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Structural Determinants in the C-terminal Domain of Apolipoprotein E Mediating Binding to the Protein Core of Human Aortic Biglycan*

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Olga Klezovitch‡§, Marilena Formato¶, Gian M. Cherchi¶, Karl H. Weisgraber‖, and Angelo M. Scanu‡**

*From the Departments of ‡Medicine and of **Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637, the ¶Department of Physiological, Biochemical, and Cellular Sciences, University of Sassari, 07100 Sassari, Italy, and the ‖Gladstone Institute of Cardiovascular Disease, Cardiovascular Research Institute, Department of Pathology, University of California, San Francisco, California 94141-9100*

Apolipoprotein (apo) E-containing high density lipoprotein particles were reported to interact *in vitro* with the proteoglycan biglycan (Bg), but the direct participation of apoE in this binding was not defined. To this end, we examined the *in vitro* binding of apoE complexed with dimyristoylphosphatidylcholine (DMPC) to human aortic Bg before and after glycosaminoglycan (GAG) depletion. In a solid-phase assay, apoE·DMPC bound to Bg and GAG-depleted protein core in a similar manner, suggesting a protein-protein mode of interaction. The binding was decreased in the presence of 1 M NaCl and was partially inhibited by either positively (0.2 M lysine, arginine) or negatively charged (0.2 M aspartic, glutamic) amino acids. A recombinant apoE fragment representing the C-terminal 10-kDa domain, complexed with DMPC, bound as efficiently as full-length apoE, whereas the N-terminal 22-kDa domain was inactive. Similar results were obtained with a gel mobility shift assay. Competition studies using a series of recombinant truncated apoEs showed that the charged segment in the C-terminal domain between residues 223 and 230 was involved in the binding. Overall, our results demonstrate that the C-terminal domain contains elements critical for the binding of apoE to the Bg protein core and that this binding is ionic in nature and independent of GAGs.

Apolipoprotein (apo)[1] E, a 34-kDa protein that plays an important role in lipid metabolism, is a component of chylomicrons, very low density lipoprotein, very low density lipoprotein remnants, and certain fractions of high density lipoprotein (HDL) particles (1). More than 90% of plasma apoE is produced by the liver. However, extrahepatic cells such as macrophages are able to synthesize and secrete apoE (2). Thrombin has been shown to cleave apoE into two domains as follows: a 22-kDa N-terminal domain (22K) and a 10-kDa C-terminal domain (10K) (3, 4). The 22K domain is responsible for the binding of apoE to the low density lipoprotein (LDL) receptor, heparin, and the cell-surface heparan sulfate proteoglycans (PGs) (4–6). The 10K domain contains the major lipid-binding region and is responsible for the tetramerization of lipid-free apoE (7, 8). In addition, the 10K domain mediates the binding of apoE to amyloid Aβ peptide (9, 10) and amyloids A and L (11).

Several studies have suggested a potential role for apoE in atherosclerosis. First, apoE is undetectable in nonatherosclerotic human arterial intima but is abundant in human lesions (12–15). Second, apoE in the arterial vessel can be expressed locally by macrophages, especially those loaded with lipid (16, 17). Third, in apoE-deficient mice the development of atherosclerosis is markedly increased even in the absence of a high fat diet (18, 19). Fourth, restoring apoE production in macrophages in apoE-deficient or irradiated wild type mice with bone marrow transplantation inhibits atherosclerosis (17), although some studies suggest that the macrophage-derived apoE promotes diet-induced atherosclerosis in male wild type mice (20). Fifth, rapid regression of atherosclerosis is also achieved by liver-directed gene transfer of apoE into apoE-deficient mice (21, 22). How apoE protects the vessel wall from atherosclerosis is yet unknown. Among the suggested mechanisms is the binding of apoE to the extracellular matrix of the artery wall (23). In this context, deposits of apoE in human atherosclerotic lesions have been reported to localize frequently with biglycan (Bg) (15), a small leucine-rich PG with a 38-kDa protein core of a known structure and two glycosaminoglycan (GAG) chains, predominantly dermatan sulfate (DS) (24, 25). Bg is synthesized by vascular endothelial and smooth muscle cells (26, 27). The function(s) of this PG is poorly understood, although several studies have indicated that it interacts with transforming growth factor-β, fibronectin, collagen, and other matrix components (24, 25). As determined by a gel mobility shift assay, it has recently been reported that HDL_3 particles rich in apoE bind to Bg, a property not exhibited by apoE-free HDL_3 (15), suggesting that the interaction was mediated by apoE (15). However, shortcomings of those studies were the lack of direct evidence for apoE binding, questions about the conformation of the Bg purified from the media of cultured human arterial smooth muscle cells in the presence of 8 M urea, and lack of insight into the mechanism of the binding. These issues are addressed in the present studies in which apoE and se-

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§ To whom correspondence should be addressed: Dept. of Medicine, University of Chicago, 5841 S. Maryland Ave., MC5041, Chicago, IL 60637. Tel.: 773-702-0570; Fax: 773-702-4534; E-mail: oklezovi@medicine.bsd.uchicago.edu.

