Reconstituted Discoidal ApoE-Phospholipid Particles Are Ligands for the Scavenger Receptor BI

THE AMINO-TERMINAL 1–165 DOMAIN OF ApoE SUFFICES FOR RECEPTOR BINDING*

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The high density lipoprotein receptor, scavenger receptor class B type I (SR-BI), recognizes lipid-bound apolipoprotein A-I (apoA-I) and other apolipoproteins. Here, we have used large scale cultures of apoE-expressing cells to purify apoE and prepare apoE containing reconstituted discoidal 1-palmitoyl-2-oleoyl-1-lysophosphatidylcholine (POPC)-apoE particles. These particles have been used to examine their binding to wild-type and mutant forms of SR-BI expressed in transfected IdLA-7 cells. Specific binding to SR-BI was determined by subtracting from the total binding, nonspecific values measured using either control untransfected IdLA-7 cells or by inhibiting SR-BI-mediated binding with a high titer antireceptor-blocking antibody. POPC-apoE particles generated using apoE2, apoE3, apoE4, or the carboxyl-terminally truncated forms apoE165, apoE202, apoE229, and apoE259 all bound tightly to wild-type SR-BI with similar affinities (Kd = 35–45 μg/ml). Binding was nearly abolished in a cell line expressing the IdLA (Q402R/Q418R) double mutant form of SR-BI that is unable to bind native high density lipoprotein but binds low density lipoprotein normally. The findings establish that apoE is a ligand for SR-BI and that the receptor binding domain is located in the amino-terminal 1–165-region of the protein. SR-BI-apoE interactions may contribute to cholesterol homeostasis in tissues and cells expressing SR-BI that are accessible to apoE-containing lipoproteins.

Apolipoprotein E (apoE)† is a 299-amino acid-long protein. ApoE, as a constituent of different lipoprotein particles, serves as a ligand for several cell receptors (apoER2, LDL receptor, LDL receptor-related protein, and VLDL receptor) and promotes their catabolism by the liver (1–5). ApoE may also be involved in cellular cholesterol efflux (6–9). These functions of apoE contribute to cell and tissue cholesterol homeostasis (6–9) and may explain why, when expressed locally in macrophages or endothelial cells, apoE protects from atherosclerosis (9–11). Mutations in apoE that prevent binding of apoE-containing lipoprotein remnants to the LDL receptor and possibly other receptors, as well as to heparan sulfate proteoglycans, are associated with type III hyperlipoproteinemia (12–18) and premature atherosclerosis (17, 19, 20).

SR-BI binds with high affinity HDL and LDL and discoidal reconstituted particles containing apoA-I (21–24). On binding lipoproteins, SR-BI mediates both selective cholesterol ester uptake from the lipoprotein to the cells (24–28) and bi-directional unesterified cholesterol movement (28–32). In addition to cholesterol esters (25), SR-BI can mediate cellular uptake from HDL of free cholesterol (31, 32), triglycerides (32, 33), phospholipids (34), and vitamin E (35–39). HDL and reconstituted discoidal HDL particles also promote cholesterol efflux from SR-BI-expressing cells (28–30, 40). Inactivation of the SR-BI gene reduces cholesterol levels in the steriodogenic tissues and bile and can promote atherosclerosis despite an associated increase in plasma HDL cholesterol levels (41, 42), thus demonstrating the beneficial physiological functions of this receptor (41, 42). Studies with transgenic mice also corroborated the role of SR-BI in cholesterol and bile acid homeostasis and in the protection against atherosclerosis (43–47).

In the current study, we have focused on the SR-BI binding of discoidal POPC-apoE particles containing the natural apoE isoforms and truncated apoE forms. The carboxyl-terminal mutants of apoE were generated to assess the contribution of the carboxyl-terminal domain of apoE to SR-BI binding. Recent studies by us established that the 1–185-region of apoE suffices for the clearance of apoE-containing lipoprotein remnants in vivo implying that this amino-terminal domain of apoE contains the necessary determinants for binding to at least some of the apoE receptors (48, 49). The current study establishes that apoE is a ligand for SR-BI and that its receptor binding domain is found within the amino-terminal 1–165 residues.

