Interaction of Nascent ApoE2, ApoE3, and ApoE4 Isoforms Expressed in Mammalian Cells with Amyloid Peptide β (1−40). Relevance to Alzheimer's Disease[†]

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ABSTRACT: Population studies have 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 Alzheimer's disease (AD), whereas apoE2 may have the opposite effect. The apoE3 and apoE4 isoforms were shown to display different binding reactivities with amyloid β peptide (Aβ) and τ protein *in vitro*. On the basis of these findings, it has been proposed that the apoE isoforms may modulate positively or negatively the formation of either the neurofibrillary tangles or the amyloid deposits in the brain of patients with AD. To study the interaction of Aβ with nascent apoE isoforms we have expressed their cDNAs in baby hamster kidney (BHK-21) cells using the Semliki Forest Virus expression system. Analysis of the secreted apoE by one- and two-dimensional gel electrophoresis and immunoblotting showed that the nascent apoE is heavily modified with carbohydrate chains containing sialic acid. A dimeric form of apoE is formed with apoE2 and apoE3 but not with apoE4 isoforms. Analysis of the interaction of nascent apoE2, apoE3, and apoE4 produced by BHK-21 cells with Aβ (1−40) under physiological conditions (pH 7.4, 37 °C) showed that the efficiency of the apoE monomer−Aβ complex formation follows the order apoE2 > apoE3 ≫ apoE4. In addition, the apoE2 dimer formed a complex with Aβ more efficiently than the apoE3 dimer. The isoform-specific differences in binding were temperature-dependent and are attenuated upon decrease of the temperature. The binding behavior of the monomeric apoE is different from that reported for plasma apoE3 and apoE4 or commercially available apoE3 and apoE4 preparations and similar to that described for apoE3 and apoE4 produced by human embryonic kidney (HEK-293) cells. It appears that the efficiency of binding between each of three main apoE isoforms and Aβ correlates inversely with the risk of developing late-onset familial AD and may indicate possible involvement of apoE in the binding and clearance of Aβ *in vivo*.

Cerebral deposition of a 39- to 42-residue long amyloid β peptide (Aβ)[1] is one of the major pathological hallmarks of Alzheimer's disease (AD). These peptides are proteolytic fragments of the type 1 integral membrane glycoprotein, β-amyloid precursor protein (APP) (Kang et al., 1987). It was demonstrated that increased production of Aβ, particularly the Aβ 1−42/43 form, can mediate its aggregation in the brain, leading to the formation of fibrillar deposits (Citron et al., 1992; Selkoe, 1996). On the other hand, it was shown that aggregated Aβ can be toxic to cultures of neurons, astrocytes, microglia, and endothelial cells (Pike et al., 1993, 1994; Lorenzo & Yankner, 1994; Meda et al., 1995; Thomas et al., 1996). Furthermore, transgenic mice overexpressing mutant forms of APP overproduce Aβ and develop AD-like pathomorphological changes in the brain (Games et al., 1995; Hsiao et al., 1996). These findings led to the hypothesis that Aβ plays an important role in the pathological cascade that leads to AD (Selkoe, 1996). Thus, the problem of binding, inactivation, and clearance of Aβ in the brain emerges as one of the possible rational approaches for the treatment of AD.

ApoE is a ubiquitous 34.2 kDa protein involved in lipid transport which has been recently implicated in the pathogenesis of AD (Namba et al., 1991; Pericak-Vance et al., 1991; Corder et al., 1993; Strittmatter et al., 1993b; Myers et al., 1996). ApoE is synthesized by the liver and most of the peripheral tissues, including astrocytes of the central nervous system (Snipes et al., 1986; Boyles et al., 1989; Diedrich et al., 1991). Following synthesis, apoE is incorporated into lipoproteins and directs their catabolism via binding with the LDL receptor, VLDL receptor, and the LDL receptor-related protein (LRP) (Pitas et al., 1980; Handelman et al., 1992; Takahashi et al., 1992; Wolf et al., 1992; Rebeck et al., 1993; Tooyama et al., 1993; Yamamoto et al., 1993; Wyne et al., 1996). In the peripheral and the central nervous system, the secretion of apoE by non-neuronal cells increases dramatically following neuronal injury, and it has been suggested that apoE participates in the growth and repair of the nervous system (Snipes et al., 1986; Boyles et al., 1989). Three major isoforms of apoE have been described in population studies: apoE2, apoE3, and apoE4, which are the products of the ϵ2, ϵ3, and ϵ4 alleles, respectively (Zannis

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[1] Abbreviations: Alzheimer's disease, AD; amyloid peptide β, Aβ; amyloid precursor protein, APP; apolipoprotein E, apoE; baby hamster kidney, BHK; CSF, cerebrospinal fluid; human embryonic kidney, HEK; lipoprotein receptor related protein, LRP; low density lipoprotein, LDL; POPC, β-oleoyl-γ-palmitoyl phosphatidylcholine; Semliki Forest Virus, SFV; very low density lipoprotein, VLDL.

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& Breslow, 1981; Zannis et al., 1993). They differ in arginine-cysteine substitution at positions 112 and 158 (Rall et al., 1982). Previous studies have established a linkage of late onset familial AD to the proximal long arm of chromosome 19 where the apoE gene maps (Pericak-Vance et al., 1991). Recently it has been shown that the risk for late onset familial AD is increased for subjects with the ϵ4 allele whereas the ϵ2 allele may confer lower risk than ϵ4 or ϵ3 (Corder et al., 1993, 1994; Saunders et al., 1993; Sorbi et al., 1993; Strittmatter et al., 1993a; St. George-Hyslop et al., 1994; Talbot et al., 1994; Myers et al., 1996). Moreover, apoE was found in the brain lesions of AD patients (Namba et al., 1991; Wisniewski & Frangione, 1992) where it is stably complexed with Aβ (Naslund et al., 1995).

The formation of complexes between Aβ and apoE *in vitro* has been reported (Ghiso et al., 1993; Strittmatter et al., 1993a,b; Wisniewski et al., 1993; LaDu et al., 1994; Zhou et al., 1996). Using apoE3 and apoE4, purified from the human plasma, Strittmatter et al. (1993b) demonstrated that apoE4 binds Aβ (1−28) more rapidly than does apoE3, although the efficiencies of binding of the two apoE isoforms seemed comparable after 6 h of incubation. However, two other studies utilizing recombinant E3 and E4 showed preferential binding of Aβ with apoE3 (LaDu et al., 1994; Zhou et al., 1996). Furthermore, Aβ−apoE4 complexes precipitated more rapidly and yielded a denser matrix of newly polymerized monofibrils than Aβ−apoE3 (Sanan et al., 1994).

In the current study we have utilized a highly efficient transient expression system based on SFV to generate and characterize the nascent apoE2, apoE3, and apoE4 isoforms. This system allowed us to produce all three isoforms simultaneously under the same experimental conditions within 24 h. Using the nascent secreted apoE from conditioned culture medium without further purification, we have demonstrated that under physiological conditions (pH 7.4, 37 °C) the efficiency of apoE−Aβ complex formation follows the order apoE2 > apoE3 ≫ apoE4 for the apoE monomers and apoE2 ≫ apoE3 for the apoE dimers. These observations may be relevant to the different contribution of apoE isoforms in the pathogenesis of AD.