[1] The abbreviations used are: apo, apolipoprotein; HDL, high density lipoprotein; 22K, the 22-kDa fragment of apoE; 10K, the 10-kDa fragment of apoE; LDL, low density lipoprotein; PG, proteoglycan; GAG, glycosaminoglycan; Bg, biglycan; DS, dermatan sulfate; DMPC, dimyristoylphosphatidylcholine; GD, GAG-depleted; BSA, bovine serum albumin; GdnHCl, guanidine hydrochloride; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; PMSF, phenylmethylsulfonyl fluoride.

lected mutants complexed with dimyristoylphosphatidylcholine (DMPC) vesicles were examined for binding to Bg extracted from human aorta and refolded into its native state. Both untreated and GAG-depleted (GD) Bg, representing the protein core, were studied. We demonstrate that apoE binds to the protein core of Bg, that critical elements of the binding reside in a charged 8-amino acid segment located in the 10K domain of apoE, and that the binding is ionic in nature and does not require the GAG component of Bg.

EXPERIMENTAL PROCEDURES

Materials—Chondroitin ABC lyase (EC 4.2.2.4) and chondroitin AC lyase (EC 4.2.2.5) were from Seikagaku Co., Tokyo, Japan. L-Amino acids, bovine serum albumin (BSA), urea, guanidine hydrochloride (GdnHCl), PMSF, Tween 20, SDS, goat anti-rabbit and anti-mouse IgG alkaline phosphatase conjugates, and ρ-nitrophenyl phosphate were purchased from Sigma. DMPC was from Avanti Polar Lipids, Inc., Alabaster, AL. Kallikrein inactivator was purchased from Calbiochem. Streptavidin conjugated to alkaline phosphatase was from Pierce. Polyclonal rabbit antisera against human Bg (LF-51) and human decorin (LF-136) were kindly provided by Dr. Larry W. Fisher (NIDCR, National Institutes of Health, Bethesda). Polyclonal rabbit antiserum against human apoE recognized both full-length apoE and the two recombinant apoE fragments, 22K and 10K. Monoclonal antibody 3H1 was characterized previously (5). The polyclonal rabbit antiserum against human apoA-I was described by Edelstein *et al.* (28).

Isolation and Purification of Bg from Human Aorta—Bg was isolated from the human post-mortem thoracic aorta of a healthy donor (female, 44 years old) who died from cerebral hemorrhage. The samples were collected within 12 h from death. After removing the adventitia and the outer media, total PGs were extracted from the aortic segments with 6 M urea, 1 M NaCl in the presence of protease inhibitors (10 mM EDTA, 5 mM benzamidine hydrochloride, 10 mM PMSF, 10 mM ϵ-aminocaproic acid, 10 mM *N*-ethylmaleimide) for 24 h at 4 °C. The extracted PGs were then dialyzed against 6 M urea for 48 h at 4 °C and further purified by ion-exchange chromatography on DEAE-Sephacel column equilibrated in 6 M urea (29). Bg, *i.e.* PG-II fraction, was separated from the other PGs by size-exclusion chromatography on a Sepharose CL-4B in the presence of 6 M urea/1 M NaCl as described by Coinu *et al.* (30). Urea solutions were prepared fresh for each experiment and filtered (0.22-μm pore) prior to use. All the chromatographic steps were conducted at 4 °C in the presence of the protease inhibitors indicated above. The purified Bg was dialyzed against 20,000 volumes of 1 mM NH_4HCO_3 for 48 h at 4 °C and subsequently lyophilized. The lyophilized product was kept at −20 °C until use. The analysis of constituent GAGs, performed after separate enzymatic degradation with chondroitinase ABC and chondroitinase AC (31), by high performance capillary electrophoresis (32) showed that they consisted of 55% DS and 45% chondroitin sulfate (29.5% of C6S and 15.5% of C4S). The purity of the Bg preparation was analyzed by a reduced 4–12% Tris glycine SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie staining (Fig. 1, *lane 3*) or an immunoblot assay with an antiserum against human Bg (dilution 1:2,000) (Fig. 1, *lane 1*) using the ECL Western Detection Reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Purified Bg migrated on the gel as a broad band of ~190–220 kDa that did not react with an anti-human decorin antibody (data not shown).