EXPERIMENTAL PROCEDURES

Materials

The Klenow fragment of DNA polymerase I, T4 ligase, polynucleotide kinase, and restriction enzymes were purchased from New England Biolabs. Calf intestinal alkaline phosphatase was purchased from Stratagene (La Jolla, CA), and Vent polymerase from Promega. Other materials for the polymerase chain reaction were obtained from PerkinElmer Life Sciences. The Sequenase sequencing kit was purchased from U. S. Biochemical Corp. The oligonucleotides were purchased from Gibco Life Technologies, Inc. Bacto-tryptone and bacto-yeast extract were obtained from Difco (Pittsburgh, PA). Dulbecco's
were labeled with a [35S]Met/Cys mixture, and the secreted protein was described previously (18). Cell clones expressing apoE2/apoE3/apoE4 (51) were transfected with the different pBMT3XEg derivatives as WT and Variant ApoE Forms—

- The correct orientation was selected, sequenced to verify the pBMT3X vector to generate the pBMT3XapoEg derivatives. Plasmids excised by pBluEexIV derivative by was used to replace the corresponding region in the pBluXapoEg derivative.
- The amplified mutant sequence was digested with XhoI site of the pBMT3X bovine papilloma virus vector.

### ApoE Production by Eukaryotic Expression Systems Using Permanent ApoE-expressing Cell Lines—

To generate media for isolation of truncated apoE forms, human astrocytoma HTB-13 cells, which do not express apoE, were infected with recombinant adenoviruses expressing apoE4—259, apoE4—229, apoE4—202, or apoE2—202 (48, 49), and the serum-free medium was collected as described previously.

### Purification of Lipid-free ApoE by Ion Exchange Chromatography Using Dextran Sulfate-Sepharose Column—

ApoE was purified from the culture medium of C127 cells expressing the apoE2, apoE3, or apoE4 genes or from the media of HTB-13 cells expressing the truncated apoE forms. The purification scheme involved dextran sulfate-Sepharose column fractionation. Briefly, 30 ml of dextran sulfate-Sepharose column was equilibrated with 20 mM Tris-HCl, pH 7.4. A total of 2 liters of apoE containing culture medium was concentrated to 75 ml in an Amicon concentrator using a membrane with a cutting molecular mass of 10 kDa, and the NaCl concentration was adjusted to 0.2 M and loaded over the column at a flow rate of ~80 ml/h. The column was eluted with 20 mM Tris-HCl, pH 7.4, at the same flow rate and fractions of 3 ml were collected. The protein purity of the pooled fractions were dialyzed extensively against 0.05 M NaH2PO4 and lyophilized. The protein yield was 10—20 mg/liter depending on the apoE variant.

### Preparation of Disordered Reconstituted HDL Particles Containing ApoE [POPC-ApoE]—

POPC was used to prepare the reconstituted discoidal reconstituted HDL particles containing the apoE variant with only minor modifications (52). POPC-ApoE particles were prepared from a molar ratio of 100:10:1:100 of POPC:cholesterol:apoE: sodium cholate. In a typical experiment, 0.14 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 5-mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM NaN3, and 0.01% EDTA buffer for vortexing for ~30 s, followed by storage on ice. The process was repeated until the phospholipid was completely suspended in the buffer. This required ~2 h. The sodium cholate was added, and the solution was placed on ice for one more hour. The apoE (usually 1 mg) was then added, and the incubation on ice continued for another hour. To remove the sodium cholate the solution was dialyzed against 5—6 liters of the 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM NaN3, and 0.01% EDTA buffer at 4 °C using a molecular mass cut-off of 12—14 kDa. Finally a gradient (8—25%) was run under native conditions at 15 °C on a Pharmacia Phast-Gel system to ascertain the size of the particle or used for electron microscopy analysis described below. The POPC-ApoE particles were stored at 4 °C under nitrogen to prevent the oxidation of lipid peroxides.

### Electron Microscopy (EM)—

POPC-apoE particles prepared with various mutant apoE forms as described above were analyzed by EM. To prepare the sample for EM, 50 µg of POPC-apoE were desalted three times using Amicon centrifugal filter devices (Microcon). The final concentration was ~1 mg/ml in deionized water. A 5-µl aliquot suspension of each vesicle was applied for 10 s to a Formvar carbon-coated 300-mesh copper grid. The carbon film surface was made hydrophilic by modified Eagle’s medium (DMEM) was provided by Invitrogen. Dextran sulfate and epoxy-activated Sepharose 6B were purchased from Pharmacia, the column was from BioRad, iso-dose beads isolation reagent and the D-salt dextran plastic desalting columns were purchased from Pierce. The sodium 125I was purchased from PerkinElmer Life Sciences. The bicinechonic acid assay kit was purchased from Pierce. Other reagents (and sources) were: fatty acid free bovine serum albumin, cholestrol, sodium cholate, and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), aprotinin, benzamidine, leupeptin, and phenylmethylsulfonyl fluoride (Sigma); dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, CA); Ham’s F-12 medium, fetal bovine serum, and trypsin/EDTA (JRH Biosciences, Lenexa, KS); and penicillin/streptomycin, glutamine, and G418 sulfate (GIBCO Life Technologies, Inc.). All other reagents were purchased from Sigma, Bio-Rad, or other standard commercial sources as previously described (22). The SR-BI receptor blocking monoclonal antibody KKB-1 was a gift of Karen Kozarsky.