EXPERIMENTAL PROCEDURES

Materials

ApoE2, apoE3, and apoE4 produced by the baculovirus expression system were purchased from Pan Vera Corporation (Madison, WI). BHK-21 cells were from ATCC (CRL 6282). All cell culture media and reagents were from Gibco BRL. T_4 DNA ligase, polynucleotide kinase, Vent DNA polymerase, and restriction enzymes were from New England Biolabs (Beverly, MA). Transformation competent bacterial DH5α cells were from Bethesda Research Laboratories. ^{35}S-methionine (1175 Ci/mmol), and Econofluor scintillation fluid were from DuPont-New England Nuclear. Reagents for automated DNA synthesis were from Applied Biosystems, Inc. The sequencing kit was from U.S. Biochemicals. Bactotryptone and bacto yeast extract were purchased from Difco. Acrylamide, sodium dodecyl sulfate (SDS), urea, and Tris were from International Biotechnologies, Inc. The *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. Maxisorp multiwell 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. Plasma apoE3 and apoE4 were a gift courtesy of Dr. K. Weisgraber (Gladstone Foundation Laboratories, San Francisco, CA). The purified plasma 0.96 mg/mL and apoE4 1.06 mg/mL was shipped frozen in dry ice. The samples were diluted to 30 μg/mL with PBS and dialyzed against 1 L PBS overnight. 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 ApoE cDNA Derivatives. The apoE cDNA we isolated previously (Zannis et al., 1984) was digested with *Aat*II, blunted with T4 polymerase and ligated to *Bgl*II linkers. Subsequently the cDNA was digested with *Dra*III, blunted with T4 polymerase, and ligated to *Bam*HI linkers. The resulting fragment was digested with *Bgl*II and *Bam*HI and was initially cloned into the corresponding sites of pBluescript SK$^+$. The apoE cDNA insert was then excised by *Hind*III and *Bam*HI digestion and cloned into the corresponding sites of the pUC-I9 derivative to generate the pUC-E3 derivative. The pUC-E3 plasmid was amplified and mutagenized by PCR using a set of external 5′ and 3′ amplification primers and a set of mutagenic primers as described (Cardot et al., 1991). One set of mutagenic primers was designed to replace Cys-112 with Arg and thus create the apoE4 cDNAs. Another set of mutagenic primers was used to replace Arg-158 with Cys and thus create the apoE2 cDNA. A typical PCR-based mutagenesis (for instance, changing Arg-158 to Cys) utilizes two separate amplifications reactions. The first reaction utilizes the 5′ external primer and the antisense mutagenic primer covering codon 158. The second utilizes the 3′ external primer and the sense mutagenic primer covering codon 158. 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 amplified fragment containing the Arg-158→Cys mutation was then excised with *Bst*EII/*Nar*I digestion and was used to replace the corresponding region in the pUC-E3 plasmid, giving rise to a new pUC-E2 plasmid. Similarly, the amplified fragment containing the Cys-112→Arg mutation was used to replace the corresponding region of the pUC-E3 plasmid, giving rise to pUC-E4 plasmid. The cDNAs of apoE2, E3, and E4 were excised by *Bam*HI and *Bgl*II digestion and cloned into the *Bam*HI site of the Semliki Forest Virus vector (pSFV-1) to generate the pSFV-E2, pSFV-E3, and pSFV-E4 derivatives. The same DNA inserts were also cloned into the *Bam*HI site of pcDNA3 (Invitrogen, Inc.) under the control of the CMV promoter to generate the pcDNA-E2, pcDNA-E3, and pcDNA-E4 derivatives.

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 1X antibiotic/antimycotic solution. Plasmids pSFV-E2, pSFV-E3, and pSFV-E4 (10 μg) were linearized by cleavage with *Spe*I, and 1 μg of each cleaved plasmid was transcribed *in vitro* under the control of the SP6 promoter using an *in vitro* transcription kit. BHK-21 cells (approximately 10^7 cells) were trypsinized, washed twice with PBS, and suspended in 1 mL of PBS. The cells were transfected by electroporation with SFV-E2, SFV-E3, and SFV-E4 RNAs or with mock SFV RNA, using two consecutive pulses at 850 V, 25 μF in a 0.4 cm cuvette. The cells were incubated in complete Glasgow medium containing 5% fetal calf serum and 10% tryptose phosphate broth for 24 h and then in serum free Glasgow medium containing 20 mM Hepes pH 7.4, 10% tryptose phosphate broth, 1X antibiotic/antimycotic solution and 1 μg/mL of aprotinin, pepstatin A, and leupeptin. The serum-free conditioned medium was harvested 48 h after electroporation.

Two-Dimensional Polyacrylamide Gel Electrophoresis and Estimation of the Relative Concentration of ApoE Isoproteins. For labeling the cells with ^{35}S methionine following electroporation, 60 mm diameter cell cultures were incubated in serum containing medium for 20 h after electroporation and then preincubated in serum-free methionine-free medium for 2 h and labeled for 2 h by the addition of 0.25 μCi of ^{35}S methionine. The culture media were collected, immunoprecipitated with polyclonal anti-apoE antibody, and analyzed by two-dimensional gel electrophoresis and autoradiography as described previously (Zannis et al., 1982, 1986). The relative concentrations of apoE isoproteins were estimated from the ^{35}S radioactivity corresponding to the individual asialo and sialo apoE spots. For this purpose the apoE spots were excised from the gel, placed in separate vials containing 30% (wt/wt) H_2O_2, and incubated at 54 °C overnight. A 2 mL aliquot of the resulting solution was mixed with 10 mL scintillation fluid (Econofluor, Dupont-NEN), and radioactivity was measured in a scintillation counter.

Preparation of Conditioned Medium and Quantitation of ApoE. Mock and apoE conditioned media were centrifuged 10 min, 2000*g* and 20 min, 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. The Maxisorp 96-well plates were coated with 6H7 IgG (anti-apoE monoclonal antibody) at 50 μL/well (1 μg/mL in 0.1 carbonate buffer pH 9.2) and stored at +4 °C overnight. The coating solution was discarded, and blocking buffer (10% nonfat dry milk in PBS, 0.1% (v/v) Tween-20) was added at 200 μL/well. The plates were incubated at room temperature for 1 h and washed three times with wash buffer (PBS, 0.1% (v/v) Tween-20). Samples and purified apoE standard diluted in blocking buffer were added at 50 μL/well and incubated at room temperature for 1 h. The plates were washed three times, and the detecting antibody (goat anti-human apoE) diluted 1/1000 in blocking buffer was added at 50 μL/well. The plates were incubated at room temperature for 1 h and washed three times, and an aliquot of 50 μL HRP-conjugated rabbit anti-goat antibody diluted 1/2000 in blocking buffer was added in each well. The plates were incubated at room temperature for 60 min and washed four times, and orthophenylenediamine dihydrochloride (OPD) substrate (10 mg of OPD in 5 mL of diluent consisting of 3.57 g of citric acid−H_2O, 17.43 g of $Na_2HPO_4 \cdot 7H_2O$, 1.33 mL of H_2O_2, 0.1 g of thimerosal per liter water pH 6.2) was added at 200 μL/well. After 30 min, the reaction was terminated by adding 50 μL of 2N H_2SO_4 per well. The absorbance was measured at 490 nm using a microtiter plate reader. Blank wells contained conditioned medium from cells transfected with pSFV-mock virus.