Preparation of GD-Bg—100 μg of protein was incubated with chondroitinase ABC in 100 μl of TBS (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl) containing 0.5 mM PMSF and 10 units/ml kallikrein inactivator for 2 h at 37 °C. The reaction was terminated by adding 1 mM $ZnCl_2$. On a reduced 4–12% Tris glycine SDS-PAGE, GD-Bg migrated as a single band of ~48 kDa that reacted with an antiserum against human Bg (Fig. 1). To remove digested GAGs, GD-Bg was dialyzed against 20,000 volumes of 1 mM NH_4HCO_3 for 48 h at 4 °C and subsequently lyophilized. The lyophilized product was kept at −20 °C until use.

Circular Dichroism Spectroscopy of Bg and GD-Bg—Lyophilized samples of either Bg or GD-Bg were dissolved in 10 mM phosphate buffer, pH 7.5, in either the presence or in the absence of 4 M GdnHCl (0.25 mg of protein/ml), incubated for 1 h at 25 °C, and used for CD spectroscopy. The CD spectra were recorded at 25 °C in a 1-mm path length on an Aviv model 62DS spectropolarimeter equipped with a temperature control unit (Aviv Associates, Inc., Lakewood, NJ). All spectra were the average of 4 scans and were corrected for background. The mean residue ellipticity was calculated as described (33). The percent secondary structure was calculated using a program provided by Aviv Inc. for Aviv CD data sets based on an algorithm of Chang *et al.* (34). As shown in Fig. 2*A*, the spectrum of Bg in 10 mM phosphate buffer exhibited a symmetrical negative trough with a minimum at 213 nm. The calculated secondary structure was 44% β-sheet, 18% β-turn, 16% α-helix, and 22% random coil. These structural parameters were no longer present in 4 M GdnHCl but were fully restored after removal of this chaotropic agent by extensive dialysis (Fig. 2*A*). The CD spectrum of GD-Bg in 10 mM phosphate buffer had a minimum at 215 nm with a broader curve than that exhibited by intact Bg (Fig. 2*B*). The predicted secondary structure of GD-Bg was 34% β-sheet, 21% β-turn, 9% α-helix, and 36% random coil; as in the case of Bg, it was markedly affected by the presence of 4 M GdnHCl and was restored upon removal of this denaturant (Fig. 2*B*). The CD spectra and the secondary structure calculation for our GD-Bg were nearly identical to those reported recently for recombinant human Bg protein core prepared by using the vaccinia virus/T7 bacteriophage expression system and purified under non-denaturing conditions (35). Taken together, our data support the conclusion that the Bg protein extracted from human aorta acquired its native conformation upon removal of the chaotropic agent utilized during the purification procedure.

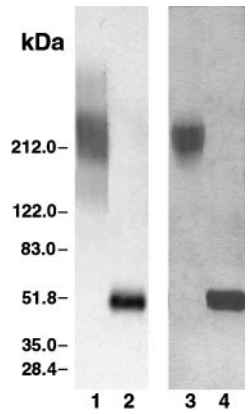

FIG. 1. **Electrophoretic analysis of human aortic Bg and GD-Bg.** Bg and GD-Bg were run on 4–12% Tris glycine SDS-PAGE under reducing conditions and either probed with an anti-Bg antibody or stained with Coomassie. *Lane 1*, Bg, 100-ng aliquot, probed with an anti-Bg antibody; *lane 2*, GD-Bg, 100-ng aliquot, probed with an anti-Bg antibody; *lane 3*, Bg, 10-μg aliquot, stained with Coomassie; *lane 4*, GD-Bg, 10-μg aliquot, stained with Coomassie.

ApoE and Fragments—ApoE3 was isolated from the delipidated *d* <1.02 g/ml human plasma lipoproteins as reported by Weisgraber *et al.* (36). The recombinant apoE3 fragments comprised residues 1–191 (22K), residues 223–299 (10K), residues 1–272, residues 1–260, residues 1–251, and apoE missing residues 186–230 (Fig. 3). They were expressed in *Escherichia coli* as described previously (37, 38). The purity of isolated apoE and the apoE fragments was assessed by 4–20% Tris glycine SDS-PAGE (NOVEX, San Diego, CA) under reducing conditions (37, 38).

