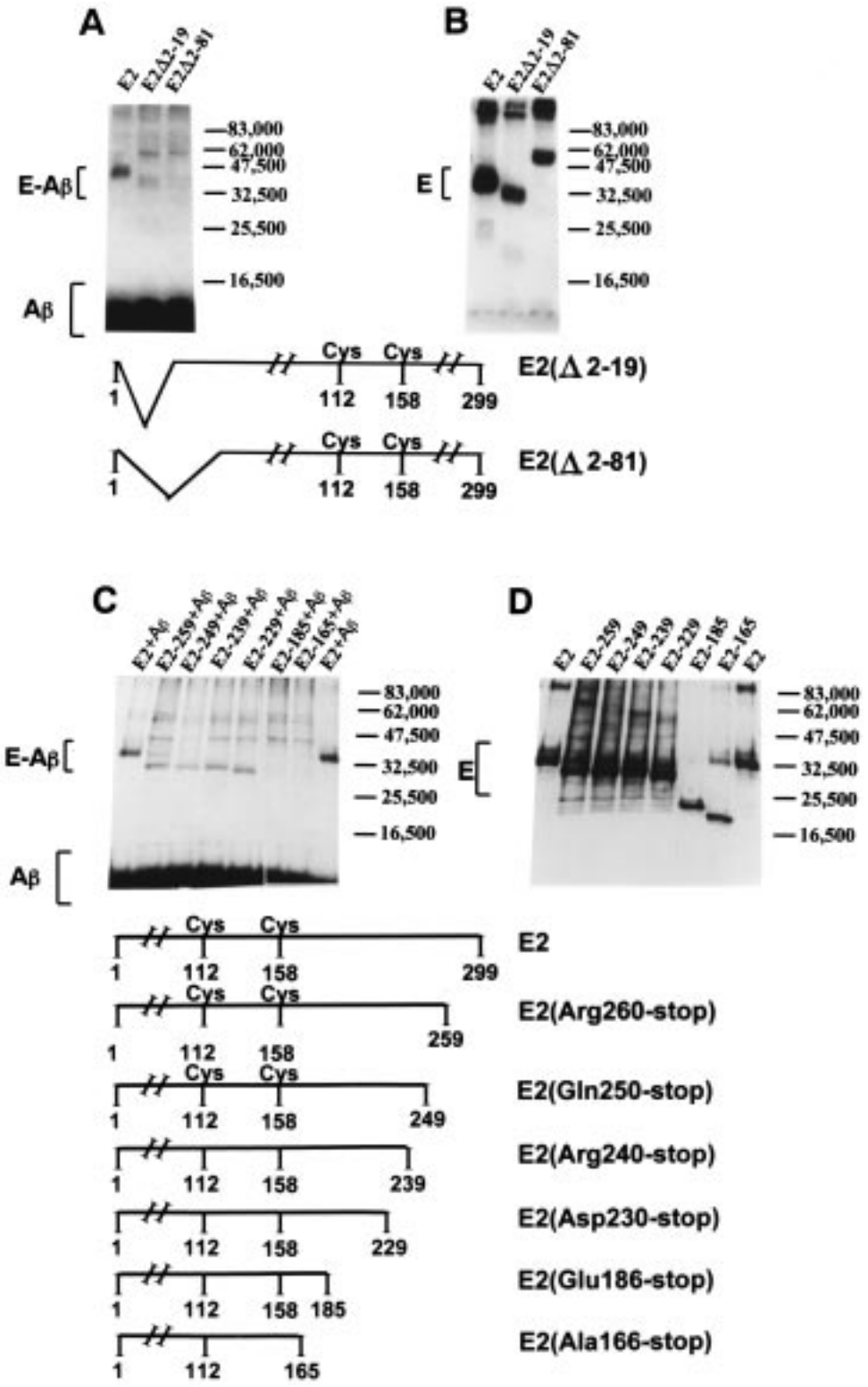

FIGURE 2: Western blotting of apoE2, truncated apoE2 variants and the apoE−Aβ complexes following interaction of different apoE forms with Aβ in solution and separation of the complexes by SDS PAGE as indicated. (A, B) Analysis of aminoterminal truncated apoE2 variants following separation of 10% SDS gel. (C, D) Analysis of carboxy terminal truncated apoE2 variants following separation of 4 to 20% gradient SDS gel. The amount of apoE sample utilized in the gradient gel is 1/4 of the quantity used in the 10% gel. The truncated apoE forms analyzed are indicated at the bottom of the figure. ApoE was expressed in BHK cells using the SFV expression system as described (*29*). The position of the prestained protein MW standards used (Bio-Rad) is indicated. The position of apoE, a dimeric form of apoE, Aβ, and apoE−Aβ complexes are shown.

putative hydrophobic domains of apoE utilized in these interactions may be similar to those utilized for binding to lipids. Alternatively, apoE could inhibit fibrillogenesis by forming apoE−Aβ complexes which can then be removed by unknown mechanisms possibly involving cell receptors (*54*−*57*). It is also possible that the direct interaction of apoE with Aβ may prevent the association of Aβ with cell membranes (*46*, *58*) which will result in cell death (*46*, *58*,

59). Thus, trapping the Aβ by apoE2 or apoE3 may have a protective effect for the neuronal cells. In this regard, it has been reported that apoE2 and apoE3 protected to a greater extent neuronal B12 cells from Aβ or hydrogen peroxide cytotoxicity than apoE4 (*60*).