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 5 min, 13000*g*, and immediately added to reaction mixtures. All aliquots were frozen and thawed only once, and the rest of the material was discarded.

Expression of ApoE cDNAs in Cos-1 Cells. Monolayers of Cos-1 cells were maintained as stocks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cos-1 cells (3×10^6) were plated on 100 mm dishes and the following day were transfected with the pcDNA-apoE vectors using the calcium phosphate DNA coprecipitation method (Graham & vander Eb, 1973). The serum-free medium was collected 48 to 72 h post-transfection.

Binding of Recombinant ApoE Isoforms with Amyloid Peptide β (Aβ). For a typical binding reaction, 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 5X 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% Tween-20 and probed successively with rabbit polyclonal anti-human Aβ (1−40) antiserum and/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.

Preparation of ApoE POPC Proteoliposomes. ApoE POPC proteoliposomes were prepared by the original sodium cholate dialysis method (Matz et al., 1982) using a molar ratio of 100:10:1:100 of POPC:cholesterol:apoE:Na cholate. In a typical experiment, 0.014 mg of cholesterol and 2.71 mg of POPC were placed in glass tubes, vortexed gently, and dried under nitrogen. The dried lipid was dissolved in a 10 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM NaN_3, and 0.01% EDTA buffer by repeated vortexing for approximately 30 s. The suspension was stored on ice for 1 h, the sodium cholate was added, and the solution was kept on ice for another 1 h. This was followed by the addition of apoE and incubation for another 1 h at 4 °C. To remove the sodium cholate the solution was twice dialyzed against 2 L of Ca^{++}- and Mg^{++}-free PBS containing NaN_3 and 0.01% EDTA buffer at 4 °C. Binding assays of the proteoliposome-bound apoE was performed in PBS adjusted to 29 mM Tris HCl pH 7.4. The final concentrations apoE and Aβ were 27 μg/mL and 100 μM respectively.

RESULTS

Features of ApoE Secreted by BHK-21 Cells. To optimize the conditions of apoE secretion by BHK-21 cells following electroporation with the SFVapoE RNA as described in Methods, the serum-free medium was collected at 24, 48, or 72 h postelectroporation. An aliquot of 10 μL was analyzed by SDS-PAGE and immunoblotting (Figure 1). This analysis established that the SFVapoE3 RNA directs the expression of full-size apoE3. The expression increases up to 48 h and then declines in a time-dependent manner. The molecular mass of the secreted apoE in lanes 2−4 is 35−39 kDa and is comparable to that of the control samples, lane 5. The expressed protein is represented by a broad band indicating post-translational glycosylation of the product (Zannis et al., 1985, 1986). A protein of an approximate M_r of 28 kDa represents a degradation product of apoE, and its relative concentration decreases upon addition of protease inhibitors in the culture medium (data not shown).

The analysis of Figure 1A established that optimal apoE production is achieved at 24−48 h post-electroporation. Usually, the level of expression estimated by ELISA was approximately 5−10 μg of apoE/mL/10^6 cells/24 h. Figure 1B shows SDS PAGE and immunoblotting of apoE2, apoE3, and apoE4 expressed by BHK-21 cells, 24−48 h postelectroporation with recombinant SFV apoE RNA. Figure 1C represents the immunoblotting of apoE2, apoE3, and apoE4 expressed by Cos-1 cells following transfection with pcDNA apoE plasmids. An apoE immunoreactive protein of high M_r was detected in the medium of cells expressing apoE2 and apoE3 but not in the medium of cells expressing apoE4. The molecular mass of this oligomeric form of apoE was estimated from the data of Figure 1C using known molecular weight standards and is approximately 95 and 91 kDa for apoE2 and apoE3, respectively. A protein band of similar size, present in plasma, was shown to represent a dimer of apoE3 which displayed an abnormal mobility on SDS gels (Weisgraber & Shinto, 1991). As shown in Figure 3B, this dimer is detectable when the protein concentration is approximately 27 μg/mL but not when the concentration is reduced to 9 μg/mL. The post-translational modification of the newly secreted apoE as well as the isoelectric point differences in apoE isoforms were established by two-dimensional gel electrophoresis of culture media of BHK-21 cells following labeling with ^{35}S Met. For this analysis, the culture media were immunoprecipitated with anti-apoE antibodies, the precipitate was mixed with plasma apoE3 and was analyzed by two dimensional polyacrylamide gel electrophoresis and autoradiography. The Coomassie blue stained gel shows the plasma apoE3 form that was included in the sample, and the autoradiogram shows the position of the newly synthesized apoE. Superimposition of the gel on the autoradiogram established the charge and size differences between the plasma apoE and the newly synthesized apoE isoforms. This analysis showed that the newly synthesized apoE2 is more acidic by one and two positive charges than apoE3 and apoE4, respectively (Figure 2A−C). It also showed that the secreted proteins are extensively modified and resemble the apoE synthesized by hepatic and other types of cells (Zannis et al., 1982, 1985, 1986). In contrast, the plasma apoE is modified lightly (Figure 2D).

Binding of Nascent ApoE2, ApoE3, and ApoE4 Secreted by Cell Cultures to the Amyloid Peptide β (Aβ) (1−40). A series of typical binding assays using apoE2, apoE3, and