### Methods

**Plasmid Construction and Generation of Expression Vector Carrying ApoE Gene Derivatives—**

Full-length apoE cDNA derivatives cloned into the HindIII and BamHI site of the pUC-19 plasmid (43) were used to generate three new sets of plasmids (I, II, and III) containing WT or mutated apoE gene sequences (Fig. 1A). Plasmid I (pBlueEexIV) contains the EcoR1-EcoRI apoE gene region consisting of exon 4 and part of the flanking introns cloned in the bluescript II KS vector. Plasmid II (pBlueXapoEg) contains the entire apoE gene between BamHI and HindIII flanked by two bioengineered XhoI sites cloned in the Bluescript II KS vector. Plasmid III (pBT3XapoEg) contains the entire apoE gene cloned in the XhoI site of the pBT3X2 bovine papilloma-virus vector. Each of the three plasmids was constructed with the apoE2, apoE3, or apoE4 exon IV sequences or with mutated sequences. The following protocol was utilized for generation of mutations. (a) Mutations are generated in exon IV by PCR amplification and mutagenesis using the pUCeXIV apoE derivatives (50) as a template and a set of two external primers spanning, for instance the Sty-BslI region, and mutagenic primers covering the region that needs to be mutagenized. The primers used are shown in Table 1. (b) The amplified mutant sequence was digested with StyI and BslI and was used to replace the corresponding apoE sequence in the pBlueExIV derivative (plasmid I). The mutated apoE sequence was excised from the pBlueExIV derivative by EcoRI digestion and was used to replace the corresponding region in the pBlueXapoEg derivative (plasmid II). (c) The entire mutated apoE gene sequence was excised by XhoI digestion and was cloned into the XhoI site of the pBT3X vector to generate the pBT3XapoEg derivatives. Plasmids with the correct orientation were selected, sequenced to verify the mutations, and utilized further.

**Generation and Characterization of Permanent Cell Lines Expressing WT and Variant ApoE Forms—**

To generate stable cell lines expressing the designated apoE forms, C127 cells derived from a mouse mammary tumor (51) were transfected with the different pBT3XapoEg derivatives as described previously (18). Cell clones expressing apoE2/apoE3/apoE4 were labeled with a [35S]Met/Cys mixture, and the secreted protein was immunoprecipitated and analyzed by two-dimensional gel electrophoresis and autoradiography to verify the known isoelectric point differences among the apoE isoforms (18).

### Table I

| Oligonucleotide Name | Oligonucleotide | Mutants |
|---------------------|----------------|---------|
| OePaS               | 5’ GCT GTT CAT GCC TGC ACC 3’ |         |
| OePaR               | 5’ GAG GGG TCG CAG CCG 3’ |         |
| E386XX               | 5’ ATT OCA GCC GGC GCC TCT 3’ | ApoE4—259 |
| E386XX              | 5’ GAC GGC TCT TGC ACC GGG AAT 3’ | ApoE4—229 |
| E236XX               | 5’ GCC GGC GGC GCC TCT 3’ | ApoE4—202 |
| E226UX               | 5’ GCC GGC CAG CAG GGG GCC 3’ | ApoE4—165 |
| E166AX               | 5’ GCC ACC GCC TCG GCC GGG GCC 3’ |         |
| E166AX               | 5’ GCC ACC TCT CCG GCC CAG CTC 3’ |         |
glow discharge in a Balzers Union CTA 010 glow discharge apparatus and used immediately. Excess POPC-apoE suspension was removed by blotting with filter paper and immediately replaced with a 5-μl droplet of 1% sodium phosphotungstate, pH 7.4. After a few seconds, excess stain was removed, and the grid was air dried. Fields of particles were photographed with a Philips CM12 electron microscope (Philips Electron Optics, Eindhoven, The Netherlands).