Incorporation of ApoE and ApoE Fragments into DMPC Vesicles—Because lipid-free apoE exists as a tetramer in solution (7, 8) and *in vivo* is associated with lipids, we studied apoE and the recombinant apoE fragments complexed with DMPC. DMPC vesicles (10 mg/ml) were prepared by sonication of dried DMPC in TBS supplemented with 1 mM EDTA (39). Each protein was combined with DMPC (1:3.75, w/w) and isolated by density gradient ultracentrifugation as described by Innerarity *et al.* (40). The hydrated densities of the resulting complexes were in the range of 1.09–1.1 g/ml, a range corresponding to that published previously for apoE and apoE fragments (4, 39–41). Fractions containing the complexes were pooled, dialyzed against TBS containing 1 mM EDTA, and stored at 4 °C until use. The comparison of the relative migration of the lipid-protein complexes on the non-denaturing 4–12% Tris glycine PAGE (NOVEX) to that of known standards gave Stokes diameters of 11–14 nm.

Preparation of Biotinylated ApoE·DMPC—ApoE was biotinylated using the ECL Protein Biotinylation kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions and complexed with DMPC as described above. The hydrated density and the Stokes diameters of the biotinylated apoE·DMPC complexes were similar to those obtained for the unlabeled apoE·DMPC.

Preparation of ApoA-I·DMPC—ApoA-I was isolated and purified from delipidated human serum HDL (*d* 1.063–1.125 g/ml) by gel filtration and ion-exchange chromatography in 8 M urea as described previ-

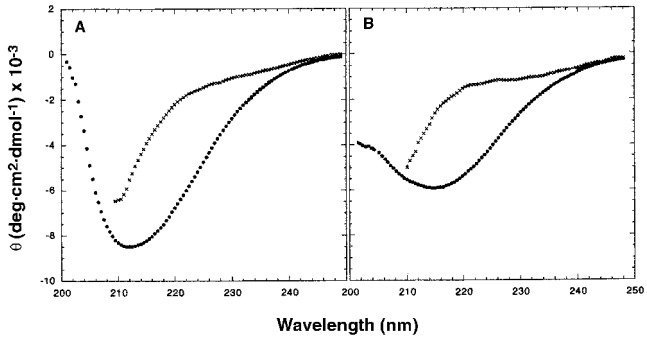

FIG. 2. **CD spectra of Bg and GD-Bg.** *A*, Bg in 10 mM phosphate buffer (···). The spectrum of Bg renatured after exposure to 4 M GdnHCl was superimposable; Bg in 10 mM phosphate buffer containing 4 M GdnHCl is indicated by *lower line*. *B*, GD-Bg in 10 mM phosphate buffer (···). The spectrum of GD-Bg renatured after exposure to 4 M GdnHCl was superimposable; GD-Bg in 10 mM phosphate buffer containing 4 M GdnHCl is indicated by *upper line*. All the spectra were the average of 4 scans and were corrected for background.

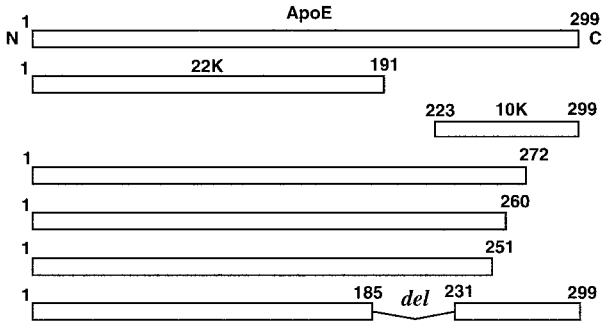

FIG. 3. **Schematic representation of the apoE fragments used in the binding studies.** The N- and C-terminals of apoE are indicated as *N* and *C*, respectively. The *numbers* indicate the number of amino acid residues. *Del* indicates the deletion of the internal apoE region.

ously by Edelstein *et al.* (28). ApoA-I was incorporated into DMPC vesicles, and the complexes were characterized as described above for apoE and the apoE fragments. The majority of the lipid-protein complex was recovered in the density range of 1.095–1.11 g/ml. Non-denaturing 4–12% Tris glycine PAGE (NOVEX) of apoA-I·DMPC indicated a uniform population of complexes with a Stokes diameter of 8.7–9.6 nm. Isolated apoA-I·DMPC was stored in TBS containing 1 mM EDTA at 4 °C until use.

