Molecular characterization of two monoclonal antibodies specific for the LDL receptor-binding site of human apolipoprotein E

Robert Raffaï,*, Roger Maurice,† Karl Weisgraber,§ Thomas Innerarity,§ Xingbo Wang,†,** Roger MacKenzie,†† Tomoko Hirama,†† David Watson,†† Eric Rassart,** and Ross Milne[1],*,†**

Lipoprotein and Atherosclerosis Group, University of Ottawa Heart Institute and Departments of Pathology and Biochemistry,* University of Ottawa, Ottawa, Canada; Institut de Recherches Cliniques de Montréal,† Montréal, Canada; Gladstone Institute of Cardiovascular Disease, Department of Pathology,§ Cardiovascular Research Institute, University of California, San Francisco, San Francisco, CA: Département des Sciences Biologiques,** Université du Québec à Montréal, Montréal, Canada; and Institute for Biological Sciences,†† National Research Council of Canada, Ottawa, Canada

Abstract Apolipoprotein E (apoE), a 299 amino acid protein, is a ligand for the low density lipoprotein receptor (LDLr). It has been established that basic amino acids situated between apoE residues 136 and 150 participate in the interaction of apoE with the LDLr. Evidence suggests that apoE is heterogeneous on lipoproteins in its conformation and in its ability to react with cell surface receptors. Our goal was to produce mAbs that could serve as conformational probes of the LDLr binding site of apoE. We used a series of apoE variants that have amino acid substitutions at residues 136, 140, 143, 144, 145, 150, 152, and 158 to identify the epitopes of two anti-human apoE monoclonal antibodies (mAbs), 1D7 and 2E8, that inhibit apoE-mediated binding to the LDLr. We show that most of the variants that have reduced reactivity with the LDL receptor also have reduced reactivity with the mAbs. The epitopes for both mAbs appear to include residues 143 through 150 and thus coincide with the LDLr-binding site of apoE. It is notable that mAb 2E8, but not 1D7, resembles the LDLr in showing a reduced reactivity with apoE ($Arg^{158} \rightarrow Cys$). While most of the receptor-defective variants involve replacement of apoE residues directly implicated in binding, substitution of Arg^{158} by Cys is thought to indirectly affect binding of apoE to the LDLr by altering the conformation of the receptor-binding site. To determine whether the similarity in specificities of the mAbs and the LDLr reflect structural similarities, we cloned and characterized the cDNAs encoding the light and heavy chains of both mAbs. Primary sequence analysis revealed that, although these two antibodies react with overlapping epitopes, their respective complementarity determining regions (CDRs) share little homology, especially those of their heavy chains. The two mAbs, therefore, likely recognize different epitopes or topologies within a limited surface of the apoE molecule. Four negatively charged amino acids were present in the second CDR of the 2E8 heavy chain that could be approximately aligned with acidic amino acids within the consensus sequence of the LDLr ligand-binding domain. This could indicate that mAb 2E8 and the LDLr use a common mode of interaction with apoE.—**Raffaï, R., R. Maurice, K. Weisgraber, T. Innerarity, X. Wang, R. MacKenzie, T. Hirama, D. Watson, E. Rassart, and R. Milne.** Molecular characterization of two monoclonal antibodies specific for the LDL receptor-binding site of human apolipoprotein E. *J. Lipid Res.* 1995. **36:** 1905–1918.

Supplementary key words monoclonal antibodies • LDL receptor • surface plasmon resonance

Apolipoprotein (apo) E is a 34-kDa protein consisting of 299 amino acids and is a functionally important constituent of chylomicrons and very low, intermediate, and high density lipoproteins (1). As a ligand for the low density lipoprotein receptor (LDLr), apoE is a major modulator of lipoprotein metabolism. The most common apoE isoforms, apoE2, apoE3, and apoE4, which are distinguishable by isoelectric focusing, are encoded by three codominantly expressed alleles. ApoE3, the most common of the three, is considered to be the wild type, whereas apoE2, apoE4, and the even rarer isoforms are considered to be variants. The most frequently observed form of apoE2 differs from apoE3 by the substitution of a cysteine for an arginine at residue 158, whereas apoE4 differs from apoE3 by the replacement of a cysteine by an arginine at residue 112. Variant

Abbreviations: LDLr, low density lipoprotein receptor; CDR, complementarity determining region; VLDL, very low density lipoprotein; SPR, surface plasmon resonance; DMPC, dimyristoylphosphatidylcholine.

[1]To whom correspondence should be addressed.

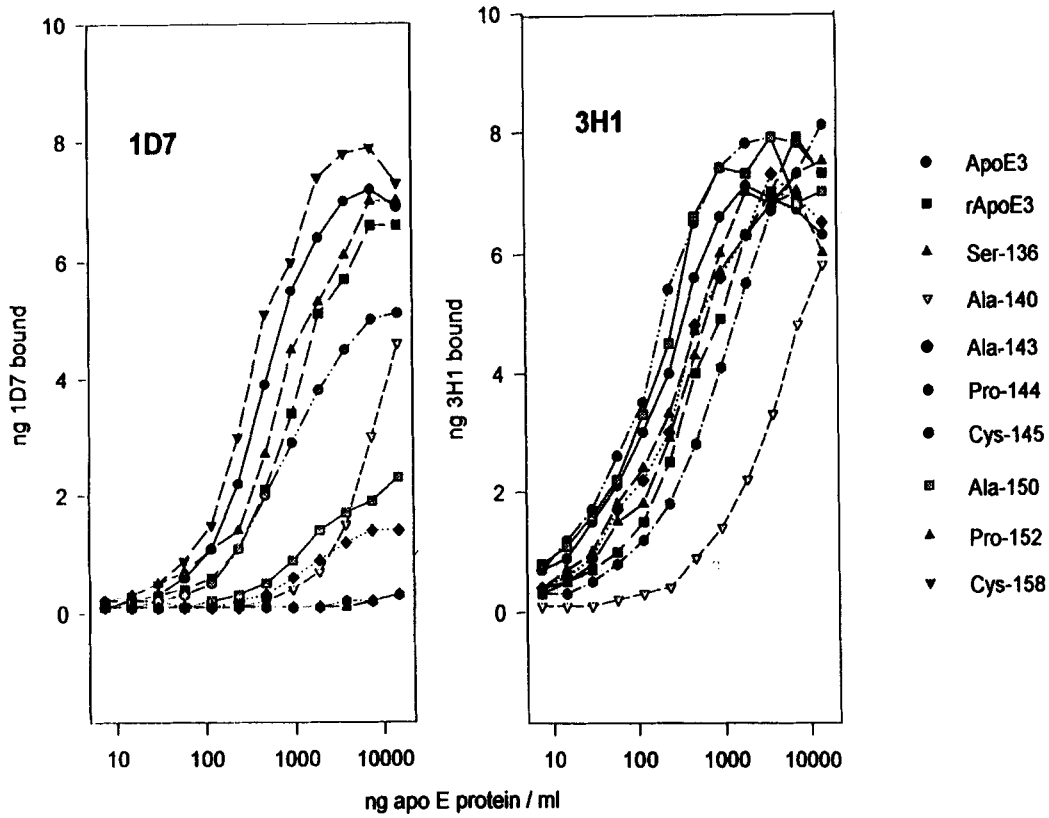

Fig. 1. Immunoreactivity of apoE variants with anti-apoE mAbs 1D7 and 3H1. Serial dilutions of apoE variants were incubated in polystyrene microwells to which anti-apoE mAb 6C5 had been adsorbed. The wells were then washed and exposed to either ^{125}I-labeled 1D7 (left) or ^{125}I-labeled 3H1 (right) and, after washing, the bound radioactivity was determined. The bound ^{125}I-labeled mAb mass was calculated from the specific activity of the labeled antibodies. Results from one experiment are shown with essentially identical results having been obtained in two other experiments.

forms of apoE, such as apoE2(Arg^{158} → Cys), that show defective binding to the LDLr are, in part, responsible for the expression of type III dyslipoproteinemia. In addition, apoE polymorphism can influence plasma lipid levels (2), postprandial lipemia (3–5), apoB metabolism (6), the lipoprotein subclass distribution of apoE (7–9), and susceptibility to Alzheimer's disease (10). Limited proteolytic digestion of apoE has shown that the molecule is composed of two structural domains (11, 12), a 22-kDa amino-terminal domain that has relatively low affinity for lipid and includes the residues responsible for binding to the LDLr and a 10-kDa carboxyl-terminal domain that binds lipid with high affinity (9). Several lines of evidence (1, 13–18) indicate that positively charged amino acids within the region of residues 136–150 directly participate in the interaction of apoE with the LDLr. The three-dimensional crystal structure of the amino-terminal 22-kDa thrombolytic fragment of human apoE has been solved (19). The fragment is folded into an elongated four-helix bundle with the positively charged amino acids implicated in receptor binding situated on the fourth helix and exposed to solvent.