The physiological significance of SDS-stable apoE−Aβ complexes remains a point of controversy. It has been shown recently that brain supernatants obtained from patients with AD (but not from control subjects) when they are immunoprecipitated with either anti-apoE or anti-Aβ antibodies give similar complexes in the 32−40 kDa range along with Aβ dimers (*61*). In addition, apoE could be immunoprecipitated with anti-Aβ antibodies and dimeric Aβ could be immunoprecipitated with anti-apoE antibodies (*61*). Another study has shown the presence of SDS-stable complexes of apoE with polymers of Aβ in hexafluoro-2-propanol extracts of brain of AD patients but not in extracts of normal brains. They suggested that the similar appearance of apoE−Aβ complexes detected by immunoblotting using either anti-Aβ or anti-apoE antibodies implied that the two antibodies recognize the same structure (*62*). In the water-insoluble complexes that can be extracted from amyloid plaques (*62*) and those that can be formed in vitro (*62*, *36*), apoE was bound along as well as between the amyloid fibrils.

It is possible that the apoE−Aβ complexes described in refs *61* and *62*, respectively, are similar in nature. The former may represent water-soluble Aβ polymers prior to their precipitation to form fibrils and the latter may represent complexes of water-insoluble apoE−Aβ fragments.

A recent study suggests that apoE−Aβ soluble aggregates exist in both normal and control brains. The aggregates found in normal brain were more abundant and more resistant to SDS than those found in AD brains. In addition, binding of Aβ to apoE in AD patients increased its sensitivity to proteolysis (*63*). Finally, it has been shown that the transport of apoE−Aβ complexes is greatly decreased as compared to the transport of Aβ alone (*64*). Thus, despite the limitations of the methodological procedures used, the detection of SDS-stable complexes may represent an important approach to understand the mechanism of sequestration clearance and/or deposition of Aβ.

The present study provides important information on the contribution of the amino and carboxy terminal domains of apoE and specific residues at position 158 and 194 in apoE−Aβ interactions. X-ray crystallography (*65*) and computer modeling (*66*) suggest that there are 10 helical regions in the apoE molecule. The carboxy-terminal deletions were designed to progressively remove helices 6−10, which define an independently folding apoE domain (*67*). These deletions produced stable apoE forms which were secreted efficiently into the culture medium, indicating that removal of portions of or the entire carboxy-terminal domain does not affect the intracellular stability of apoE. Stable apoE forms could be obtained by deletion of residues 2−19 which contain a short helical region (residues 2−6) or by deletion of residues 2−81 which contain the short amino-terminal helix as well as two additional helices. Further deletions produced unstable apoE forms that could not be secreted from the cells (data not shown). It is also interesting that the (2−81) apoE mutant exists predominantly as a dimer, suggesting that removal of residues 2−81 promoted dimerization of apoE. Analysis of apoE−Aβ interactions showed that truncated apoE forms extending up to residue 229 retain SDS-stable apoE−Aβ binding, whereas apoE forms extending to residues 185 and 165 did not bind to Aβ. Previous findings indicated that the residues of apoE extending between amino acids 244 to 272 are required for binding to Aβ (*30*). ApoE-Aβ binding assays showed that the amino-terminally truncated apoE2 mutant (Δ2−19) retains reduced binding to Aβ whereas the Δ2−81 apoE mutant did not bind to Aβ. Biochemical and structural studies (*65*, *67*) combined with secondary structure prediction (*66*) indicated that apoE contains two independently folded domains which are separated by a hinge region between residues 165 and 215. In vitro mutagenesis has established that ionic interactions between Arg-61 and glutamic 255 are responsible for the preference of apoE4 for VLDL (*68*). Although the relative environment of Arg-61 has been proposed to be different in apoE2 and apoE3 than that of apoE4 (*69*), it is interesting that the deletion of a segment that contains Arg-61 in apoE Δ2−81 in apoE2 abolished binding to Aβ and promoted dimerization of apoE2.

The rationale of the point mutations in apoE was to define residues that may account for the differences in Aβ binding among the three naturally occurring apoE isoforms. The present study showed that changing one Arg residue of apoE4 at position 158 into cysteine restores SDS-stable binding of apoE4 (112Arg, 158Cys) to levels similar to those observed for apoE2. Previous studies also showed that apoE3, which has Cys at position 112 and Arg at position 158, binds more efficiently to Aβ than apoE4 which has arginines in both positions (*29*). The finding indicates that the presence of at least one Cys in the apoE molecule is important to obtain SDS-stable apoE−Aβ complex formation. It is possible that the presence of cysteine not only in positions 112 and 158 but in other residues as well promotes the formation of stable apoE−Aβ complexes. This question will be addressed by the analysis of other apoE2 variants carrying cysteines in other positions. In contrast to the contribution of the Cys residues to apoE−Aβ binding, substitution of the Thr194 (*22*, *23*) by Ala, which abolishes the O-glycosylation of the protein, did not affect the formation of SDS-stable apoE−Aβ complexes. Previous studies have established that plasma apoE exists mostly (over 75%) in the nonsialylated form, whereas the newly secreted apoE exists mostly (over 80−90%) in the sialylated form (*27*, *29*, *33*). The present study enhances our understanding on the domains and residues of apoE, which contribute to apoE−Aβ interaction. Mapping of these domains may provide insights into the molecular mechanisms which lead to plaque formation and potentially new approaches to inhibit this process.

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