Binding Experiments—Solid-phase assays were performed essentially as described by Klezovitch *et al.* (42). Briefly, the microtiter plates (Beckman Instruments, Fullerton, CA) were coated with 100 μl of Bg or GD-Bg (10 μg/ml in TBS) for 2 h at 37 °C. Nonspecific binding sites were blocked with 1% BSA in TBS for 1 h at room temperature. After three washes with TBST (TBS supplemented with 0.1% BSA and 0.02% Tween 20), various protein concentrations of either of the following apoE·DMPC, 22K·DMPC, 10K·DMPC, or apoA-I·DMPC were added to the wells in TBS and incubated for 1 h at 37 °C. After incubation, the wells were washed three times with TBST. The bound protein was detected by using either polyclonal rabbit anti-apoE or anti-apoA-I serum in TBST (each at 1:2,000 dilution) for 1 h at room temperature. At this time, the wells were washed three times with TBST and the goat anti-rabbit IgG alkaline phosphatase conjugate in TBST (dilution 1:2,000) was added for 1 h at room temperature. After washing with TBST, ρ-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer, pH 9.8) was added, and the color development was followed at 405 nm on a microtiter reader, Biomek 100 (Beckman Instruments). When the biotinylated apoE was used in the system, the binding was detected with a streptavidin conjugated to alkaline phosphatase (dilution 1:2,000). Absorbance data were then transformed into moles by using standard curves established for each ligand. Molarity of the protein·DMPC complexes was calculated based on the molarity of the corresponding protein component. Scatchard analysis was carried out by plotting the amount of ligand bound (*x* axis) *versus* the ratio of bound to free ligand (*y* axis). The B_{max} was obtained from the *x* intercept, and K_d was the ratio of the B_{max} over the *y* intercept.

Gel Mobility Shift Assay—A constant amount (0.3 μg) of either of the following apoE·DMPC, 22K·DMPC, 10K·DMPC or apoA-I·DMPC was incubated for 1 h at 37 °C with 5 μg of GD-Bg in a final volume of 30 μl of TBS. Two μl of glycerol were added, and the samples were analyzed by non-denaturing 4–12% Tris glycine PAGE (NOVEX) at 4 °C, following by immunoblotting with either an anti-apoE (dilution 1:5,000) or an anti-apoA-I (dilution 1:2,000) rabbit polyclonal antibody.

Quantitative Analyses—Protein concentrations of Bg, GD-Bg, apoE, the apoE fragments, and apoA-I were determined by the DC protein assay (Bio-Rad) according to the manufacturer's instructions.

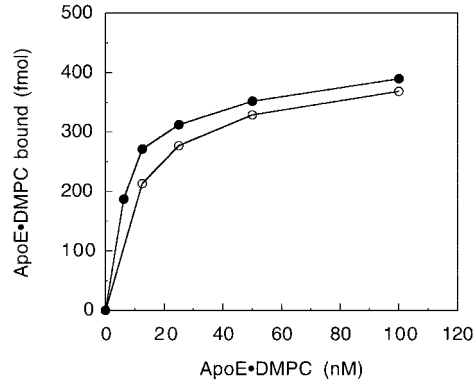

FIG. 4. **Binding of apoE·DMPC to immobilized Bg and GD-Bg.** ApoE·DMPC at the indicated concentration (0–100 nM in terms of apoE) was incubated with immobilized Bg (●) and GD-Bg (○) for 1 h at 37 °C. The amount of apoE·DMPC bound was determined with an anti-apoE antibody as described under "Experimental Procedures." The data presented are means of two independent experiments each conducted in duplicate.

RESULTS

Binding of ApoE·DMPC to Immobilized Bg and GD-Bg—ApoE·DMPC exhibited concentration-dependent saturable binding to both Bg and GD-Bg (Fig. 4) with similar apparent K_d values of 7.5 and 11.3 nM, respectively. The B_{max} values were also similar, 413 and 405 fmol, respectively. Since GAG depletion did not affect the binding parameters, we concluded that the interaction of apoE·DMPC with Bg occurs via its protein core. In order to verify binding specificity, we studied the binding of another α-helical apolipoprotein, apoA-I, incorporated into DMPC vesicles. Under the conditions of our binding assay, apoA-I·DMPC failed to interact with either form of Bg (data not shown).

The binding of apoE·DMPC to GD-Bg was calcium-independent (Table I) and occurred under physiological ionic conditions, *i.e.* in the presence of 0.15 M NaCl. In contrast, at a high concentration of salt (1 M NaCl), the binding was dramatically decreased (Table I), suggesting the ionic nature of the interaction between apoE and the Bg protein core.