The mAb 1D7 is one of a group of anti-human apoE mAbs that we first reported in 1981 (20). Of the six mAbs, only 1D7 is capable of blocking apoE-mediated binding to the LDLr (17). Antibody 1D7 has subsequently been used by ourselves and others to distinguish between apoE- and apoB-mediated lipoprotein binding to cell surface receptors (21–23) and as a probe for the identification of the receptor-binding (17) and heparin-binding sites of apoE (24). To define the fine specificity of 1D7, we have now determined its reactivity with a series of apoE variants that differ in their affinities for the LDLr. In parallel with 1D7, we have similarly analyzed the reactivity of a second anti-apoE mAb, 2E8, which also blocks binding of apoE to the LDLr. In both cases the respective epitopes of 1D7 and 2E8 appear to coincide with the LDLr-binding site on apoE. We have furthermore determined the nucleotide sequence of the cDNAs coding both antibodies and have demonstrated that the deduced amino acid sequence of the antibody combining site of 2E8 shows homology to the ligand binding consensus sequence of the LDLr. The results are discussed in terms of the potential mechanisms of binding of apoE to both antibodies and the LDLr.

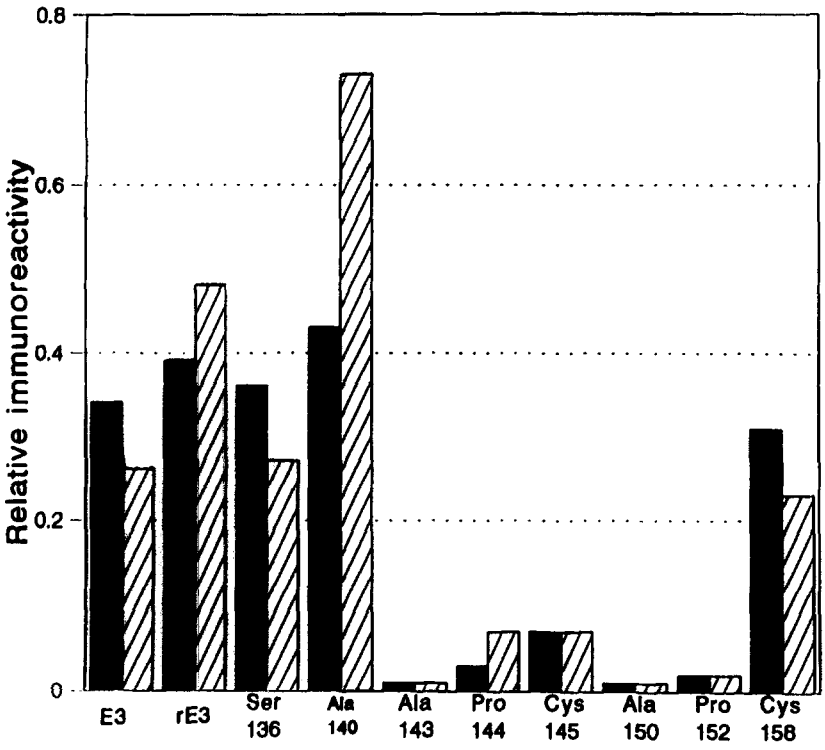

Fig. 2. Relative reactivity of mAb 1D7 with apoE variants. The relative immunoreactivity of apoE variants (■) with mAb 1D7 was calculated from the results presented in Fig. 1 according to the formula: ng apoE necessary to have 3 ng of ^{125}I-labeled 3H1 bound/ng apoE necessary to have 3 ng of ^{125}I-labeled 1D7 bound. Results are also presented from the same experiment for apoE variants that had been incorporated into DMPC vesicles (cross hatched bars).

In triglyceride-rich lipoproteins, apoE appears to be heterogeneous with respect to its conformation or accessibility, with only a subpopulation of molecules being capable of mediating binding to the LDLr (25). It has been reported that expression of the 1D7 epitope on very low density lipoproteins (VLDL) may correlate with the ability of the particle to bind to the LDLr (26). Thus, 1D7 may be specific for a conformational state of apoE that permits interaction of apoE with the LDLr or may react with a subpopulation of molecules whose receptor binding site is exposed. Our goal is to identify or produce mAbs that would recognize the same conformational structure on apoE as is recognized by the LDLr. These antibodies will be used as conformational probes to study the physical and chemical parameters that modulate expression of the LDL receptor binding site of apoE.

EXPERIMENTAL PROCEDURES

Monoclonal antibodies

The production and characterization of mAbs 1D7, 3H1, and 6C5 have been described previously (17, 20, 24). Hybridoma 2E8 was the product of a fusion between spleen cells of a mouse immunized with purified human apoE3 and the non-secreting plasmacytoma cell line SP2-O. The mAb 2E8 was characterized according to the same criteria that were used for the other anti-apoE mAbs (17). The IgG fraction containing the mAbs was purified from the ascites of hybridoma-bearing mice by affinity chromatography on Protein A-Sepharose (27). IgG was labeled with ^{125}I as previously described (28).

Preparation of apoE and production of apoE variants

ApoE3, apoE2(Arg^{158} → Cys), and apoE2(Arg^{145} → Cys) were purified from plasma as reported by Weisgraber, Rall, and Mahley (29). The generation of the apoE variants apoE(Arg^{136} → Ser), apoE(His^{140} → Ala), apoE(Lys^{143} → Ala), apoE(Leu^{144} → Pro), apoE(Arg^{150} → Ala), and apoE(Ala^{152} → Pro), their expression in *E. coli*, and their purification have also been described (18). For comparison of purified plasma apoE and apoE produced by *E. coli*, purified, bacterially expressed apoE3 was included in some experiments (30). For certain experiments, the isolated apoE was incorporated into dimyristoylphosphatidylcholine (DMPC) vesicles (31).

Sandwich apoE radioimmunometric assay

Polystyrene wells (Removawells, Dynatech Laboratories, Alexandria, VA) were coated by an overnight incubation at room temperature with 100 µl of 6C5 IgG at a concentration of 10 µg/ml in 5 mM glycine, pH 9.2. After washing with 0.15 M NaCl containing 0.025%

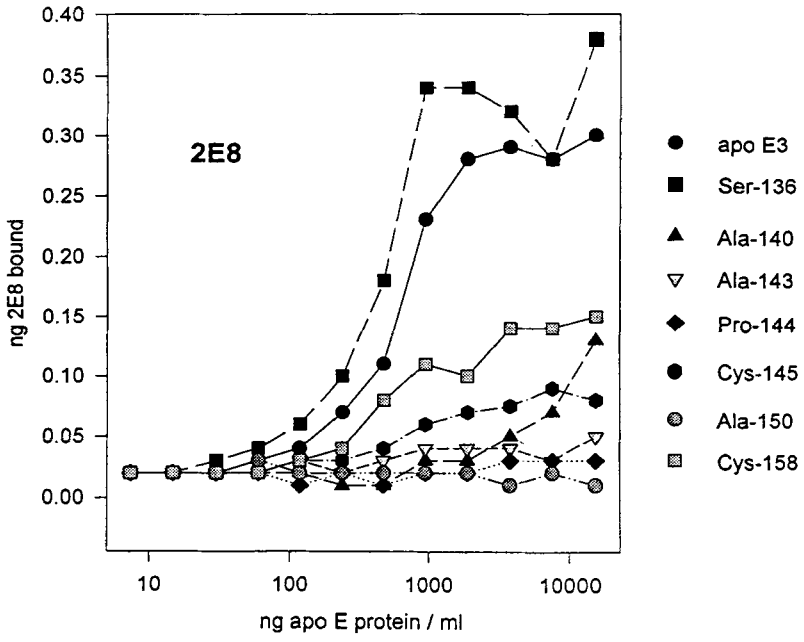

Fig. 3. Immunoreactivity of apoE variants with anti-apoE mAbs 2E8. Serial dilutions of apoE variants were incubated in polystyrene microwells to which anti-apoE mAb 6C5 had been adsorbed. The wells were then washed and exposed to ^{125}I-labeled 2E8 and, after washing, the bound radioactivity was determined. The bound ^{125}I-labeled mAb mass was calculated from the specific activity of the labeled mAb.

Tween 20 (NaCl-Tween), the wells were saturated by a 30-min incubation with a 1% (w/v) solution of bovine serum albumin in phosphate-buffered saline (PBS-BSA). The wells were emptied and 100 µl of dilutions of apoE or apoE-DMPC complexes in PBS-BSA were added and allowed to incubate overnight at room temperature. After washing with NaCl-Tween the wells were filled with 100 µl of ^{125}I-labeled 1D7 or ^{125}I-labeled 3H1 diluted to 100 ng/ml in PBS-BSA. After an overnight incubation, the wells were washed and bound radioactivity was determined. The calculation of IgG mass bound was based on the specific activity of the ^{125}I-labeled IgG.

Surface plasmon resonance

The kinetics of 3H1, 1D7, and 2E8 binding to apoE and apoE variants were determined by surface plasmon resonance (SPR) using a BIAcore biosensor system (Pharmacia Biosensor). This technology allows for interactions to be continuously monitored in real time and generates binding profiles, or sensorgrams, from which rate constants can be derived (32). Primary amine groups of apoE3 and five variants (apoE(Arg^{136} → Ser), apoE(His^{140} → Ala), apoE(Lys^{143} → Ala), apoE(Arg^{150} → Ala), and apoE(Arg^{158} → Cys)) were covalently coupled to research grade CM5 sensor chips (Pharmacia Biosensor) using the amine coupling kit supplied by the manufacturer. The proteins were diluted to 20 µg/ml in 10 mM sodium acetate, pH 4.5, and 40-µl aliquots were injected over the activated chip surface. Unreacted moieties were blocked with ethanolamine. All measurements were carried out in HEPES-buffered saline (HBS) which contained: 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Surfactant P-20 (Pharmacia Biosensor). Analyses were performed at 25°C and the binding of each antibody was tested at seven concentrations. Immobilizations and binding assays were carried out at a flow rate of 3 µl/min. Sensor chip surfaces were regenerated with 100 mM HCl and surface integrity was periodically checked with control antibody.