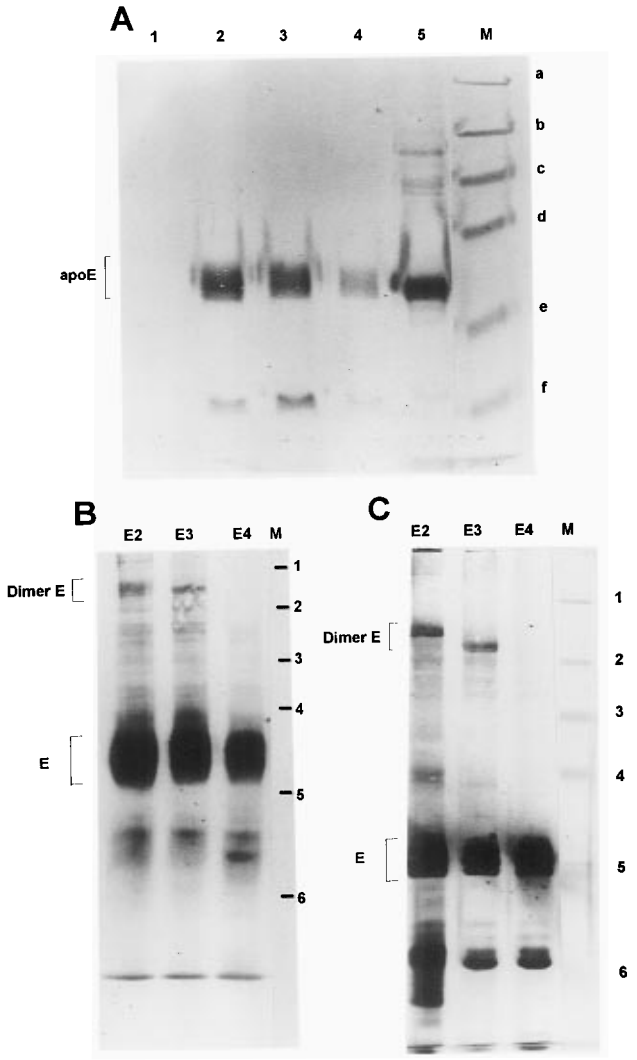

FIGURE 1: Expression of recombinant SFVapoE vectors in BHK-21 or Cos-1 cells. Panel A: SDS PAGE and immunoblotting of apoE3 secreted into the medium of BHK-21 cells following electroporation. After transfer the membranes were blocked with 1% BSA-TBST for 1 h. The membranes were then treated with anti-apoE, secondary antibody conjugated to horseradish peroxidase, and a solution of 3′3′-diaminobenzidene. Lane 1: medium obtained from control BHK-21 cells electroporated with the SFV RNA (without E3 cDNA copy). Lanes 2, 3, 4: media obtained 0−24, 24−48, 48−72 h, respectively, after the cells were switched to serum-free media. Lane 5: 0.5 μg of recombinant apoE3 obtained by the baculovirus expression system (purchased from Pan Vera Corp.). Lane M: protein molecular weight standards. The molecular weights (New England Biolabs) are as follows: a or 1 = β-galactosidase, M_r = 175 kDa; b or 2 = paramyosin, M_r = 83 kDa; c or 3 = glutamic dehydrogenase M_r = 62 kDa; d or 4 = aldolase, M_r = 47.5 kDa; e or 5 = triose phosphate isomerase, M_r = 32.5 kDa; f or 6 = lactoglobulin, M_r = 25 kDa. Panel B: SDS PAGE and immunoblotting of apoE2, apoE3, and apoE4 secreted into the medium of BHK-21. Media were collected between 24−48 h following electroporation with recombinant apoE2, apoE3, or apoE4 containing SFV mRNA. Lanes 1, 2, 3: 10 μL media from BHK-21 cells electroporated with SFV apoE2 SFV apoE3, or SFV apoE4 RNA, respectively. Panel C: SDS PAGE and immunoblotting of apoE2, apoE3, and apoE4 secreted into the medium of Cos-1 cells 48−72 h following transfection with pcDNA apoE expression vectors. Lanes 1, 2, 3: 10 μL media from Cos-1 cells transfected with recombinant apoE2 or apoE3 or apoE4 containing pcDNA vectors. The percent polyacrylamide gels utilized were 10% for panels A and B and 7.5% for panel C. The M_r of the dimers was calculated from panel C based on the relative mobility (R_f) of the MW standards a to d. The R_fs are a = 0.1, b = 0.23, c = 0.35, d = 0.45; the R_fs of E2 and E3 dimers are 0.17 and 0.19, respectively.

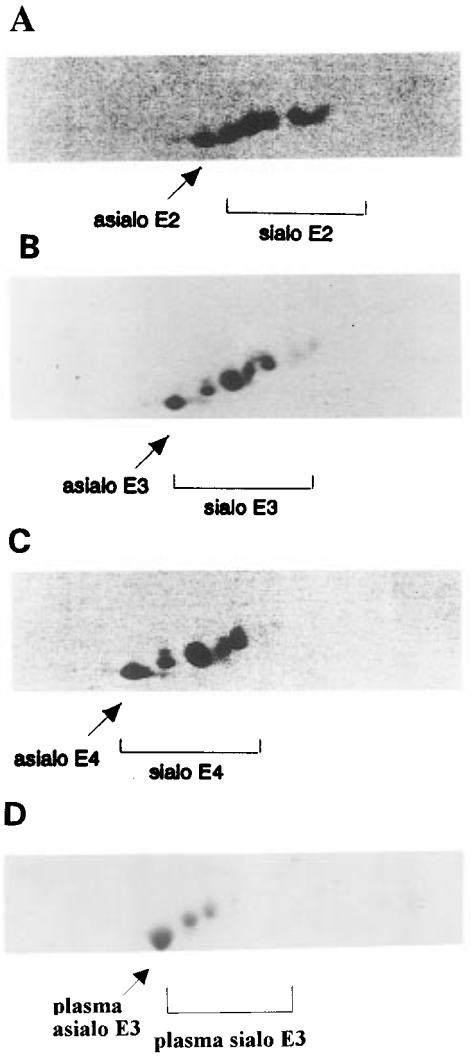

FIGURE 2: Two-dimensional gel electrophoresis and autoradiography of apoE secreted by BHK-21 cells expressing apoE2, apoE3, and apoE4 and comparison with plasma apoE3. For this analysis, ^{35}S-labeled apoE secreted by the different cell clones was labeled with ^{35}S-methionine. The radiolabeled apoE was immunoprecipitated with anti-apoE antibodies and analyzed by 2D polyacrylamide gel electrophoresis and autoradiography. Panels A to C show the autoradiograms of ^{35}S-labeled apoE2, apoE3 and apoE4, respectively, secreted by BHK-21 cells. The relative isoelectric point differences of apoE2, apoE3, and apoE4 were established by comparison with marker plasma apoE which was included in the sample. Panel D: Two-dimensional gel of plasma apoE3 stained with Coomassie brilliant blue.

apoE4 proteins obtained from three independent transfections is shown in Figure 3A,B. In panel A the filter was treated with anti-Aβ antibodies; in panel B, the same filter was treated with anti-apoE antibodies. This analysis shows that native recombinant apoE2, apoE3, and apoE4 isoforms produced by BHK-21 cells form stable complexes with Aβ in solution. The diffused nature of the complexes indicates that the sialated apoE isoforms participate in complex formation with Aβ. The size of the complex has an apparent M_r of 38−42 kDa, suggesting a 1:1 stoichiometry of apoE:Aβ. As determined by densitometry of the films corresponding to the blots, developed with anti-Aβ antiserum, the ratio between apoE−Aβ complexes is approximately 6:5:1 for E2:E3:E4, respectively (average of three independent experiments; see Figure 3A).