**Iodination of ApoE**—ApoE was labeled by 125I using the Iodo-Beads (53) iodination reagent and Na 125I (PerkinElmer Life Sciences). Each reaction used one mCi of 125I and three beads and 1 mg of apoE. The reaction was carried out in Tris-HCl buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.01% EDTA). An aliquot of 100 μl of Tris-HCl buffer was added to the 125I container and mixed. The diluted radioactive 125I solution was transferred to an Eppendorf tube containing 1–2 mg of apoE in the form of POPC-apoE particles in Tris-HCl salt buffer. The final volume was adjusted to 900 μl using Tris-HCl salt buffer. The sample was placed in a lead pig. Just prior to use, beads were washed with 500 μl of Tris HCl salt buffer per bead and dried on a filter paper. This washing step removes any loose particles and reagent from the beads. The beads were added to the reaction solution and kept at room temperature for 45 min with mixing every 5–10 min. The reaction was terminated by removing the solution from the reaction vessel. The 125I-labeled apoE was separated from the unincorporated Na125I using Pierce’s Presto Desalting Columns (Pierce, Inc.). Ten fractions (0.5 ml each) were collected, and 1 μl of each fraction was used for determination of the 125I counts. Ten μl of each fraction were used to measure the protein concentration by bicinchoninic acid protein assay (54). Another 10 μl of each fraction were used for PAGE gel analysis of the sample. The specific activity was calculated based on the protein concentration and the 125I counts and expressed as cpm/ng protein. Specific activities of 1000–1500 cpm/ng protein were obtained.

**Radioreceptor Binding Assay**—ldlA-7 is an LDL receptor-deficient Chinese hamster ovary cell mutant (55, 56), which expresses very little SR-BI protein or HDL binding/selective uptake activity (25, 57). The
FIG. 3. A–G, concentration-dependent binding of $^{125}$I-POPC-apoE4 complexes to confluent monolayers of IdIA [mSR-BI] and IdIA [Q402R/Q418R]. A, IdIA-7 cells and IdIA [mSR-BI] cells in the microtiter wells were washed and incubated with various concentrations of $^{125}$I-POPC-apoE4. Total binding to IdIA [mSR-BI] cells and total binding to untransfected IdIA-7 cells was obtained experimentally. The specific
LDIA-7 cells and the LDLa[mSR-BI] cells, which are LDLa-7 cells stably transfected with murine SR-BI cDNA (25, 57), were maintained in monolayer culture in Ham’s F12 medium containing 5% fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM glutamine. All incubations with cells were performed at 37 °C in a humidified 5% CO2, 95% air incubator.

SR-BI activity at 37 or 4 °C was assessed by measuring cell association of radiolabeled ligands. Briefly, on day 0 cells (both LDLa-7 and LDLa [mSR-BI] or LDLa-7 and LDLa [Q402R/Q418R]) were plated at concentrations of 4.5–5 × 10⁵ cells/well in 24-well dishes in complete F12 medium. On day 2, the monolayers were washed twice with Ham’s F12 medium and then refed with 0.4–0.5 ml of medium (Ham’s F-12 containing 0.5% (w/v) fatty acid-free bovine serum albumin, 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM glutamine) with the indicated radiolabeled ligands (125I-POPC-apoE). Eight different concentrations, ranging from 0.5–100 ng/ml, were used, and the experiments were performed in duplicate. After a 1.5 h incubation at either 37 or 4 °C, the cells were washed twice at 4 °C with buffer B (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl) containing 2 mg/ml fatty acid-free bovine serum albumin, followed by one rapid wash with buffer B alone. The cells were then solubilized with 0.1 N NaOH (300 µl each well).

Aliquots of 200 µl were used for radioactivity determinations, and 25 µl were used for determination of the protein concentration using the bicinchoninic acid assay. The specific binding was obtained by subtracting the binding of the untransfected cells (LDLa-7) from the binding of the receptor-expressing cell lines LDLa[mSR-BI] or LDLa [Q402R/Q418R] (28). Receptor binding studies to the untransfected and transfected cells were also performed in the presence and absence of the SR-BI blocking monoclonal antibody KKB-1 (28). Receptor blocking experiments were carried out as described previously (28). Binding parameters $K_B$ and $E_{max}$ were determined on the basis of the specific binding curve using the Prism program (GraphPad Software, Inc.). The specific binding (cell association) values of the saturation curves is expressed as ng of apoE in the complex associated with the cells per mg of total cell protein.