It has been previously shown that positively charged lysine and arginine residues are involved in the protein-protein interaction of apoE with the LDL receptor (43, 44) and lipoprotein lipase (45). On this premise, we incubated apoE·DMPC (50 nM with respect to apoE) with GD-Bg in the absence or presence of either L-lysine or L-arginine, each at a concentration of 0.2 M. Both amino acids partially inhibited the binding of apoE·DMPC to GD-Bg to a similar extent (Table I). When the negatively charged amino acids, L-aspartic or L-glutamic, were used in the system, the binding was also decreased, although to a lesser extent (Table I). Of note, uncharged amino acids L-glycine, L-proline, or L-valine had no effect on the binding (Table I). These results suggest that the interaction of apoE with the Bg protein core is charge-dependent.

Binding of 22K and 10K to Immobilized GD-Bg—In order to define the region on apoE responsible for the binding to the Bg protein core, we studied the two major fragments of apoE, 22K (N-terminal) and 10K (C-terminal) complexed with DMPC. The

TABLE I

Factors affecting the binding of apoE·DMPC to immobilized GD-Bg

ApoE·DMPC, at the apoE concentration of 50 nM, was incubated with immobilized GD-Bg in the presence of the indicated concentrations of one of the following: Ca^{2+}, NaCl, L-lysine, L-arginine, L-aspartic acid, L-glutamic acid, L-glycine, L-proline, or L-valine. The amount of apoE·DMPC bound was determined with a rabbit polyclonal anti-apoE antibody as described under "Experimental Procedures."

	Binding
	%
ApoE·DMPC	100[a]
+ Ca^{2+}, 10 mM	93.8 ± 4.7 (3)
+ NaCl, 1 M	13.4 ± 4.3 (6)
+ L-Lysine, 0.2 M	51.5 ± 6.7 (6)
+ L-Arginine, 0.2 M	46.7 ± 9.7 (6)
+ L-Aspartic acid, 0.2 M	59.6 ± 1.4 (3)
+ L-Glutamic acid, 0.2 M	76.1 ± 3.9 (6)
+ L-Glycine, 0.2 M	93.9 ± 7.8 (3)
+ L-Proline, 0.2 M	112.2 ± 6.2 (3)
+ L-Valine, 0.2 M	94.5 ± 4.1 (3)

[a] The data are means ± S.D. The numbers of independent experiments conducted in duplicate are shown in parentheses.

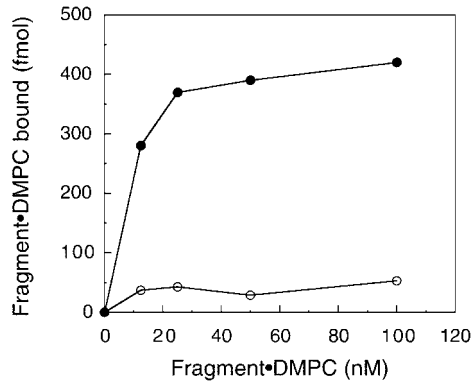

FIG. 5. **Binding to immobilized GD-Bg of apoE fragments incorporated into DMPC vesicles.** 22K·DMPC (○) and 10K·DMPC (●) were incubated with immobilized GD-Bg at the indicated protein concentration (0–100 nM) for 1 h at 37 °C, and the amount bound was determined with an anti-apoE antibody as described under "Experimental Procedures." The data presented are means of two independent experiments each conducted in duplicate.

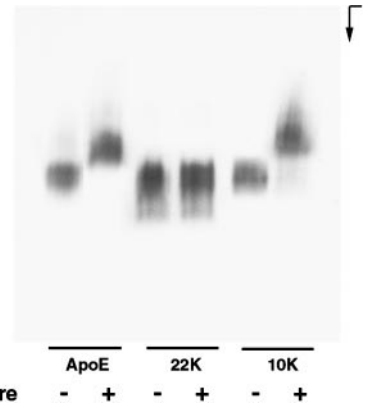

FIG. 6. **Western blot analysis of apoE·DMPC and the fragments after incubation in TBS in the absence or in the presence of GD-Bg.** A constant amount (0.3 μg) of apoE·DMPC (apoE), 22K·DMPC (22K), or 10K·DMPC (10K) was incubated for 1 h at 37 °C in the absence or presence of 5 μg of GD-Bg (core) in a final volume of 30 μl of TBS. The samples were analyzed by non-denaturing 4–12% Tris glycine PAGE at 4 °C, followed by immunoblotting with a rabbit polyclonal anti-apoE antibody. The *arrow* indicates the origin of the gel and the direction of the electrophoretic migration.