We also observed complex formation between apoE dimers and Aβ. The intensity of the apoE2 dimer−Aβ complex formed was much higher than that for E3 dimer−Aβ complex when the concentrations of the dimers were normalized (Figure 3A,B). Dimers of apoE4 are not detectable. The binding behavior of the nascent apoE secreted by BHK-21 cells is quantitatively different from plasma apoE as well as apoE produced by the baculovirus expression system. In the case of plasma apoE the intensity of apoE Aβ complex formed with apoE3 monomers is approximately 2-fold greater than that of the complexes formed with apoE4. In the case of the apoE produced by the baculovirus expression system the intensity of the apoE−Aβ complex formed with apoE4 monomers is approximately 2-fold greater than that with apoE3 monomer at either 37 or 24 °C (Figure 4A). Faint apoE−Aβ complexes between dimers or oligomers of apoE3 and apoE4 are also present in plasma and the commercial preparation produced by the baculovirus expression system (Figure 4A,B). We have analyzed the binding of apoE−POPC proteoliposomes prepared by the sodium cholate dialysis method with Aβ using new lots of commercially available preparations of apoE2, apoE3, and apoE4 (PanVera Corp.). This analysis indicated that complexing of apoE with POPC diminishes the efficiency of apoE−Aβ complex formation for all three isoforms (Figure 5A,B). It should be noted that this preparation of apoE represented a different lot than the apoE used in Figure 4 and contained large quantities of aggregated apoE and apoE fragments. The overall complex formed with this preparation including high mobility complexes of apoE with Aβ follows the order apoE2 > apoE3 ≫ apoE4. This order of complex formation was maintained when apoE was complexed with POPC. The apoE−POPC−Aβ interactions require further analysis with apoE obtained from mammalian cells.

Temperature Dependence of ApoE−Aβ Interactions. The Aβ binding reactivity of apoE2, apoE3, and apoE4 isoforms secreted by BHK-21 cells was also studied at different temperatures ranging from 37 to 16 °C (Figure 6A,B). This analysis showed that at 37 °C, the intensity of complex formed follows the order apoE2 > apoE3 ≫ apoE4. At 24 °C, the efficiencies of apoE−Aβ complex formation decreases and the difference between E2 and E3 is less pronounced. Incubation at 16 °C attenuates complex formation for all three isoforms (Figures 3A, 6A).

DISCUSSION

Background. Apolipoprotein E is a ubiquitous protein initially recognized for its role in cholesterol transport (Shore & Shore, 1973). ApoE-containing lipoproteins are recognized by the LDL receptor, the LDL receptor related protein (LRP), and the VLDL receptor thus contributing to their uptake by the liver and the peripheral tissues (Pitas et al., 1980; Handelman et al., 1992; Takahashi et al., 1992; Wolf et al., 1992; Rebeck et al., 1993; Tooyama et al., 1993; Yamamoto et al., 1993; Wyne et al., 1996). There are six common human apoE phenotypes which result from three apoE alleles in a single genetic locus (Zannis & Breslow, 1981). Several population studies have shown that the apoE alleles affect the plasma lipid levels in the general population (Davignon et al., 1988; Dallongeville et al., 1992). Homozygosity of the ϵ2 allele and some other rare apoE mutations are associated with type III hyperlipoproteinemia (Zannis et al., 1993).

Implication of ApoE in the Pathogenesis of AD. Recent population studies have shown that the ϵ4 allele is a risk factor for late onset familial AD (Saunders et al., 1993;

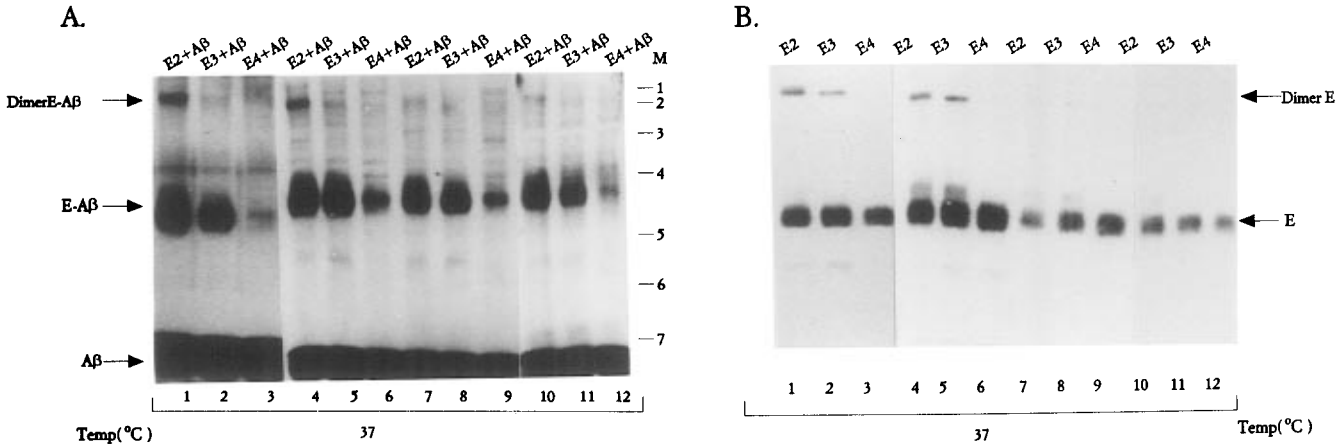

FIGURE 3: Western blotting of apoE2, apoE3, and apoE4 secreted by BHK-21 cells following interaction with Aβ. In panel A, the membrane was treated with monoclonal antibodies against Aβ. In panel B, the membrane was treated with monoclonal antibodies against apoE. Lanes 1−3, 4−9, and 10−12 demonstrate the results of independent electroporation and binding experiments. The amounts of apoE utilized were as follows: lanes 1−3, 0.6 μg; lane 4, 0.6 μg; lanes 5 and 6, 0.72 μg (the concentrations of apoE were increased to achieve comparable amounts of apoE2 and apoE3 dimers in order to better assess their differential binding properties to Aβ); lanes 7−9 and 10−12, 0.2 μg. M indicates the position of the protein MW standards (BioRad prestained). 1 = myosin, M_r = 202 kDa; 2 = β-galactosidase, M_r = 133 kDa; 3 = BSA, M_r = 71 kDa; 4 = carbonic anhydrase, M_r 41800 kDa; 5 = soybean trypsin inhibitor, M_r = 30.6; 6 = lysozyme, M_r = 17.8 kDa; 7 = aprotinin, M_r = 6.9 kDa. The position of apoE, a dimeric form of apoE, Aβ, and complexes of Aβ with the monomeric and dimeric form of apoE are shown. The interaction of apoE with Aβ was performed at 37 °C and pH 7.4 for 2 h as explained in Methods. After transfer, the membranes were blocked with 1% BSA-TBST for 1 h at room temperature. The membranes were treated successively with anti-apoE or anti-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.