**Immunoreceptor Assay Using ELISA—**For the immunoreceptor association experiments, the Maxisorb 96-well plates were coated with anti-human apoE polyclonal antibody (1 µg/ml) (Biodesign) diluted 1:800 in carbonate/buffered saline (Tris-HCl, pH 8.5, 0.01 M NaCl) at 4 °C overnight. The coating solution was aspirated, and the well was washed three times with washing buffer (0.05% Tween 20 in phosphate-buffered saline) at 200 µl/well. Blocking buffer (10% nonfat dry milk in washing buffer) was then added at 200 µl/well. The plates were incubated at room temperature for 1.5 h and washed three times with washing buffer. Cell lysates were diluted in phosphate-buffered saline to a final concentration of 100 µl, added in each well, and the plates were incubated at room temperature for 1 h. To obtain a standard curve, POPC-apoE particles containing different amounts of apoE, mixed with 25 µg of the lysate of blank cells (LDLa-7 or LDLa[apoER-2]) that were not treated with rHDL particles or any lipoprotein, were added to a total volume of 100 µl and were added to different wells. After the plates were washed with washing buffer three times, the secondary antibody (rabbit anti-human IgG, peroxidase-conjugated) (Biodesign) diluted 1:500 in blocking buffer was added at 100 µl/well. The plates were incubated at room temperature for 1 h and washed with washing buffer three times. An aliquot of 200 µl of o-phenylenediamine dihydrochloride substrate (o-phenylenediamine dihydrochloride, 0.4 mg/ml; urea hydrogen peroxide, 0.4 mg/ml; phosphate-citrate buffer 0.05 M) was added to each well. After 30 min of incubation at room temperature, the reaction was terminated by adding 50 µl of 2 N H2SO4 per well. The absorbance was measured at 490 nm using a microtiter plate reader. A polynomial second order regression was used as the best fit for the standard curve.

The specific binding was obtained by subtracting the binding of the LDLa-7 cells from the binding of the apoER-2-expressing cells from each experimental point, and it is expressed as ng of cell-associated POPC-apoE particles per mg of total cell protein. The cell-associated ligands were analyzed as a function of concentration by nonlinear regression by the program Prism using a single binding isotherm.

**Competition Assays—**Competition assays were carried out as described above for direct association experiments, except that on day 2, unlabeled competitor protein in the concentration range 0.5–100 μg/ml was added in addition to the labeled ligand. The average percent cell association for the competition assays was calculated relative to the control values. The I50 value corresponds to binding of 5 µg of [125I]-labeled HDL or 5 µg of [125I]-POPC-apoE and it represents the absence of competitor. Two independent competition experiments were performed in duplicate.

**RESULTS**

**Expression, Production, Purification, and Characterization of WT and Variant apoE Forms—**To characterize the apoE isoforms and the mutant apoE forms, cell clones expressing WT and variant apoE genes were labeled with [35S]Met/Cys, immunoprecipitated, and analyzed by one- or two-dimensional polyacrylamide gel electrophoresis and autoradiography using 10 µg of VLDL from a subject with apoE4/4 phenotype as an internal marker. The Coomassie Brilliant Blue-stained gel obtained from this analysis showed the position of the apoE4 that was included in the sample, and the autoradiogram showed the position of the newly synthesized apoE. Superimposition of the gel on the autoradiogram establishes the charge and size differences between the apoE4 and the newly synthesized variant apoE forms as described previously (data not shown). Fig. 1B shows SDS-PAGE analysis of the full-length and truncated apoE forms. ApoE was purified from the culture medium of apoE-expressing cells as described under “Experimental Procedures.” Fig. 1C shows SDS-PAGE analysis of different purified apoE forms.

**Generation, Characterization, and EM Analysis of Reconstituted POPC-ApoE Particles—**The wild-type and different mutant apoE proteins were reconstituted in particles containing POPC and cholesterol at a ratio of 100:10:1 of POPC:cholesterol:apoE, using the sodium cholate dialysis method (52). The sodium cholate dialysis method allowed the formation of discoidal particles with all the WT and the mutant apoE forms tested. The POPC-apoE particles were negatively stained with potassium phosphotungstate, overlaid on carbon-coated grids, and photographed with a Philips CM12 electron microscope. Fig. 2, A–G shows formation of the [POPC-apoE] particles with all the WT and mutant apoE forms. Under the negative staining conditions used, these particles form the typical “rouleaux,” indicating that they are discoidal and have the thickness of a phospholipid bilayer. The number of rouleaux observed depends on the concentration of the sample on the carbon grid. In less concentrated samples, a large number of round particles that lie flat on the grid were also observed. These particles did binding was determined by subtracting the values of the binding of the LDLa-7 cells from the corresponding values of binding to the LDLa [mSR-BI] cells. $B$ and $C$, LDLa-7 cells and LDLa [mSR-BI] cells in the microtiter wells were washed and incubated with various concentrations of [125I]-POPC-apoE4in the presence and absence of receptor-blocking monoclonal antibodies. Total binding to LDLa [mSR-BI] and LDLa-7 cells was obtained experimentally. The specific binding to the LDLa [mSR-BI] cells (B) or to LDLa-7 cells (C) was determined by subtracting the values of binding to each of the two cell lines in the presence of receptor-blocking monoclonal antibodies. Note that there is specific binding in the LDLa [mSR-BI] cells (B) but not in the LDLa cells (C). $D$, concentration-dependent binding of [125I]-POPC-apoE4 complexes to confluent monolayers of LDLa cells expressing WT mouse SR-BI-designated LDLa [mSR-BI] or mutant mouse SR-BI-designated LDLa [Q402R/Q418R] cells. Cells in the microtiter wells were washed and incubated with various concentrations of [125I]-POPC-apoE4. Total binding to LDLa [mSR-BI] or to LDLa [Q402R/Q418R] cells and total binding to untransfected LDLa-7 cells were obtained experimentally. The data for the WT and the mutant receptor were obtained by 40% subtracting the values of binding to the LDLa-7 cells from the corresponding values of binding to the LDLa [mSR-BI] or LDLa [Q402R/Q418R] cells. The specific activity of [125I]-POPC-apoE4 was in the range of 1000–1500 cpn/mg protein. The experiments in A–D were performed by the radioreceptor assay at 37 °C. $E$, $F$, and $G$ represent control experiments. In $E$, the binding at 37 °C was measured using ELISA assay as described under “Experimental Procedures.” Specific binding was determined as explained above. In $F$ and $G$, the binding at 4 °C was measured using the radioreceptor assay as described above and in A and under “Experimental Procedures.” Note that the binding parameters obtained under different experimental conditions are similar (Table II).
not seem to pack hexagonally on the grid during aggregation (a characteristic of spherical structures), providing further evidence that these particles were, in fact, discoidal. To further characterize the size of the discoidal POPC-apoE cross-linking experiments were performed with particles isolated by gel filtration using sodium suberate. This analysis showed the presence of two apoE2 or apoE2–202 molecules per particle. The average size of POPC particles, determined from the EM pictures, was 174 ± 49 Å (data not shown).