10K·DMPC complexes exhibited saturable and concentration-dependent binding to immobilized GD-Bg (Fig. 5) with calculated K_d and B_{max} of 7.7 nM and 459 fmol, respectively. These values were similar to those obtained with full-length apoE. In contrast, there was no significant binding with the 22K·DMPC complexes (Fig. 5). These observations indicate that the 10K domain is responsible for the binding of apoE to the protein core of Bg.

Gel Mobility Shift Assay—To determine if apoE and its fragments interact with the Bg protein core in solution under physiological salt conditions, we incubated apoE and its fragments, 22K and 10K, all complexed with DMPC, with GD-Bg in TBS and subsequently examined the electrophoretic mobility of each resulting complex by native 4–12% Tris glycine PAGE. As shown in Fig. 6, GD-Bg retarded the migration of both full-length apoE and 10K but had no effect on 22K. Moreover, the incubation of apoA-I·DMPC with GD-Bg under similar experimental conditions did not alter the electrophoretic behavior of this complex (data not shown). These results indicate that the binding of apoE to the Bg core in solution is specific and involves the apoE 10K domain.

Effect of Monoclonal Antibody 3H1 on the Binding of Biotinylated ApoE·DMPC to Immobilized GD-Bg—In order to verify that the 10K domain mediates the interaction of apoE with the Bg protein core, biotinylated apoE·DMPC (50 nM with respect to apoE) was preincubated with monoclonal antibody, 3H1, which recognizes residues 243–272 (ascites fluid, 1:100 dilution) for 1 h at 25 °C prior to the binding to GD-Bg. The amount of bound biotinylated apoE·DMPC was determined with a streptavidin-alkaline phosphatase conjugate as described under "Experimental Procedures." Preincubation with 3H1 caused a marked decrease of the binding of biotinylated apoE·DMPC to GD-Bg to 33.9 ± 5.8% (n = 3) of the control value. In turn, the preincubation with a monoclonal antibody against an irrelevant antigen, apo(a), had no effect on this binding. These results further support the involvement of the C-terminal domain in the binding of apoE to the Bg protein core.

Competition Studies—In order to define further the structural elements in the 10K domain of apoE responsible for its binding to the Bg protein core, DMPC complexes of apoE fragments terminating at residues 272, 260, or 251, apoE with deletion of residues 186–230, 10K, and 22K were tested for their ability to compete with biotinylated apoE for binding to immobilized GD-Bg. Biotinylation did not affect the binding of apoE·DMPC to either Bg or GD-Bg (data not shown). As expected, 10K was as potent inhibitor as full-length apoE (Table II). Moreover, the apoE fragments terminating at residues 272, 260, or 251 were equally efficient in inhibiting the binding to GD-Bg (Table II). In contrast, neither 22K nor apoE (Δ residues 186–230) had a significant effect on the binding (Table II). Taken together, these results suggest that the region between residues 223 and 230 contains critical elements for the binding of apoE to the protein core of Bg.

DISCUSSION

The results of the current studies demonstrate that full-length apoE complexed with DMPC binds to the protein core of aortic Bg and that the GAG component is not or only minimally involved in the binding. This dominant protein-protein mode of interaction is consistent with the results of previous studies showing that apoE binds poorly to DS and chondroitin sulfate (46, 47), the main GAGs of Bg. Our studies have also demonstrated that the binding to the Bg protein core occurs via the C-terminal 10K domain of apoE. This observation receives indirect support from our preliminary studies showing that binding is unaffected by apoE phenotypes that are determined

TABLE II
Capacity of full-length apoE and apoE fragments complexed with DMPC to compete with biotinylated apoE·DMPC for the binding to immobilized GD-Bg

ApoE was biotinylated and subsequently incorporated into DMPC vesicles as described under "Experimental Procedures." Biotinylated apoE·DMPC (apoE*), at the apoE concentration of 50 nM, was incubated with immobilized GD-Bg in the presence of one of the following apoE forms complexed with DMPC reported in the table below, each at the protein concentration of 1 μM. The amount of apoE* bound was determined with a streptavidin-alkaline phosphatase conjugate as described under "Experimental Procedures."