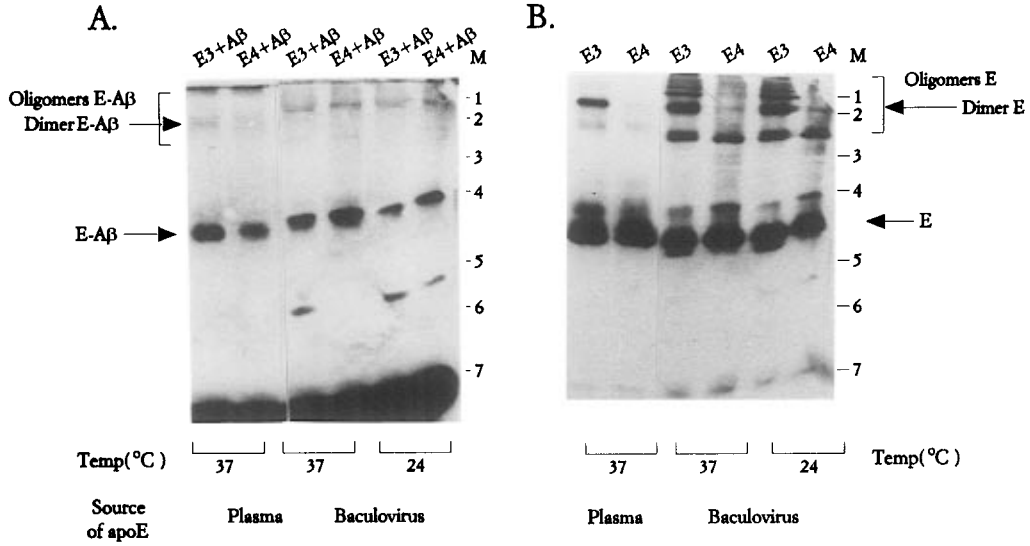

FIGURE 4: Western blotting of apoE3 and apoE4 purified from plasma or produced by expression in insect cells using the baculovirus expression system (Pan Vera) as indicated in the figure. A 1 μg amount of apoE was utilized in all experiments. In panels A and B, membranes were treated as described in panels A and B of Figure 3, respectively. M indicates the position of the protein MW standards as in Figure 3. The position of the apoE or Aβ and apoE−Aβ complexes and the temperature of apoE−Aβ interaction is indicated.

Strittmatter et al., 1993a; Corder et al., 1994). To date, the inheritance of either ϵ2 or ϵ3 or ϵ4 alleles is considered the major genetic factor determining the susceptibility to late-onset AD (for review, see Shellenberg, 1995).

At least four hypotheses have been advanced to explain these recent findings:

The first hypothesis implies that isoform-specific differences in apoE−Aβ interactions may modulate the pathological impact of Aβ. The second hypothesis implies that the inability of apoE4 to bind τ or Map2C proteins may destabilize microtubules and trigger the formation of τ-derived paired helical filaments which may trigger a pathological cascade leading to AD (Huang et al., 1994; Strittmatter et al., 1994). The third hypothesis implies a role of apoE in neuronal repair and remodeling (Poirier et al., 1995; Weisgraber & Mahley, 1996; Arendt et al., 1997). It has been suggested that isoform-specific differences in the lipid transport system may influence the efficiency of synaptogenesis and neuronal cell remodeling and thereby may affect compensatory reinnervation following neuronal cell loss (Poirier, 1994). The fourth hypothesis implicates apoE isoforms in phospholipid homeostasis and cholinergic functions (Poirier et al., 1995). Finally, it is possible that a combination of any of the four proposed causes or other, yet unknown, isoform-specific apoE functions may be relevant to the pathogenesis of AD.

The first hypothesis is the best substantiated to date by a variety of experimental findings as follows. Alzheimer's disease is linked to chromosome 19, where the apoE gene maps (Pericak-Vance et al., 1991). ApoE is associated with

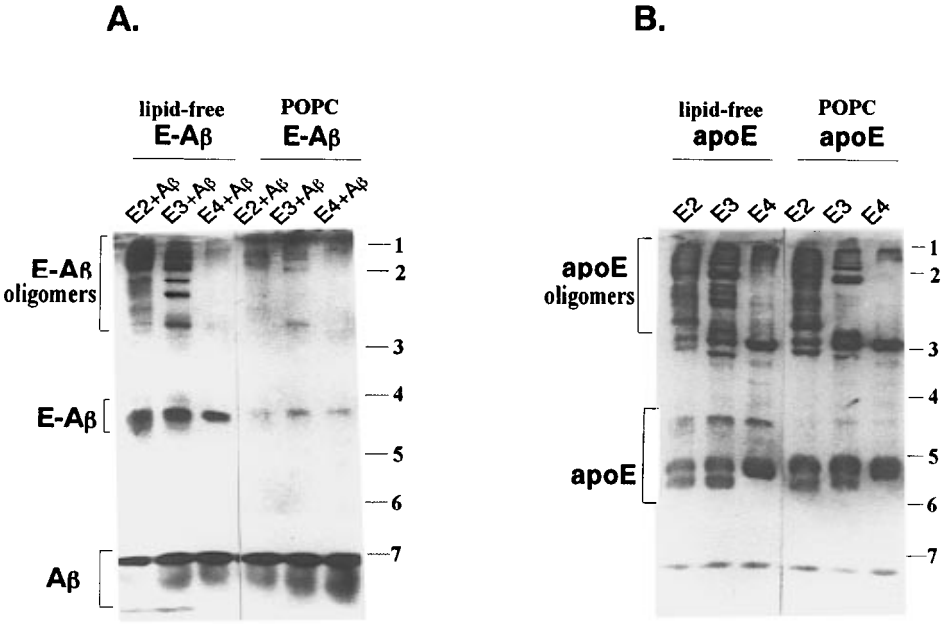

FIGURE 5: Western blots of lipid-free and POPC-complexed apoE2, apoE3, and apoE4 (Pan Vera Corp.) following interaction with Aβ. Panel A: the filter was treated with monoclonal antibodies against Aβ. Panel B: the same filter was treated with monoclonal antibodies against apoE. After transfer the membranes were treated as in Figure 3. The protein bands were detected by exposing the blots to Biomax film. A 1 μg amount of apoE was utilized in all experiments. M indicates the position of the protein MW standards as in Figure 3. The position of free and complexed apoE and Aβ forms is indicated. Aggregates at the top of Figure 5A may represent complexes of apoE with more than one molecule of Aβ.