Association and dissociation rate constants were calculated by non-linear fitting of the sensorgram data (33) using the BIAevaluation 2.0 software (Pharmacia Biosensor). The dissociation rate constant is derived using the equation:

$$R_t = R_{to}\, e^{-koff(t-to)}$$

where R_t is the response at time t, R_{to} is the amplitude of the response, and k_{off} is the dissociation rate constant. The association rate constant can be derived using the equation:

$$R_t = \frac{k_{on}\, C\, R_{max}\, (1-e^{-(k_{on}C+k_{off})t})}{k_{on}\, C+k_{off}}$$

where R_t is the response at time *t*, R_{max} is the maximum response, C is the concentration of ligate in solution, k_{on}

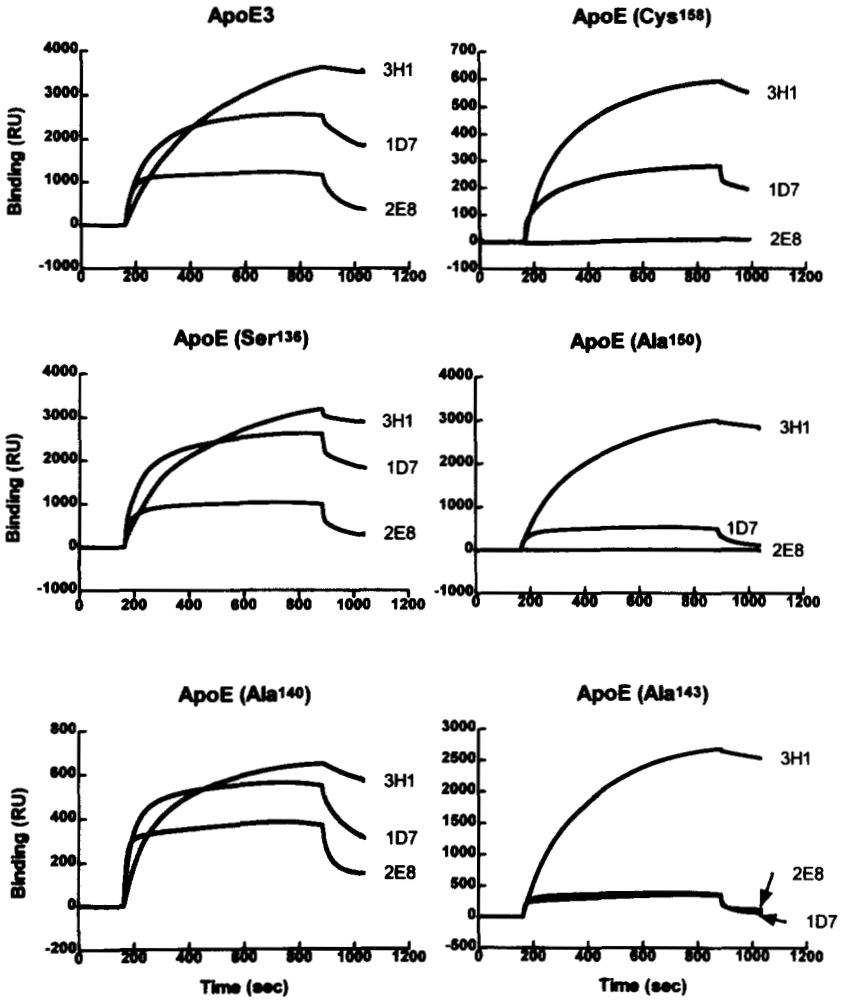

Fig. 4. Sensorgrams of apoE variants with 1D7, 2E8, and 3H1 using SPR. These sensorgrams depict the kinetics of 1D7, 2E8, and 3H1 binding to apoE3, apoE(Arg^{136} → Ser), apoE(His^{140} → Ala), apoE(Lys^{143} → Ala), apoE(Arg^{150} → Ala), and apoE(Arg^{158} → Cys). The association phase of each sensogram begins at approximately 180 sec and the dissociation phase at 850 sec. The results that are illustrated were obtained with concentrations of 150 nM, 150 nM, and 50 nM for 1D7, 2E8, and 3H1, respectively.

is the association rate constant, and k_{off} is the dissociation rate constant.

Molecular cloning and characterization of 1D7 and 2E8 heavy and light chain cDNAs

Total RNA from 1D7 and 2E8 hybridoma cells was extracted according to the acid-guanidinium thiocyanate phenol chloroform method (34) and poly(A+) RNA was purified by oligo-dT affinity chromatography. Northern blot analysis was performed to ensure the presence of light and heavy chain immunoglobulins (35). The constant region of a mouse kappa light chain (36) and the first constant region of an IgG1a heavy chain (37) were used as molecular probes for hybridization. A 1D7 cDNA library was created by using 5 μg of poly(A+) mRNA as template for reverse transcription with an oligo(dT) primer. Following second strand synthesis, Not1/EcoR1 adaptors (Pharmacia) were added and the products were cloned into pUC18. Recombinant plasmids were transformed into DH5α competent bacteria by heat shock treatment. Colonies were screened by colony lift hybridization using the same radiolabeled IgG1a and kappa constant region probes that were used for northern analysis (38).

The cDNA encoding the 2E8 light chain was amplified by the polymerase chain reaction (PCR) (39), after reverse transcription of hybridoma mRNA, using one primer complementary to the 3′ end of the kappa translated sequence (5′-GCG CCG TCT AGA ATT AAC ACT CAT TCC TGT TGA A-3′), and the other corresponding to the nucleotides encoding the amino terminal residues of the mature light chain (5′-CCA GGT CCG AGC TCG

TABLE 1. Association (k_{on}) and dissociation (k_{off}) rate constants of mAbs 3H1, 1D7, and 2E8 with apoE variants as determined by surface plasmon resonance

ApoE Variant		Antibody 3H1	1D7	2E8
ApoE3	k_{on}	$1.3 \times 10^5 \pm 2.6 \times 10^3$	$1.2 \times 10^5 \pm 8.2 \times 10^3$	$3.0 \times 10^5 \pm 1.7 \times 10^4$
	k_{of}	$3.1 \times 10^{-4} \pm 1.5 \times 10^{-5}$	$2.2 \times 10^{-3} \pm 1.3 \times 10^{-4}$	$9.2 \times 10^{-3} \pm 1.5 \times 10^{-3}$
	K_d	2.4 nM	19 nM	25 nM
ApoE(Arg^{136} → Ser)	k_{on}	$1.3 \times 10^5 \pm 9.3 \times 10^3$	$1.0 \times 10^5 \pm 6.8 \times 10^3$	$1.2 \times 10^5 \pm 4.7 \times 10^3$
	k_{off}	$3.6 \times 10^{-4} \pm 1.8 \times 10^{-5}$	$1.8 \times 10^{-3} \pm 7.0 \times 10^{-5}$	$8.3 \times 10^{-3} \pm 3.2 \times 10^{-4}$
	K_d	2.9 nM	18 nM	69 nM
ApoE(His^{140} → Ala)	k_{on}	$2.1 \times 10^5 \pm 9.5 \times 10^3$	$2.0 \times 10^5 \pm 4.8 \times 10^3$	$6.6 \times 10^5 \pm 6.5 \times 10^4$
	k_{off}	$1.0 \times 10^{-3} \pm 8.0 \times 10^{-5}$	$4.9 \times 10^{-3} \pm 2.1 \times 10^{-4}$	$1.2 \times 10^{-2} \pm 4.4 \times 10^{-4}$
	K_d	5.0 nM	24 nM	18 nM
ApoE(Lys^{143} → Ala)	k_{on}	$1.3 \times 10^5 \pm 7.2 \times 10^3$	$2.1 \times 10^5 \pm 1.3 \times 10^4$	< 1000
	k_{off}	$3.9 \times 10^{-4} \pm 2.2 \times 10^{-5}$	$1.5 \times 10^{-2} \pm 3.6 \times 10^{-4}$	$3.7 \times 10^{-3} \pm 2.0 \times 10^{-4}$
	K_d	2.9 nM	73 nM	N.D.
ApoE(Arg^{150} → Ala)	k_{on}	$7 \times 10^5 \pm 5.3 \times 10^3$	1.;tb2.0 $\times 10^5 \pm 1.8 \times 10^4$	N.D.
	k_{off}	$3.1 \times 10^{-4} \pm 1.1 \times 10^{-5}$	$1.2 \times 10^{-2} \pm 5.2 \times 10^{-4}$	$7.7 \times 10^{-4} \pm 6.2 \times 10^{-5}$
	K_d	1.8 nM	60 nM	N.D.
ApoE(Arg^{158} → Cys)	k_{on}	$1.7 \times 10^5 \pm 5.4 \times 10^3$	$6.9 \times 10^4 \pm 4.9 \times 10^3$	N.D.
	k_{off}	$9.5 \times 10^{-4} \pm 6.5 \times 10^{-5}$	$1.9 \times 10^{-3} \pm 1.1 \times 10^{-4}$	N.D.
	K_d	5.4 nM	28 nM	N.D.