**High Affinity Binding of Discoidal Reconstituted POPC-ApoE Particles to SR-BI**—Binding is reduced in an SR-BI mutant. HDL and reconstituted discoidal HDL particles containing apoA-I, apoA-II, or apoCIII bind with high affinity to SR-BI, whereas lipid-free apoA-I and pre-β HDL are poor ligands (22, 23). In the current study, we have examined the binding of reconstituted POPC-apoE particles to SR-BI. To determine the specific binding of the POPC-apoE ligands to SR-BI, binding studies were performed using both SR-BI-expressing (ldlA[mSR-BI]) transfected cells and their control untransfected cells (ldlA-7). Both approaches were necessary since the SR-BI-dependent association of the particles with the control cells was high, and high background was clearly manifest in the transfected cells (see below). This is presumably due to the presence of other apoE binding sites. The specific binding curve and the binding parameters $K_a$ and $B_{\text{max}}$ for ldlA[mSR-BI] cells were determined by subtracting the binding values for the untransfected cells from the corresponding values from the transfected cells (Fig. 7A). To ensure that the differences obtained reflected the overexpression of mSR-BI in the transfected cells and not a fortuitous increase in expression of the endogenous mouse SR-BI, we performed receptor binding studies in the untransfected and transfected cells in the presence of SR-BI receptor-blocking antibodies. Specific binding was observed only in the transfected cells (Fig. 3, B and C). The binding parameters obtained by using the SR-BI receptor-blocking antibodies and by subtracting the binding curves of the untransfected from those of mSR-BI-expressing cell line were similar (Fig. 3, A and B). The ligand-receptor specificity was also assessed in cells expressing a mutant mSR-BI (double substitution of arginines for glutamines at positions 402 and 418, designated ldlA [Q402R/Q418R]) (28). Even after correction for the lower level of mutant receptor expression in ldlA [Q402R/Q418R] cells relative to wild-type receptor expression in ldlA[mSR-BI] cells, the ability of this mutant to bind native HDL was dramatically reduced relative to that of the wild-type receptor (28). We have shown recently that POPC-apoA-I particles bind more tightly to SR-BI than native HDL (22, 23) and that the binding of these particles to this mutant receptor is more readily detected than the binding of native HDL. The specific binding affinities for the POPC-apoE particles to this mutant were clearly reduced ($K_a = 185 \mu g/ml$) for the mutant SR-BI as compared with wild-type SR-BI (38 \mu g/ml) (Table II). To establish whether iodination of apoE changes its binding properties to SR-BI, we have determined receptor binding using an ELISA assay. This control experiment showed that the binding parameters for apoE2 and apoE2–202 were similar to those obtained when iodinated apoE was used in binding studies ($K_a = 45.5 \pm 8$ and $35.5 \pm 4.5 \mu g/ml$ (Fig. 3E and Table II)). Similar results were obtained when binding experiments of apoE2, apoE4, and apoE2–202 were performed at 4 °C (Fig. 3, F and G and Table II). It is interesting that the nonspecific binding at 4 °C was reduced, whereas the SR-BI-specific binding was not altered considerably.