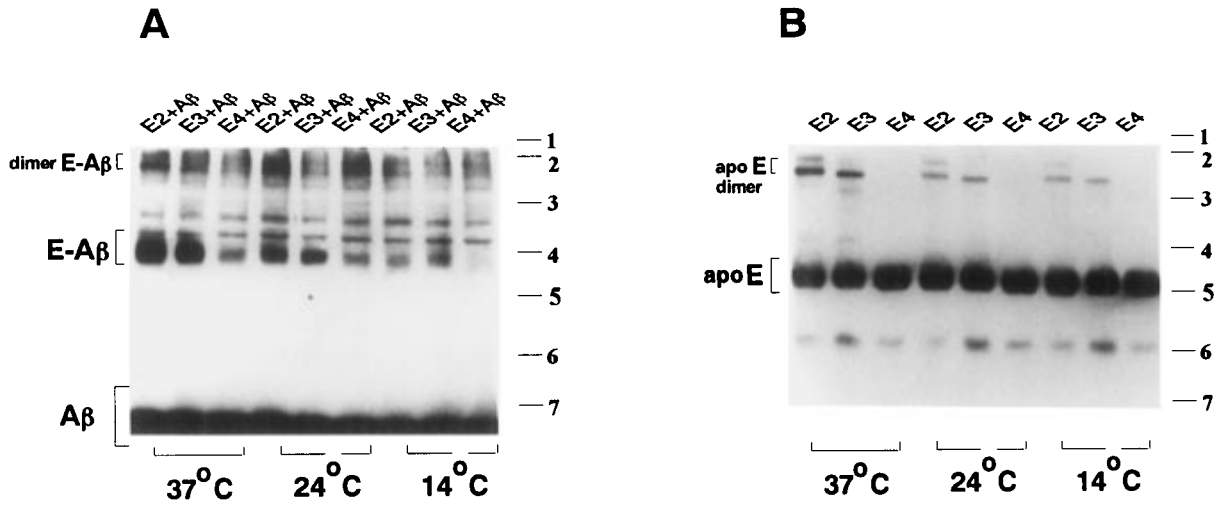

FIGURE 6: Western blots of apoE2, apoE3, and apoE4 produced by BHK-21 cell cultures following interaction with Aβ at different temperatures. Panel A: the filter was treated with monoclonal antibodies against Aβ. Panel B: the same filter was treated with monoclonal antibodies against apoE. After transfer the membranes were treated as in Figure 3. The protein bands were detected by exposing the blots to Biomax film. A 0.6 μg amount of apoE was utilized in all experiments. M indicates the position of the protein MW standards as in Figure 3. The position of free and complexed apoE and Aβ forms is indicated. Aggregates at the top of Figure 6A may represent complexes of apoE with more than one Aβ molecule.

the senile plaques and a congophilic angiopathy of Alzheimer's disease which contain Aβ amyloid fibers (Namba et al., 1991; Wisniewski & Frangione, 1992; Strittmatter et al., 1993a). Several groups have reported independently that apoE can promote fibrillogenesis of Aβ *in vitro*. These studies showed that apoE4 is a more potent amyloid-promoting factor than apoE3 (Ma et al., 1994; Sanan et al., 1994; Wisniewski et al., 1994; Castano et al., 1995). Studies by Ma et al. (1994) demonstrated that the relative isoform-specific amyloidogenic potential of apoE isoforms follows the order E4 > E3 > E2. In contrast, Evans et al. (1995) showed that apoE3 and apoE4 kinetically inhibit Aβ fibrillogenesis *in vitro* and that apoE3-dimer is a more potent inhibitor than the apoE or apoE4 monomers. It is noteworthy that all these studies utilized purified apoE from different sources, including human plasma or recombinant apoE expressed in *E. coli* and insect cells. The possible mechanisms of apoE-mediated modulation of Aβ fibrillogenesis or other potential neurotoxic effects of Aβ may involve the formation of stable (or semistable) apoE$-$Aβ complexes. Such complexes may be removed subsequently by receptor dependent (Pitas et al., 1980; Handelman et al., 1992; Takahashi et al., 1992; Wolf et al., 1992; Rebeck et al., 1993; Tooyama et al., 1993; Yamamoto et al., 1993; Wyne et al., 1996) or receptor independent processes. Demonstration of Aβ-apoE SDS-stable complexes *in vitro* (Strittmatter et al., 1993b; LaDu et al., 1994; Zhou et al., 1996) and *in vivo* (Naslund et al., 1995) can thereby provide the necessary link between this line of experiments and the pathogenesis of AD.

In vitro experiments have shown that apoE in cerebrospinal fluid can form complexes with the amyloid peptide β (Aβ) (Strittmatter et al., 1993a; Wisniewski et al., 1993). It was also shown that plasma apoE4 binds more efficiently to Aβ (1−28) as compared to apoE3 *in vitro*, and that the complex formed is stable following boiling for 5 min in 5% SDS. Differences were also found in the pH dependence of the plasma apoE−Aβ binding (Strittmatter et al., 1993b). Interestingly, recent studies showed that newly secreted apoE3 by HEK-293 cells interacts more strongly with Aβ than apoE4 over a large pH range 4 to 9 (LaDu et al., 1994). However, purification of apoE attenuated the differences in binding of the two apoE isoforms toward Aβ (LaDu et al., 1995). Similar results were obtained for apoE3 and apoE4 expressed by Raw-264 macrophages (Zhou et al., 1996).

The isoform-specific differences in the reactivity of apoE3 and apoE4 with Aβ reported by different groups may be attributed to different properties of the apoE utilized. Such properties may depend on the source of the protein and the presence of additional modulating factors such as lipids, and conformational as well as modification differences between newly synthesized and purified forms (LaDu et al., 1995).

Increased Reactivity of ApoE2 Monomers and Dimers Toward Aβ. Population studies have shown convincingly that subjects with either E3/2 or E3/3 phenotype have a decreased risk for development of late onset familial AD as compared to subjects with apoE4/4 or E3/4 phenotype (Corder et al., 1993; Strittmatter et al., 1993a; Chartier-Harlin et al., 1994). Although there are limited data, it appears that the ϵ2 allele may confer even greater protection against Alzheimer's disease as compared to the ϵ3 allele (Corder et al., 1994; Lannfelt et al., 1994). Since biochemical data pertinent to the interaction of apoE2 toward Aβ are lacking, the present study was undertaken to compare the reactivity of the naturally occurring apoE2, apoE3 and apoE4 forms toward Aβ. Taking into account the possibility that the reactivity of apoE toward Aβ may be sensitive to the conformational stability of the protein, the experiments were performed with apoE newly secreted by cultures of BHK-21 cells using the SFV-RNA transient expression system. The culture medium containing nascent apoE was collected 24−48 h postelectroporation and utilized for binding studies.

Analysis of the apoE secreted into the culture media of BHK-21 cells expressing the apoE2, apoE3, and apoE4 cDNAs revealed the following features of the protein that may be relevant to its reactivity toward Aβ. The nascent apoEs exist mostly (80−90%) in the modified (sialylated) form as compared to the plasma apoE which consists of approximately 80% of the unmodified form. The apoE−Aβ complex formed with the apoE secreted by BHK-21 cells is a diffused band. This suggests that both the sialo and asialo apoE forms are reactive toward Aβ. Although the current analysis does not permit a comparison of the relative reactivity of the different unmodified and modified apoE forms to Aβ, overall the efficiency of apoE−Aβ complex formation with the different isoforms follows the order apoE2 > apoE3 ≫ apoE4. ApoE2 and apoE3 but not apoE4 form dimers in the culture medium which can be detected by electrophoretic techniques. The apparent M_r of apoE3 and apoE2 dimers were estimated to be 91 and 95 kDa, respectively, whereas the expected M_r of the dimers are 70 to 80 kDa. The efficiency of formation of apoE2 dimer−Aβ complex is much greater than that of apoE3 dimer−Aβ complex.