The values k_{on} and k_{off} represent the means calculated from determinations at seven different antibody concentrations; N.D., association and/or dissociation rate constants could not be calculated.

TGA TGA CCC AGT CTC CA-3′). Both the 5′ and 3′ region primers were based on previously compiled sequences (40) and contain, respectively, Sac1 and Xba1 restriction sites indicated in bold, which facilitate subsequent cloning of PCR amplification products for eventual expression. After amplification, the material was gel purified and a 700 base-pair fragment corresponding to two immunoglobulin domains was extracted using Gene Clean (Bio 101, La Jolla, CA) and subcloned, after Klenow and kinase treatment, into p-Bluescript(KS+) (Stratagene, San Diego, CA) digested with SmaI (38). After reverse transcription of total RNA that was isolated from the 2E8 hybridoma, the 2E8 heavy chain cDNA was amplified by anchored PCR (41). Terminal deoxynucleotidyl transferase was used to add a 5′ overhang consisting of guanosine nucleotides to a first strand cDNA template. A first round of amplification using an oligo(dC) and a 3′ primer complementary to codons 224–232 located in the hinge region (5′-AGG CTT ACT AGT ACA ATC CCT GGG CAC A-3′) and which contains an Spe1 site indicated in bold, was carried out and the products were separated on a low melting point agarose gel (FMC Bioproducts, Rockland, ME). The gel containing fragments with molecular weights ranging from 300 to 800 base pairs was excised and the products were used directly as template for a second round of PCR using the oligo dC and an internal 3′ constant region primer (5′-GGC AGC AGA TCC AGG GGC-3′) corresponding to codons 125–130. This second amplification gave a specific 500 base pair product that was isolated and sub-cloned into p-Bluescript. All the cloned cDNAs were sequenced using the dideoxy chain termination method (42).

Amino acid sequence sequence analysis and cleavage with CNBr at methionine residues

Protein and peptide samples for sequencing were purified by SDS-PAGE and electroblotted onto sequencer stable PVDF membrane as described previously (43). Automated gas-phase sequencing was performed on an Applied Biosystems 475A protein sequencing system (Foster City, CA) incorporating a model 470A gas-phase sequencer equipped with an on-line model 120A PTH analysis module. Cleavage of peptide bonds adjacent to methionine residues was effected by using CNBr. Freeze-dried protein was dissolved in 500 μl of 88% (v/v) formic acid followed by the addition of solid CNBr. The reaction vial was flushed with argon, sealed and incubated in the dark at room temperature for 24 h. The reagent and solvent were removed under a stream of argon and the remaining material was suspended in Milli-Q water and freeze dried.

RESULTS

Fine specificity of mAb 1D7

We have previously shown that mAb 1D7 reacts with a cyanogen bromide fragment of apoE composed of residues 126–218 and with a synthetic peptide that includes apoE residues 139–169 (17). To more precisely map the 1D7 epitope, we have taken advantage of a

panel of recombinant apoE variants produced by site-directed mutagenesis and synthesized in a bacterial expression system (18). For these studies, a solid phase apoE sandwich immunometric assay was developed. The mAb 6C5 was used as the anchor and bound apoE was detected with either ^{125}I-labeled 1D7 or ^{125}I-labeled 3H1 (**Fig. 1**). Elsewhere, evidence has been presented that the epitopes for anti-apoE mAbs 6C5 and 3H1 are entirely or partially situated between residues 1–13 and 243–272, respectively (24). Antibodies 6C5 and 3H1 do not compete with each other for binding to immobilized apoE nor do they compete with 2E8 and 1D7 (R. Milne and R. Raffai, unpublished results). As the mutagenesis was confined to the receptor-binding region of apoE, the introduced amino acid substitutions should not influence binding of the variants to either 6C5 or to ^{125}I-labeled 3H1. Recombinant apoE3 is somewhat less immunoreactive with both 1D7 and with 3H1 than is purified plasma apoE3 (Fig. 1). This could be attributable to some protein denaturation or a decreased binding of recombinant apoE to 6C5 due to the presence of an amino-terminal methionine that is not present in mature plasma apoE. The differences in immunoreactivity observed between native and recombinant apoE3 are, however, minor in comparison with the decreases in 1D7 immunoreactivity that resulted from amino acid substitutions in apoE. Amino acid substitutions at residues 143, 144, 150, or 152 almost totally eliminated binding of 1D7 and, as reported previously, the natural apoE variant apoE(Arg^{145} → Cys) showed reduced 1D7 immunoreactivity. In contrast, normal 1D7 reactivity was seen with apoE(Arg^{136} → Ser) and apoE(Arg^{158} → Cys) variants. ApoE(His^{140} → Ala) showed a large decrease in 1D7 binding compared to apoE3 (Fig. 1); however, when normalized for 3H1 binding (**Fig. 2**), this difference was lost. While it is possible that a substitution at residue 140 could modulate both the 1D7 and 3H1 epitopes, it is more probable that there is reduced binding to the anchor mAb, 6C5, perhaps as a result of denaturation or amino-terminal proteolysis of the molecule. While lipid-free apoE is not a ligand for the LDLr, binding activity is restored when the apoE is incorpo-

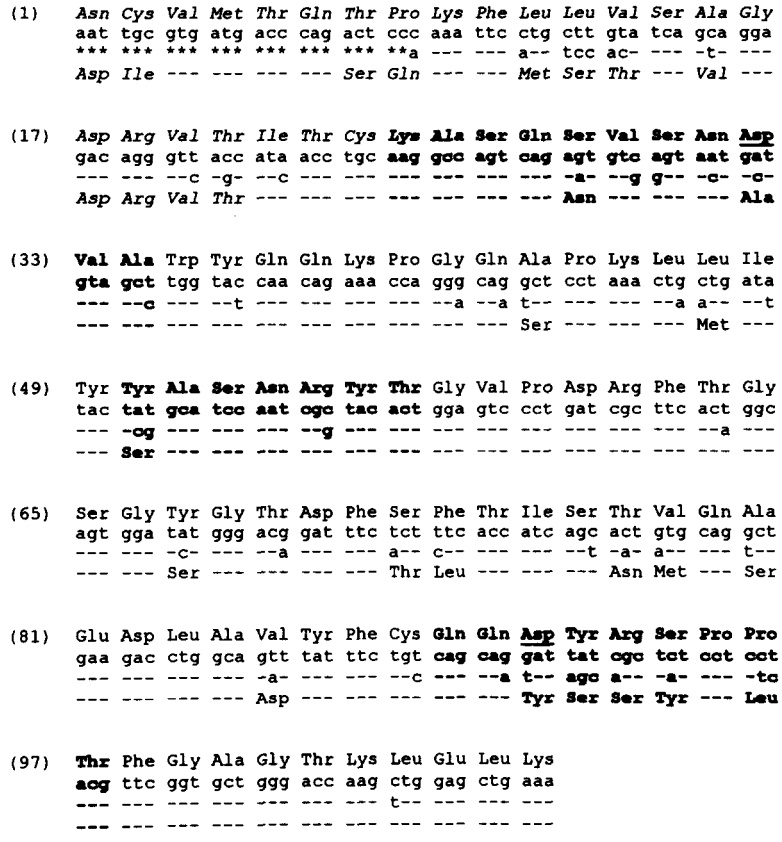

Fig. 5. Nucleotide and deduced protein sequences of 1D7 and 2E8 light chains. The nucleotide and deduced protein sequences of the variable domains of 1D7 (upper) and 2E8 (lower) light chains are presented. CDRs are indicated in bold print and negatively charged residues within the CDRs are underlined. Amino acid residues represented in italics were confirmed by protein sequence determination. The asterisks indicate the nucleotides within the degenerate PCR primer that were used for cloning the 2E8 light chain cDNA. The corresponding amino acids were determined by direct protein sequencing. Codons are numbered and CDRs are identified according to Wu and Kabat (47).