We have previously shown that under normal cell culture ligand-binding conditions, LDL is a relatively poor inhibitor of HDL binding, possibly because SR-BI exhibits multiple classes of ligand binding sites (24, 28). Fig. 4 shows that the binding of 125I-labeled HDL to SR-BI is effectively competed by HDL, less effectively by POPC-apoE, and much less effectively by LDL. The findings are consistent with the $K_a$ values of POPC-apoE particles for SR-BI (22) (Table II) as well as with the reduced binding of POPC-apoE4 to the ldlA [Q402R/Q418R] cells (Fig. 3D) (28).

**The Natural ApoE Isoforms and Mutant ApoE Forms Have Similar Affinities for SR-BI**—Receptor binding is maintained when the carboxyl-terminal residues 166–299 are deleted. To address the question of whether specific domains or residues of apoE are involved in receptor binding, we have generated several carboxyl-terminally truncated apoE forms extending to residues 259, 229, 202, and 165. The purpose of the mutations was to assess the importance of the carboxyl-terminal region of apoE to the SR-BI-specific binding. Previous studies have shown that residue Arg-158 is important for the binding of apoE containing lipoproteins to the LDL receptor (58–61). Similarly in vitro experiments have shown that residues in the 171–183 region of apoE contribute directly or indirectly to LDL receptor binding (60). Analysis of the binding of the truncated variant apoE forms to SR-BI showed the truncated apoE forms

*2 Liu, T., Krieger, M., Kan, H.-Y., and Zannis, V. I. (2002) J. Biol. Chem. 277, 21576–21584.*
FIG. 5. A–H, concentration-dependent binding of $^{125}\text{I}$-POPC-apoE4 and POPC-apoA-I complexes containing different apoE isoforms, mutant and truncated apoE forms, and apoA-I to confluent monolayers of IDIA [mSR-BI] cells. A–G, Cells in the microtiter wells were washed and incubated with various concentrations of $^{125}\text{I}$-POPC-apoE4. Total binding to IDIA [mSR-BI] cells and total binding to untransfected IDIA-7 cells was obtained experimentally. The specific binding shown in this figure was determined by subtracting the values of binding to the IDIA-7 cells from the corresponding values of binding to the IDIA [mSR-BI] cells. The specific activity of apoE was in the range of 1000 to 1500 cpm/ng. Two to four independent experiments were performed in duplicate for each apoE form. The average $K_d$ and $B_{max}$ values thus determined are shown in Table II. The apoE forms used are A, apoE2; B, apoE3; C, apoE4; D, apoE4–259; E, apoE4–229; F, apoE4–202; G, apoE4–165. H shows the specific binding curve of non-radiolabeled POPC-apoA-I to IDIA [mSR-BI] cells determined by an immunoreceptor assay as described (22). The apoA-I was obtained using a baculovirus expression system (22).
bind with similar affinities ($K_d$, 35–45 μg/ml) (Fig. 5, A–G and Table II). This indicates that the amino-terminal residues 1–165 of apoE contain the determinant region for binding to SR-BI. Fig. 5H shows for comparison the binding of POPC-[apoA-I] particles to ldlA [mSR-BI] cells determined by an immunoreceptor assay as described previously (22). The affinity of POPC-apoA-I particles (3.8 ± 0.4 μg of protein/ml) is an order of magnitude greater than that of the POPC-apoE particles.

**DISCUSSION**

**Background**—In the current study, we have focused on interactions of SR-BI with another potential ligand, the apolipoprotein E, which plays an important role for cholesterol homeostasis in the circulation and in the brain (6–9, 62). Apolipoprotein E has remarkable structural similarities with apoA-I. Both proteins contain 11- or 22-amino-acid-long repeats that are organized in amphipathic α helices. ApoE is a ligand for several cell receptors that serve to deliver cholesterol to cells (1–5). ApoE may also play an important role in cholesteryl efflux (6–9). It was shown initially that apoE-containing lipoprotein particles with γ electrophoretic mobility (γLp-E) are very effective in removing excess cholesterol from cholesterol-loaded cell cultures (6). Plasma of mice lacking apoE has a reduced capacity to promote cholesteryl efflux from macrophages. However, this defect was corrected by selective expression of apoE in macrophages of the E/−/− mice (7).

Cholesteryl efflux may account for the protective role of apoE against atherosclerosis when it is expressed locally in the arterial wall by macrophages and endothelial cells (9–11). Numerous studies have also implicated SR-BI in cholesteryl efflux *in vitro* (28–30, 40). It has been shown that transiently transfected cells and permanent cell lines expressing SR-BI promote net cholesteryl efflux from cells to the HDL and rHDL cholesterol acceptors, but not to lipid-free apoA-I (28, 30). In a variety of cell lines studied, the rate of cholesteryl efflux correlates with the levels of SR-BI expression (30). Potential interactions of apoE-containing lipoproteins with SR-BI could contribute to cholesteryl ester delivery as well as to cholesteryl efflux from cells that express SR-BI (63).