It was shown previously that apoE3 homodimers purified from the blood of homozygous patients have an abnormally slow electrophoretic mobility on SDS gel (Weisgraber & Shinto, 1991). This observation raises the interesting possibility that the apoE dimer but not the monomer may form SDS-stable secondary structures which may be responsible for the observed abnormal electrophoretic mobility. Thus the different mobilities of apoE2 and apoE3 dimers may indeed reflect conformational differences among the two isoforms which may account for their difference in reactivity toward Aβ.

In agreement with recent reports (LaDu et al., 1994, 1995; Zhou et al., 1996), the reactivity of purified apoE3 and apoE4 obtained from plasma or using the baculovirus expression system was different from that observed with the native apoE secreted by BHK-21 cells. Dimers and other oligomers formed with plasma apoE as well as apoE produced by the baculovirus expression system have diminished reactivity toward Aβ.

Here we also show that apoE−Aβ complex formation depends on the reaction temperature. Decrease of the temperature led to attenuation of complex formation and resulted in approximately equal binding efficiency for all three apoE isoforms. It is possible that the decrease in temperature may induce conformational changes of hydrophobic proteins such as Aβ and apoE. The equally pronounced decrease in Aβ-binding for all three isoforms may indicate that apoE2, apoE3, and apoE4 share the same principal mechanism of binding and that the differential binding at 37° may be caused by overall conformational differences between apoE2, apoE3, and apoE4.

Several factors may account for these differences. As reported previously and demonstrated in the present study, apoE produced by mammalian cells exists in different forms, monomers, dimers, unmodified, modified, lipid-free, lipid-bound, etc. (Zannis et al., 1982, 1985, 1986; Hussain et al., 1989). These apoE forms may have different conformations or properties and therefore different reactivities toward Aβ. Several observations suggest that the conformation of apoE may be very important in promoting apoE−Aβ interactions. These include the difference in the binding to Aβ of the newly secreted, and presumably native, apoE2, apoE3, and apoE4 monomers as compared to the plasma or commercially available apoE preparations, the changes in the reactivity of apoE following purification, or by changes of pH and temperature (Strittmatter et al., 1993; LaDu et al., 1995) and the increased reactivity of the apoE2 dimers with Aβ as compared to the apoE3 dimers. ApoE2 has two cysteine residues at positions 112 and 158 whereas apoE3 has only the 158 cysteine. X-ray crystallography of the 22 kDa amino terminal portion of the human apoE which contains several 22-residue repeats (Li et al., 1988) has shown that it forms a four-helix bundle (Wilson et al., 1991). X-ray crystallography also showed that substitution of Cys for Arg158 in apoE2 or substitution of Cys for Arg112 in apoE4 are associated with conformational changes in the 3D structure of apoE. These changes may account for the different reactivities of apoE isoforms toward Aβ (Wilson et al., 1993; Dong et al., 1994). Alterations in apoE conformation may also occur by binding to different discoidal or spherical lipoprotein particles as compared to the conformation of apoE in solution. In the discoidal particles apoE helices spread out to cover through their hydrophobic surfaces the periphery of the discoidal particle (Innerarity et al., 1979). Infrared

spectroscopy showed that the axis of the helices is parallel to the acyl chains of the phospholipids (Hefelewald et al., 1990) whereas in the spherical particles the helices must bend to accommodate their hydrophobic surfaces on the surface of the spherical particle. ^{14}C NMR spectroscopy showed different pK_a values for Lys residues of apoE in lipoproteins as compared to DMPC discs, indicating that the microenvironment of apoE in discs and lipoproteins is different (Lund-Katz et al., 1993). The conformation of apoE may also be affected by the lipid composition of the particles as well as potential participation of other proteins in the formation of proteoliposomes. Similar differences have been for instance observed apoA-I and account for different subpopulations of particles with β electrophoretic mobility (Davidson et al., 1994). These conformation differences may also result in different reactivities of apoE isoforms toward Aβ. In the current study the reactivity of commercially available apoE (PanVera Corp.) complex to POPC toward Aβ was reduced. Higher mobility complexes of apoE with Aβ were observed with the apoE preparation used in this specific experiment. The intensity of these complexes is greater for apoE2 and apoE3 than apoE4 both in lipid-free and lipid-bound apoE (Figure 5A,B). It is possible that naturally occurring populations of apoE proteoliposomes in the CSF or apoE aggregates in extracellular deposits may have different reactivities toward Aβ. A definitive answer to these questions will require systematic characterization of the lipoprotein particles present in the CSF. Important in apoE−Aβ interaction may also be the conformation as well as the length of the Aβ peptide.

Implications of ApoE Aβ Interactions in the Pathogenesis of AD. The binding data obtained with nascent apoE secreted by mammalian cells in this as well as in two previous studies (LaDu et al., 1994, 1995; Zhou et al., 1996) and the epidemiological data show that strong binding of nascent apoE to Aβ correlates inversely with the risk of developing Alzheimer's disease (Corder et al., 1993, 1994; Saunders et al., 1993; Sorbi et al., 1993; Strittmatter et al., 1993a; Chartier-Harlin, 1994; Lannfelt et al., 1994; St. George-Hyslop et al., 1994; Myers et al., 1996). ApoE4, which interacts weakly with Aβ, predisposes to AD relative to apoE2 and apoE3. Thus it is possible that apoE2 and apoE3 secreted by astrocytes in the brain *in vivo* associate more efficiently with Aβ than with apoE4. Such association might prevent the polymerization of the Aβ and the formation of amyloid fibers and the amyloid plaque in the brain. The demonstration that there is an increase in the number but not the size of the plaques in the brain of E4/AD as compared to E3/AD patients (Hyman et al., 1995) may reflect the fact that more efficient binding of apoE3 as compared to apoE4 may decrease the amount of free Aβ available for nucleation and fiber formation. Although the concentration of Aβ utilized in the present studies is several orders of magnitude higher than its physiological concentration in the brain milieu (Seubert et al., 1992), it is still possible that under certain conditions local concentration of Aβ in certain sites of the brain may rise and cause polymerization and fiber formation. In the presence of apoE2 or apoE3, a soluble apoE−Aβ complex may be formed which may be removed subsequently by cell receptors. Candidates for such removal are the LDL and the VLDL receptors and the LRP (Pitas et al., 1980; Handelman et al., 1992; Takahashi et al., 1992; Wolf et al., 1992; Rebeck et al., 1993; Tooyama et al., 1993; Yamamoto et al., 1993; Wyne et al., 1996). Reduction in Aβ or increase in the apoE2 or apoE3 concentration in the brain may then have a protective role against Alzheimer's disease. Such hypotheses may be addressed with existing or new animal models expressing appropriate forms of apoE and APP isoforms (Games et al., 1995; Higgins et al., 1995; Hsiao et al., 1996). Both the genetic and the biochemical data suggest an important role of apoE in the pathogenesis of Alzheimer's disease. Systematic analysis of the interactions of apoE with its intra- and extracellular protein ligands may provide new insights on the molecular events which lead to the pathogenesis of the disease.

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