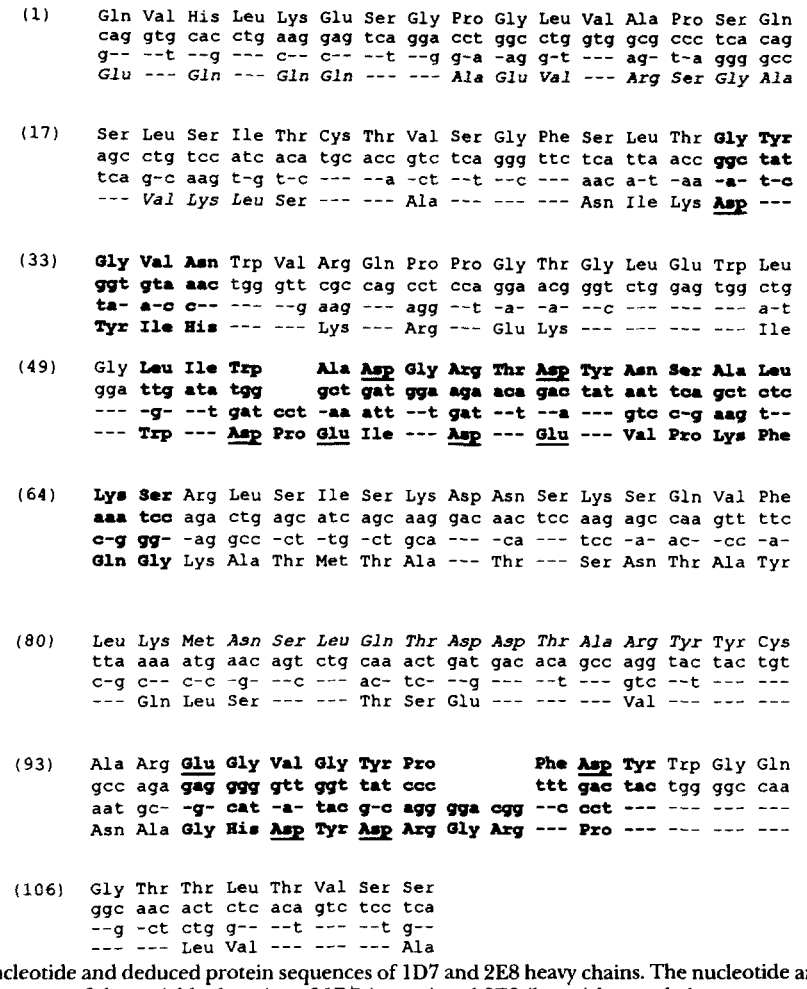

Fig. 6. Nucleotide and deduced protein sequences of 1D7 and 2E8 heavy chains. The nucleotide and deduced protein sequences of the variable domains of 1D7 (upper) and 2E8 (lower) heavy chains are presented. CDRs are indicated in bold print and negatively charged residues within the CDRs are underlined. Amino acid residues represented in italics were confirmed by protein sequence determination. Codons are numbered and CDRs are identified according to Wu and Kabat (47).

rated into lipid vesicles (44). As it has been suggested that binding to lipid may induce a conformational change in apoE, we compared the 1D7 immunoreactivity of the apoE variants in a lipid-free and a lipid-bound form. However, as shown in Fig. 2, incorporation of the apoE into DMPC vesicles did not significantly change the observed fine specificity of 1D7.

Fine specificity mAb 2E8

A second anti-human apoE mAb, 2E8, has been identified that blocks binding of apoE to the LDLr (T. Innerarity and K. Weisgraber, unpublished results). While the affinity of 2E8 for apoE is lower than that of 1D7, it can compete with 1D7 for binding to immobilized apoE and shows a pattern of reactivity similar to that of 1D7 with apoE proteolytic fragments (T. Innerarity and K. Weisgraber, unpublished results). We have used the apoE variants to determine the fine specificity of 2E8 as was done for 1D7. The results in **Fig. 3** confirm the lower affinity of 2E8 for apoE compared to 1D7 but also demonstrate that, in general, the two antibodies have similar specificity with respect to the apoE variants. One notable difference, however, is that 2E8, unlike 1D7, shows decreased reactivity with apoE2(Arg^{158} → Cys). Antibody 2E8 does not discriminate between lipid-bound and free apoE nor does incorporation of apoE into lipid vesicles change its relative reactivities with apoE3 and apoE2(Arg^{158} → Cys) (results not shown).

Surface plasmon resonance

In order to confirm the binding specificities of 1D7 and 2E8 that were determined by sandwich RIA, and to establish association and dissociation rate constants for the antibodies with the different mutants, we have stud-

ied their interactions using surface plasmon resonance (SPR). ApoE3 and the five variants, apoE(Arg^{136} → Ser), apoE(His^{140} → Ala), apoE(Lys^{143} → Ala), apoE(Arg^{150} → Ala), and apoE(Arg^{158} → Cys) were immobilized and were subjected to a pulse of antibody. Sensorgrams depicting the binding of the antibodies to the apoE variants as a function of time are shown in **Fig. 4.** The control antibody 3H1 reacted well with all of the variants including apoE(His^{140} → Ala), indicating that the reduced reactivity observed by RIA may well have been due to a reduced capture of this mutant by the anchor antibody 6C5. The binding of 1D7 to the variants revealed the same fine specificity as that determined using the sandwich RIA. Again 1D7 had reduced reactivity with apoE(Lys^{143} → Ala) and apoE(Arg^{150} → Ala). In the case of 2E8, the results obtained using SPR are also consistent with those obtained using the sandwich RIA. Notably, the reduced of reactivity of 2E8 with apoE(Lys^{143} → Ala), apoE(Arg^{150} → Ala), and apoE(Arg^{158} → Cys) have been confirmed.

This methodology has also allowed us to determine the association and dissociation rate constants as well as the affinity of the antibodies for the individual apoE variants (**Table 1**). From Table 1, it is apparent that the lower affinity of 2E8 compared to 1D7 and 3H1 for apoE3 is the result of a high dissociation rate constant. Moreover, the reduction of affinity of 1D7 and 2E8 for individual apoE variants can reflect changes in the association and/or the dissociation rate constants. For example, the reduced affinities of 1D7 for apoE(Lys^{143} → Ala) and apoE(Arg^{150} → Ala), compared to that for apoE3, are largely due to an increased dissociation rate constant whereas the reduced affinity of 2E8 for apoE(Lys^{143} → Ala) reflects a reduced k_{on}. Reactivity of 2E8 with certain of the variants was too low to allow calculation of rate constants and affinities. As expected, the binding kinetics of 3H1 were similar with all of the apoE variants.

LDL receptor

195 210
Cys Asp Gly Gly Pro Asp Cys Lys Asp Lys Ser Asp Glu Glu Asn Cys

Trp Ile Asp Pro Glu Ile Gly Asp Thr Glu Tyr Val
50 60

2E8 heavy chain CDR2

Fig. 7. Comparison of the putative ligand binding sites of the LDLr and the heavy chain CDR2 of 2E8. Residue numbers 195 to 210 (cysteine-rich repeat number 5) of the human LDLr (upper) are compared to those present in the heavy chain CDR2 of mAb 2E8. Negatively charged residues are underlined.

Molecular cloning and characterization of the heavy and light chain cDNA of mAbs 1D7 and 2E8

The cDNAs encoding the entire light and the Fd regions (variable and first constant domains of the heavy chains) of mAbs 1D7 and 2E8 have been cloned. A series of cDNA light chain clones were identified that had an identical nucleotide sequence and an intact reading frame (**Fig. 5**). In the case of the 1D7 heavy chain, a single message was cloned from the cDNA library and corresponded to the expected protein (**Fig. 6**). This was also the case for the 2E8 light (Fig. 5) and heavy chains (Fig. 6) that were cloned using PCR. Partial protein sequences were obtained from the variable domains of each of the antibodies and, in each case, the amino acid sequence corresponded to the amino acid sequence deduced from the nucleotide sequence of the respective cDNA clones. In the process of cloning the 1D7 light chain from a cDNA library, two non-functional light chains were identified. Both had deleterious frame shifts within the nucleotides encoding the variable region and would represent products of non-productive kappa gene rearrangements (45).

Comparison of the variable region sequences of the two mAbs with those in the database of Kabat et al. (46) indicated that the rearranged 1D7 heavy chain is a member of the mouse heavy chain variable region subgroup I(B) and that codons 103–117 were derived from the mouse heavy chain J-minigene, MUSJH2 (47). Its D-minigene could not be identified. The 2E8 heavy chain gene is a member of the mouse heavy chain variable region subgroup II(C) and includes sequences derived from the DSP2.2R + 2 D-minigene (codons 99 to 103) (47) and from the MUSJH3 J-minigene (codons 106–120) (47). Sequencing of the nucleotides encoding the C_{H1} showed that both heavy chains were of the γ1 subclass, thus confirming serological identification. The 1D7 and 2E8 light chains are both members of the mouse kappa variable subgroup V, and both contain sequences derived from the MUSJK5 kappa J-minigene (47).

As the two anti-apoE mAbs have similar fine specificities, it may be expected that they share homology in the six hypervariable or complementarity determining regions (CDRs). Amino acids that constitute the three CDRs of the light chain and the three CDRs of the heavy chain interact directly with the epitope and are responsible for antibody specificity. Comparison of the primary structure of the light chains of 1D7 and 2E8 shows considerable homology for the CDR1 and CDR2 with most of the differences occurring in CDR3. The 1D7 light chain contains two negatively charged residues in CDR1 and CDR2, respectively, whereas the 2E8 light chain has no negatively charged residues within its CDRs. There is little homology between the heavy chain

CDRs of the two mAbs, and CDR2 and CDR3 of 2E8 contain one and two additional amino acids, respectively, as compared to the corresponding CDRs of 1D7. The heavy chain CDRs of 1D7 include four acidic amino acids whereas those of the 2E8 heavy chain contain seven acidic residues.