	Binding
	%
ApoE*	100[a]
+ ApoE (1–299)	32
+ ApoE (223–299)	23
+ ApoE (1–272)	29
+ ApoE (1–260)	22
+ ApoE (1–251)	22
+ ApoE (1–191)	92
+ ApoE (Δ186–230)	90

[a] The data are means of two independent experiments each conducted in duplicate.

by sequence variability in the N-terminal domain of apoE.[2] The fact that the C-terminal domain contains three stretches of α-helices (1) led us to consider that this structural motif may be responsible for the binding and that helical apolipoproteins with similar amphipathic characteristics might exhibit a comparable binding property. However, human apoA-I, a representative apolipoprotein, complexed with DMPC, did not bind to the Bg protein core. A clearer definition of the binding mode emerged from the study of C-terminal truncated forms of apoE. The results from these studies demonstrated that the segment between residues 223 and 230, Ser-Arg-Thr-Arg-Asp-Arg-Leu-Asp, contained elements critical for the binding suggesting that the 3 positively and 2 negatively charged amino acid residues are involved in the ionic interaction between apoE and the Bg protein core. This interpretation is supported by the results showing that L-aspartic acid and L-arginine were inhibitors of the binding. On the basis of these data we anticipate that the apoE-binding site on the Bg protein core resides in one of the stretches of charged amino acids flanking the leucine-rich domains.

In human atherosclerotic lesions, apoE accumulates predominantly with Bg, although it is likely that other extracellular matrix components contribute to apoE retention. In this regard, Huang *et al.* (48) demonstrated that apoE has a high avidity for laminin *in vitro* and also co-localizes with this protein in the neuromuscular junction, probably via protein-protein interactions. Currently it is unclear how much of the apoE that accumulates in the extracellular matrix is derived from plasma apoE-containing lipoproteins and how much from resident macrophages. Most of the information on the subject, which may not necessarily apply to man, has been obtained from studies in apoE-deficient mice. In that atheroma model, apoE is predominantly derived from macrophages (17), although potential minor contributions by small size apoE-containing lipoproteins like γ-LpE and pre-β-LpE have been suggested (21).

The anti-atherogenic role of apoE has emerged predominantly from the studies demonstrating that severe spontaneous arterial lesions develop in mice with a targeted deletion of the apoE gene. Although the onset of those lesions may be partially attributable to the development of an atherogenic plasma lipoprotein profile, it is apparent that the anti-atherogenic effect of apoE may be independent of plasma lipoprotein changes. For instance, recent studies by Linton and Fazio (17) have shown that macrophages lacking apoE may directly contribute to the atherogenic process and, conversely, that apoE secreted by macrophages has a direct anti-atherogenic effect in the absence of changes in plasma lipoproteins. However, the mechanism whereby apoE exerts its anti-atherogenic action is still unclear. Lipoprotein retention by the extracellular matrix is currently viewed as an important step in early atherogenesis (49). Thus, factors that prevent such a retention would have anti-atherogenic potential. In the case of apoE the studies by Saxena *et al.* (23) have suggested that the anti-atherogenic role of this apolipoprotein may be related, at least in part, to its ability to interfere with the lipoprotein lipase-dependent retention of LDL in the sub-endothelial matrix. This would involve in particular DS-PGs, *i.e.* decorin and Bg, both of which increase in concentration as the atheromatous lesions progress (50–52). Moreover, apoE/Bg protein core interaction may reduce the ability of the core protein to subsequently bind potentially "atherogenic" factors. These factors include collagen I, fibronectin, transforming growth factor-β (24, 25), and according to the results of our preliminary studies, apo(a).[2] In addition, it has been suggested that apoE bound by the vascular extracellular matrix may favor reverse cholesterol transport, inhibit platelet aggregation, modulate local lymphocyte function, or influence the growth and phenotypic expression of surrounding smooth muscle cells (16, 53, 54).

The fact that *in vitro* apoE is readily cleaved in its hinge region (residues 165–220) by several enzymes in the serine protease family (55) raises the question of whether in atherosclerotic plaques the immunolocalized apoE represents either a full-length entity or fragments thereof. In the latter respect, it should be noted that in the artery wall, apoE is only found in the inflammatory regions where the atheromatous lesions are located and where active metalloproteinases capable of cleaving *in vitro* apoE are also present.[3] It remains to be established whether fragments of apoE are indeed present in either experimental or human atheroma and whether they play a role in the postulated anti-atherogenic role of apoE.

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Structural Determinants in the C-terminal Domain of Apolipoprotein E Mediating Binding to the Protein Core of Human Aortic Biglycan

Olga Klezovitch, Marilena Formato, Gian M. Cherchi, Karl H. Weisgraber and Angelo M. Scanu

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