**Specific Binding of Reconstituted POPC-ApoE Particles to SR-BI**—Cells contain numerous apoE-recognizing receptors. The most prominent are the LDL receptor (1, 2, 64), the LDL receptor-related protein (3), the VLDL receptor (5), and the apoE receptor-2 (4). IdlA-7 do not have the LDL receptor (55, 56) but do have other apoE-recognizing receptors. We observed high background binding, presumably due to the presence in these cells of other apoE-recognizing receptors. To establish specific binding of apoE-containing lipoproteins to SR-BI, it was necessary to subtract the background binding of apoE to all these receptors in a Chinese hamster ovary cell line that overexpresses the mouse SR-BI (IdlA [mSR-BI]). Binding experiments using apoE4-established specific binding of POPC-apoE particles to IdlA [mSR-BI] occurs with $K_d$ = 38 μg/ml and $B_{max} = 1700$ ng/mg cell protein.

To ensure that the specific values determined were valid, we measured the SR-BI-dependent binding using SR-BI receptor-blocking antibodies. The specific binding curves and the binding parameters obtained by both procedures were essentially identical ($K_d$ = 39 μg/ml and $B_{max} = 1800$ ng/mg cell protein). Similar binding parameters were obtained when the binding experiments were performed at 4 °C or when receptor binding was determined by ELISA.

The binding of POPC-apoE4 was affected by a double mutation in SR-BI that eliminated most of the binding of HDL but not of LDL (28). This observation suggests that there are similarities in the modes of binding of apoA-I containing native HDL and POPC-apoE4. This conclusion is supported by competition experiments, suggesting that the recognition site for apoE on the SR-BI is more like that of the site that binds HDL than the site that binds LDL.

**Effects of ApoE Mutations on the Binding of POPC-ApoE Particles to SR-BI**—Binding of apoE to the LDL receptor is affected by mutations in residue 158 as well as by substitutions of charged residues in the 140–150 region of apoE (58–60). In the current study, we examined the binding of three naturally occurring apoE forms (apoE2 [C112/C158], apoE3 [C112/R158], and apoE4 [R118/R158]) and several truncated apoE forms. These truncations produced the amino-terminal segments of apoE, which extend to residues 259, 229, 202, or 165. In previous studies we have used the cell lines expressing mouse SR-BI to determine the ligand specificity of the receptor using ligands containing human apoA-I (22). The use of this heterologous system in the present study offers the advantage of the availability of the large number of mouse SR-BI and human apoE mutations, which are not available for the human SR-BI and the mouse apoE. Although mouse and human apoE, as well as mouse and human SR-BI, have considerable sequence similarities, one can not exclude the possibility for species differences in the receptor ligand specificity due to the use of a heterologous system.

As shown in Fig. 5, A–H and Table II the binding parameters of POPC-apoE particles to SR-BI are similar for the WT apoE and the truncated apoE forms (range of $K_d$, 35–45 μg/ml). The findings suggest that the amino-terminal region 1–165 of apoE contains the necessary determinants for its recognition by SR-BI and that Arg-158 is not critical for SR-BI receptor binding. Similar studies with the LDL receptor have shown previously that Arg-158 as well as the carboxyl-terminal 171–183 apoE region are important for the binding of apoE-containing liposomes to the LDL receptor (60). The receptor-binding properties of POPC-apoE particles are similar to those involving rHDL-apoA-I particles. In the case of apoA-I, deletion of residues 186–243 did not affect binding of POPC-apoA-I to SR-BI (22). The interactions of SR-BI with HDL and LDL are associated with selective lipid uptake (24–28, 31–34) as well as cholesteryl efflux (28–30, 40). Recent studies indicated that SR-BI is expressed by astrocytes but not by microglia in the brain (63), indicating that interactions of apoE-containing lipoproteins with SR-BI may contribute to brain cholesterol homeostasis. Reconstituted POPC/apoE particles containing apoE have the ability to deliver cholesteryl esters to cells (32). The role of apoE-containing lipoproteins in SR-BI-mediated cholesteryl efflux and selective lipid uptake in the brain and the impact of apoE mutations on these processes remains to be determined.

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Reconstituted Discoidal ApoE-Phospholipid Particles Are Ligands for the Scavenger Receptor BI: THE AMINO-TERMINAL 1–165 DOMAIN OF ApoE SUFFICES FOR RECEPTOR BINDING
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