DISCUSSION

We present evidence that the 1D7 and 2E8 epitopes coincide with the LDLr-binding site on apoE that is situated between residues 136 and 150. We cannot, however, exclude the possibility that the epitopes of 1D7 and 2E8 are located elsewhere on the molecule and may be disrupted by changes in conformation due to the amino acid substitutions that characterize the different apoE variants that we have tested. Nevertheless, it should be noted that a recent study that evaluated site-directed mutagenesis as a method for epitope mapping revealed that mutations which caused a greater than 10-fold change in apparent antibody binding affinity (using methods analogous to those presented in Fig. 2) were correctly assigned to the epitope when confirmed by X-ray crystallography (48). From results presented here and elsewhere (16, 49), it is probably that Arg^{142}, Lys^{143}, Arg^{145}, Lys^{146}, and Arg^{150} form part of the 1D7 and 2E8 epitopes. Furthermore, the lack of reactivity of apoE(Leu^{144} → Pro) and apoE(Ala^{152} → Pro) may indicate that either Leu^{144} and Ala^{152} are also directly implicated in the epitopes or that a local conformational change induced by the introduction of a proline at these positions eliminates mAb binding. The relatively moderate decrease in reactivity of the apoE(Arg^{145} → Cys) variant would be consistent with the Arg^{145} side chain making only a minor contribution to the binding energy of the mAb-apoE complex in the case of both mAbs. While the fine specificity of 2E8 is similar to that of 1D7, it is nevertheless notable that 2E8 and 1D7 differ in their relative affinities for apoE2(Arg^{158} → Cys) and apoE3, with 2E8 having little affinity for apoE2(Arg^{158} → Cys) (Figs. 1 and 3 and Table 1). Our results, therefore, indicate that the epitopes for 1D7 and 2E8 overlap with each other and with the LDLr binding site on apoE.

Incorporation of apoE into lipid vesicles does not change its immunoreactivity with either of the mAbs. It has been proposed that, in the presence of lipid, the four-helix bundle of the amino terminal domain of apoE opens up to generate the receptor-active conformation (50). While this would represent a major alteration in tertiary structure, the changes in the secondary structure of the individual α-helices may be minor. The reactivity of 1D7 with apoE synthetic peptides as short as 30 amino acids (17) would indicate that its epitope

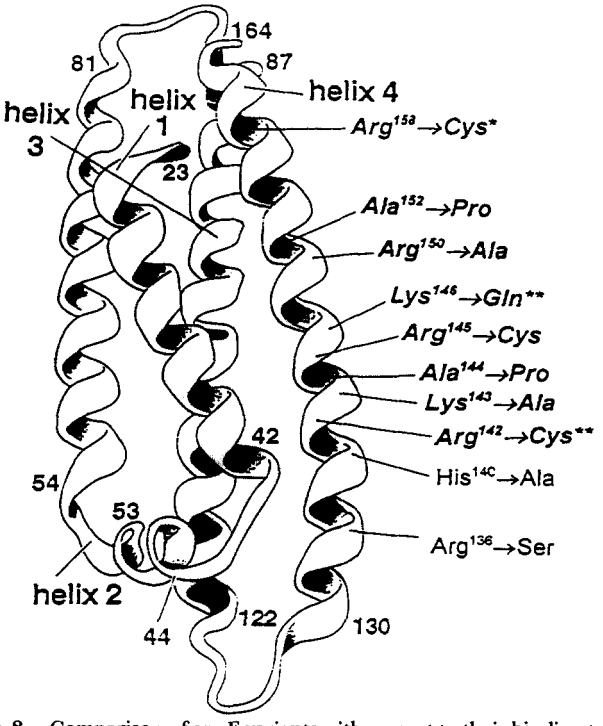

Fig. 8. Comparison of apoE variants with respect to their binding to mAbs 1D7 and 2E8 and to the LDLr. The positions of amino acid substitutions that have been shown to decrease affinity of apoE for the LDLr are indicated with respect to their position in the four-helix bundle that constitutes the amino terminal 22-kDa fragment of apoE. Those variants that also have reduced reactivity with mAbs 1D7 and/or 2E8 are represented in bold italics. Of these, apoE(Arg^{158} → Cys) (*) showed reduced reactivity with 2E8 but not with 1D7. ApoE(Arg^{142} → Cys) and apoE(Lys^{146} → Gln) (**) showed reduced reactivity with 1D7 but were not tested with 2E8.

does not require elaborate tertiary structure and is not composed of disparate regions of apoE primary structure. In the case of 1D7, the epitope may thus be continuous or, as we have suggested earlier (17), composed of one face of the α-helix that constitutes the secondary structure of this region.

It has been determined that the ligand-binding domain of the LDLr is situated at the amino-terminus and is made up of seven imperfect repeats of a 40-amino acid cysteine-rich sequence that is characterized by a conserved cluster of acidic amino acids in the carboxy-terminal portion of the repeat (51). Repeats 3 through 7 appear to contribute to apoB-mediated binding of LDL, whereas only repeat 5 appears to be critical for apoE-mediated binding of β-VLDL (52). Binding of apoE and apoB-100 to the LDLr is thought to involve ionic interactions between basic amino acids of the apolipoproteins and acidic residues of the receptor (53, 54). Chemical modifications of arginine and lysine residues in apoB-100 and apoE (13, 54) and substitution of neutral amino

acids for basic residues in the putative receptor-binding region of apoE reduce affinity of the apolipoproteins for the LDLr. Reductive methylation of lysine residues in apoE and apoB, which maintains the positive charge of the modified residues, also eliminates binding to the LDLr. This would suggest that not only the positive charge but also the structure of the basic residues contribute to the recognition of the ligands by the receptor. This is further supported by the observation that cysteamine treatment of apoE(Arg^{112}, Cys^{142}) restores a positive charge at residue 142 without rendering the molecule capable of binding to the LDLr (49). Moreover, the acid-mediated dissociation between the receptor and β-VLDL is not dependent on a simple titration of charged residues within the ligand binding domain (55).

While basic residues located within the LDLr binding site of apoE contribute to the epitopes of both mAbs 1D7 and 2E8, the lack of similarity between the primary structures of the respective CDRs of the two mAbs indicates that they recognize a similar region on apoE differently. It has been observed that oriented dipoles rather than counter charges are preferentially used in stabilizing charged residues in the formation of antigen-antibody complexes (56). Nevertheless, both mAbs do contain acidic residues within their respective CDRs that could potentially contribute to the binding energy of the antibody-antigen complex through the formation of ionic bonds with basic residues of apoE in a manner similar to that which has been proposed for the interaction between apoE and the LDLr. The two mAbs differ in the distribution of these negatively charged amino acids amongst their respective CDRs. In the case of 1D7, the six acidic residues are distributed in the CDRs of both the heavy and light chains whereas, in 2E8, the seven acidic residues are restricted to the heavy chain CDRs. Assuming that aspartic and glutamic residues of the two antibodies do make a major contribution to the binding energy of their respective immune complexes, this could indicate that 1D7 makes use of both chains for binding apoE, whereas, in 2E8, it is primarily the heavy chain that is responsible for antigen binding. It should, however, be emphasized that the surface of contact between antigen and antibody is large, and hydrogen bonds, van der Waals forces, as well as electrostatic interactions likely contribute to the binding energy.

ApoB, the major protein of LDL, can compete with apoE for binding to the LDLr. Comparison of the primary structures of apoE and apoB has revealed a short sequence of apoB composed of residues 3359 through 3367 that resembles residues 140–150 of apoE with respect to the relative positions of basic amino acids (57). It has been proposed that this characteristic distribution of basic residues could indicate that both apolipoproteins may be forming similar ionic interactions in their association with the ligand binding site of the LDLr. We have made an analogous comparison of the primary structures of the ligand-binding repeats of the LDLr and the CDRs of mAbs 1D7 and 2E8. Of the seven acidic residues in the heavy chain CDRs of 2E8, four are clustered in CDR2. The spacing of the four aspartic and glutamic residues within CDR2 of the 2E8 heavy chain bears some resemblance to that of the acidic residues in the ligand-binding repeats of the LDLr (**Fig. 7**). Therefore, the CDR2 of the 2E8 heavy chain may mimic the postulated ionic interaction between the consensus sequence repeats of the LDLr and apoE. While the overall architecture of an antibody and that of the LDL receptor are clearly different, it is interesting to note that an anti-idiotypic mAb prepared against a neutralizing anti-reovirus hemagglutinin mAb recognized the mammalian cell surface reovirus receptor and, in this case, the molecular mimicry resulted from homology in primary structure between the CDRs of the anti-idiotypic mAb and the viral hemagglutinin (58). In addition, it has been shown that certain anti-integrin antibodies that bind to the ligand binding site of platelet integrin $\alpha_{II}\beta_3$ contain the "Arg-Gly-Asp" integrin recognition motif in their antigen binding sites (59). Moreover, the activity of one such inhibitory antibody could be emulated by synthetic peptides whose synthesis was based on the primary structure of the heavy chain CDR3 of the mAb.

It is interesting to compare the structural requirements for apoE-mediated binding to the LDLr with those for 1D7 and 2E8 immunoreactivity (**Fig. 8**). Substitutions that result in the loss of a positive charge at residues 142, 143, 145, 146, or 150 produce a decrease in the ability of the variant to bind to both antibodies and to the receptor (17, 18, 49, 60, 61). Similarly, the introduction of proline residues at positions 144 and 152 decreases both antibody and receptor binding by either the replacement of a critical residue for binding or by disruption of apoE secondary or tertiary structure (18). In contrast, several of the variants (e.g., apoE(Arg^{136} → Ser) and apoE(Arg^{158} → Cys)) are defective with respect to LDLr binding but bind to 1D7 with high affinity (18, 60). The binding of antibody 2E8 to apoE(Arg^{158} → Cys), on the other hand, is severely impaired (Fig. 3, Table 1). It is thought that Arg^{158} is not directly implicated in binding to the LDLr but its replacement by a Cys in apoE(Arg^{158} → Cys) induces a conformational change in the receptor binding domain, probably situated between residues 136 and 150 (31). In the crystal structure of the amino terminal domain of apoE3, Arg^{158} forms salt bridges with Glu^{96} and Asp^{154} and may help to stabilize the pairing of helices 3 and 4 (19). In apoE(Arg^{158} → Cys), Arg^{150} forms a salt bridge

with Asp^{154} (62). The putative conformational change responsible for the decrease in binding affinity of apoE($Asp^{158} \rightarrow$ Cys) for the LDLr may result from these reorganized salt bridges. LDLr-binding and 1D7 immunoreactivity can also be distinguished by other criteria. Rat and mouse apoE bind with high affinity to the human LDLr but have little 1D7 immunoreactivity. Finally, as discussed above, only lipid-bound apoE is recognized by the LDLr (44) whereas neither mAb differentiates between free and lipid-bound apoE. Thus, while the apoE LDLr-binding domain and the 1D7 and 2E8 epitopes may coincide or overlap, the respective conformational elements that are recognized by the antibodies and the receptor are not identical.

It appears that apoE can be present in two forms of triglyceride-rich lipoproteins, one of which is resistant to cleavage by proteases and is not recognized by the LDLr and a second that is protease-sensitive and capable of binding to the LDLr (25). ApoE may be converted from the first to the second form by either change in conformation or in accessibility that is induced by lipolysis of the particle (23). As it has been proposed that 1D7 may distinguish between these two forms (26), it may be a useful probe of the LDLr-binding site of apoE. Here we have identified a number of apoE residues that are critical for binding of the 1D7 and 2E8 mAbs and have demonstrated that the epitopes for both mAbs overlap with the LDLr-binding site on apoE. How each of these residues contributes to the antibody-antigen interaction should become more apparent once the three-dimensional crystal structures of apoE mAb complexes have been solved. By mutagenesis of the two anti-apoE mAbs, we are now attempting to identify residues within the CDRs that are important in antigen binding and to produce variants that even more closely resemble the LDLr in their interactions with apoE. These antibodies will be used to determine the physical and chemical properties of lipoproteins that modulate the conformation of the apoE LDL receptor binding site. In addition, they will be used as immunogens in order to produce anti-idiotypic mAbs that will cross react with the LDLr.

We thank Mr. Marc Desforges, Ms. Bingyi Han, Mr. Christophe Marcel, and Ms. Thanh Dung Nguyen for excellent technical assistance and Drs. Benoit Barbeau, David Gould, Dan Sparks, and Zemin Yao, and Mr. Xingyu Wang for helpful discussions and advice. This investigation was supported by grants from the Heart and Stroke Foundation of Ontario and the Foundation des Maladies du Coeur du Québec. Ross Milne is a Scientist of the Medical Research Council of Canada.

Manuscript received 14 December 1994 and in revised form 31 May 1995.

REFERENCES

1. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* **240:** 622–630.
2. Davignon, J., R. E. Gregg, and C. F. Sing. 1988. Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis.* **8:** 1–21.
3. Brenninkmeijer, B. J., P. M. J. Stuyt, P. N. M. Demacker, A. F. H. Stalenhoef, and A. van't Laar. 1987. Catabolism of chylomicron remnants in normolipidemic subjects in relation to the apoprotein E phenotype. *J. Lipid Res.* **28:** 361–370.
4. Weintraub, M. S., S. Eisenberg, and J. L. Breslow. 1987. Dietary fat clearance in normal subjects is regulated by genetic variation in apolipoprotein E. *J. Clin. Invest.* **80:** 1571–1577.
5. Brown, A. J., and D. C. K. Roberts. 1991. The effect of fasting triacylglyceride concentration and apolipoprotein E polymorphism on postprandial lipemia. *Arterioscler. Thromb.* **11:** 1737–1744.
6. Demant, T., D. Bedford, C. J. Packard, and J. Shepard. 1991. Influence of apolipoprotein E polymorphism on apolipoprotein B-100 metabolism in normolipemic subjects. *J. Clin. Invest.* **88:** 1490–1501.
7. Gregg, R. E., L. A. Zech, E. J. Schaefer, D. Stark, D. Wilson, and H. B. Brewer, Jr. 1986. Abnormal in vivo metabolism of apolipoprotein E_4 in humans. *J. Clin. Invest.* **78:** 815–821.
8. Steinmetz, A., C. Jakobs, S. Motzny, and H. Kaffarnik. 1989. Differential distribution of apolipoprotein E isoforms in human plasma lipoproteins. *Arteriosclerosis.* **9:** 405–411.
9. Weisgraber, K. H. 1990. Apolipoprotein E distribution among human plasma lipoproteins: role of the cysteine–arginine interchange at residue 112. *J. Lipid Res.* **31:** 1503–1511.
10. Strittmatter, W. J., A. M. Saunders, D. Schmechel, M. Pericak-Vance, J. Enghild, G. S. Salvesen, and A. D. Roses. 1993. Apolipoprotein E: high-avidity binding to β-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc. Natl. Acad. Sci. USA.* **90:** 1977–1981.
11. Wetterau, J. R., L. P. Aggerbeck, S. C. Rall, Jr., and K. H. Weisgraber. 1988. Human apolipoprotein E3 in aqueous solution. I. Evidence for two structural domains. *J. Biol. Chem.* **263:** 6240–6248.
12. Aggerbeck, L. P., J. R. Wetterau, K. H. Weisgraber, C-S. C. Wu, and F. T. Lindgren. 1988. Human apolipoprotein E3 in aqueous solution. II. Properties of the amino-and carboxyl-terminal domains. *J. Biol. Chem.* **263:** 6249–6258.
13. Mahley, R. W., T. L. Innerarity, R. E. Pitas, K. H. Weisgraber, J. H. Brown, and E. Gross. 1977. Inhibition of lipoprotein binding to cell surface receptors of fibroblasts following selective modification of arginyl residues in arginine-rich and B apoproteins. *J. Biol. Chem.* **252:** 7279–7287.
14. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1978. Role of the lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. *J. Biol. Chem.* **253:** 9053–9062.
15. Innerarity, T. L., E. J. Friedlander, S. C. Rall, Jr., K. H.

Weisgraber, and R. W. Mahley. 1983. The receptor-binding domain of human apolipoprotein E. Binding of apolipoprotein E fragments. *J. Biol. Chem.* **258:** 12341–12347.
16. Dyer, C. A., and L. K. Curtiss. 1991. A synthetic peptide mimic of plasma apoE that binds the LDL receptor. *J. Biol. Chem.* **266:** 22803–22806.
17. Weisgraber, K. H., T. L. Innerarity, K. J. Harder, R. W. Mahley, R. W. Milne, Y. L. Marcel, and J. T. Sparrow. 1983. The receptor-binding domain of human apolipoprotein E. Monoclonal antibody inhibition of binding. *J. Biol. Chem.* **258:** 12348–12354.
18. Lalazar, A., K. H. Weisgraber, S. C. Rall, H. Giladi, T. I. Innerarity, A. Z. Levanon, J. K. Boyles, B. Amit, M. Gorecki, R. W. Mahley, and T. Vogel. 1988. Site-specific mutagenesis of human apolipoprotein E. Receptor binding activity of variants with single amino acid substitutions. *J. Biol. Chem.* **263:** 3542–3545.
19. Wilson, C., M. R. Wardell, K. H. Weisgraber, R. W. Mahley, and D. A. Agard. 1991. Three-dimensional structure of the LDL receptor-binding domain of human apolipoprotein E. *Science.* **252:** 1817–1822.
20. Milne, R. W., P. Douste-Blazy, Y. L. Marcel, and L. Retegui. 1981. Characterization of monoclonal antibodies against human apolipoprotein E. *J. Clin. Invest.* **68:** 111–117.
21. Hui, D. Y., T. L. Innerarity, R. W. Milne, Y. L. Marcel, and R. W. Mahley. 1984. Binding of chylomicron remnants and β-very low density lipoproteins to hepatic and extrahepatic lipoprotein receptors. A process independent of apolipoprotein B-48. *J. Biol. Chem.* **259:** 15060–15068.
22. Marcel, Y. L., M. Hogue, P. K. Weech, J. Davignon, and R. W. Milne. 1988. Expression of apolipoprotein B epitopes in lipoproteins. Relationship of conformation and function. *Arteriosclerosis.* **8:** 832–844.
23. Sehayek, E., U. Lewin-Velvert, T. Chajek-Shaul, and S. Eisenberg. 1991. Lipolysis exposes unreactive endogenous apolipoprotein E-3 in human and rat plasma very low density lipoprotein E-3. *J. Clin. Invest.* **88:** 553–560.
24. Weisgraber, K. H., S. C. Rall, Jr., R. W. Mahley, R. W. Milne, Y. L. Marcel, and J. T. Sparrow. 1986. Human apolipoprotein E: determination of the heparin binding sites of apolipoprotein E3. *J. Biol. Chem.* **261:** 2068–2076.
25. Bradley, W. A., S-L. C. Hwang, J. B. Karlin, A. H. Y. Lin, S. C. Prasad, A. M. Gotto, and S. H. Gianturco. 1984. Low-density lipoprotein receptor binding determinants switch from apolipoprotein E to apolipoprotein B during conversion of hypertriglyceridemic very-low-density lipoprotein to low-density lipoprotein. *J. Biol. Chem.* **259:** 14728–14735.
26. Sacks, F. M., and G. P. Krukonis. 1987. Subtypes of normolipidemic VLDL, which has the receptor binding region of apolipoprotein E exposed, causes most of the uptake of VLDL by U937 human macrophages. *Arteriosclerosis.* **7:** 544a.
27. Ey, P. L., S. T. Prowse, and C. R. Jenkin. 1978. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulin from mouse serum using protein-A Sepharose. *Immunochemistry.* **15:** 429–436.
28. Milne, R. W., R. Théolis, Jr., R. B. Verdery, and Y. L. Marcel. 1983. Characterization of monoclonal antibodies against human low density lipoprotein. *Arteriosclerosis.* **3:** 23–30.
29. Weisgraber, K. H., S. C. Rall, Jr., and R. W. Mahley. 1981. Human E apoprotein heterogeneity. Cysteine-arginine interchanges in the amino acid sequence of the apo-E isoforms. *J. Biol. Chem.* **256:** 9077–9083.
30. Vogel, T., K. H. Weisgraber, M. L. Zeevi, H. Ben-Artzi, A. Z. Lavanon, S. C. Rall, Jr., T. L. Innerarity, D. Y. Hui, J. M. Taylor, D. Kanner, Z. Yavin, B. Amit, H. Aviv, M. Gorecki, and R. W. Mahley. 1985. Human apolipoprotein E expression in *Escherichia coli:* structural and functional identity of the bacterially produced protein with plasma apolipoprotein E. *Proc. Natl. Acad. Sci. USA.* **82:** 8696–8700.
31. Innerarity, T. L., K. H. Weisgraber, K. S. Arnold, S. C. Rall, Jr., and R. W. Mahley. 1984. Normalization of receptor binding of apolipoprotein E2. Evidence for modulation of the binding site conformation. *J. Biol. Chem.* **259:** 7261–7267.
32. Jösson, U., L. Fägerstam, B. Ivarsson, B. Johnsson, R. Karlsson, K. Lundh, S. Löfås, B. Persson, H. Roos, I. Rönnberg, S. Sjölander, E. Stenberg, R. Ståhlberg, C. Urbaniczky, H. Östlin, and M. Malmqvist. 1991. Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *Bio Techniques.* **11:** 620–627.
33. O'Shannessy, D. J., M. Brigham-Burke, K. K. Soneson, P. Hensley, and I. Brooks. 1993. Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance: use of nonlinear least squares analysis methods. *Anal. Biochem.* **212:** 457–468.
34. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
35. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* **77:** 5201–5205.
36. Max, E. E., J. V. Maizel, and P. Leder. 1981. The nucleotide sequence of a 5,5-kilobase DNA segment containing the *k* immunoglobulin J and C region genes. *J. Biol. Chem.* **256:** 5116–5120.
37. Honjo, T., M. Obata, Y. Yamawaki, T. Kataoka, T. Kawakami, N. Takahashi, and Y. Mano. 1979. Cloning and complete nucleotide sequence of the mouse immunoglobulin gamma 1 chain gene. *Cell.* **18:** 559–568.
38. Sambrook, J., E. F. Fritch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
39. Mullis, K. B., and F. A. Faloona. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155:** 335–350.
40. Huse, W. D., L. Sastry, S. A. Iverson, A. S. Kang, M. Alting-Mees, D. R. Burton, S. T. Benkovic, and R. A. Lerner. 1989. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science.* **246:** 1275–1281.
41. Loh, E. Y., J. F. Elliot, S. Cwirla, L. L. Lanier, and M. M. Davis. 1989. Polymerase chain reaction with single-sided specificity: analysis of T cell receptor δ chain. *Science.* **243:** 217–220.
42. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74:** 5463–5467.
43. Laycock, M. V., T. Hirama, S. Hasnain, D. Watson, and A. C. Storer. 1989. Purification and characterization of a digestive cystein proteinase from the American lobster (*Homarus americus*). *Biochem. J.* **263:** 439–444.

44. Innerarity, T. L., R. E. Pitas, and R. W. Mahley. 1979. Binding of arginine-rich (E) apoprotein after recombination with phospholipid vesicles to the low density lipoprotein receptors of fibroblasts. *J. Biol. Chem.* **254:** 4186–4190.
45. Walfield, A., E. Selsing, B. Arp, and U. Storb. 1981. Misalignment of V and J gene segments resulting in a nonfunctional immunoglobulin gene. *Nucleic Acids Res.* **9:** 1101–1108.
46. Kabat, E. A., T. T. Wu, M. Reid-Miller, H. M. Perry, and K. S. Gottesman. 1991. *In* Sequences of Immunological Interest. US Public Health Service, National Institutes of Health, Bethesda, MD.
47. Kurosawa, Y., and S. Tonegawa. 1982. Organization, structure, and assembly of immunoglobulin heavy chain diversity DNA segments. *J. Exp. Med.* **155:** 201–218.
48. Prasad, L., S. Sharma, M. Vandonselaar, J. Wilson-Quail, J. S. Lee, E. B. Waygood, K. S. Wilson, Z. Dauter, and L. T. J. Delbaere. 1993. Evaluation of mutagenesis for epitope mapping. Structure of an antibody–protein antigen complex. *J. Biol. Chem.* **268:** 10705–10708.
49. Horie, Y., S. Fazio, J. R. Westerlund, K. H. Weisgraber, and S. C. Rall. 1992. The functional characteristics of a human apolipoprotein E variant (cysteine at residue 142) may explain its association with dominant expression of type-III hyperlipoproteinemia. *J. Biol. Chem.* **267:** 1962–1968.
50. Weisgraber, K. H. 1994. Apolipoprotein E: structure–function relationships. *Adv. Protein Chem.* **45:** 249–302.
51. Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russell, and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* **1:** 1–39.
52. Esser, V., L. E. Limbird, M. S. Brown, J. L. Goldstein, and D. W. Russell. 1988. Mutational analysis of the ligand binding domain of the low density lipoprotein receptor. *J. Biol. Chem.* **263:** 13282–13290.
53. Brown, M. S., T. F. Deuel, S. K. Basu, and J. L. Goldstein. 1978. Inhibition of the binding of low-density lipoprotein to its cell surface receptor in human fibroblasts by positively charged proteins. *J. Supramol. Struct.* **8:** 223–234.
54. Basu, S. K., R. G. W. Anderson, J. L. Goldstein, and M. S. Brown. 1977. Metabolism of cationized lipoproteins by human fibroblasts. *J. Cell Biol.* **74:** 119–135.
55. Davis, G. C., J. L. Goldstein, T. C. Südhof, R. G. W. Anderson, D. W. Russell, and M. S. Brown. 1987. Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region. *Nature.* **326:** 760–765.
56. Mian, I. S., A. R. Bradwell, and A. J. Olson. 1991. Structure, function and properties of antibody binding sites. *J. Mol. Biol.* **217:** 133–151.
57. Knott, T. J., S. C. Rall, T. L. Innerarity, S. F. Jacobson, M. S. Urdea, B. Levy-Wilson, L. M. Powell, R. J. Pease, R. Eddy, H. Nakai, M. Byers, L. M. Priestly, E. Robertson, L. B. Rall, C. Betsholtz, T. B. Shows, R. W. Mahley, and J. Scott. 1985. Human apolipoprotein B: structure of carboxyl-terminal domains, sites of gene expression and chromosomal localization. *Science.* **230:** 37–43.
58. Bruck, C., M. S. Co, M. Slaoui, G. N. Gaulton, T. Smith, B. N. Fields, J. I. Mullins, and M. I. Greene. 1986. Nucleic acid sequence of an internal image-bearing monoclonal anti-idiotype and its comparison to the sequence of the external antigen. *Proc. Natl. Acad. Sci. USA.* **83:** 6578–6582.
59. Smith, J. W., D. Hu, A. Satterthwait, S. Pinz-Sweeney, and C. F. Barbas III. 1994. Building synthetic antibodies as adhesive ligands for integrins. *J. Biol. Chem.* **269:** 32788–32795.
60. Rall, S. C., Jr., K. H. Weisgraber, T. L. Innerarity, and R. W. Mahley. 1982. Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects. *Proc. Natl. Acad. Sci. USA.* **79:** 4696–4700.
61. Rall, S. C., Jr., K. H. Weisgraber, and R. W. Mahley. 1982. Human apolipoprotein E. The complete amino acid sequence. *J. Biol. Chem.* **257:** 4171–4178.
62. Wilson, C., T. Mau, K. H. Weisgraber, M. R. Wardell, R. W. Mahley, and D. A. Agard. 1994. Salt bridge relay triggers defective LDL receptor binding by a mutant apolipoprotein. *Structure.* **2:** 713–718.