The Receptor Binding Domain of Apolipoprotein E, Linked to a Model Class A Amphipathic Helix, Enhances Internalization and Degradation of LDL by Fibroblasts†

Geeta Datta, Manjula Chaddha, David W. Garber, Byung Hong Chung, Ewan M. Tytler, Nassrin Dashti, William A. Bradley, Sandra H. Gianturco, and G. M. Anantharamaiah*

Departments of Medicine, Biochemistry, and Molecular Genetics and Atherosclerosis Research Unit, University of Alabama at Birmingham Medical Center, Birmingham, Alabama 35294

Received May 26, 1999; Revised Manuscript Received September 3, 1999

ABSTRACT: Human apolipoprotein E (apo E) consists of two distinct domains, the lipid-associating domain (residues 192−299) and the globular domain (residues 1−191) which contains the LDL receptor (LDLR) binding site (residues 129−169). To test the hypothesis that an arginine-rich apo E receptor binding domain (residues 141−150) is sufficient to enhance low-density lipoprotein (LDL) uptake and clearance when covalently linked to a class A amphipathic helix, a peptide in which the receptor binding domain of human apo E, LRKLRKRLLR (hApoE[141−150]), is linked to 18A, a well-characterized high-affinity lipid-associating peptide (DWLKAFYDKVAEKLKEAF), we synthesized the peptide hApoE[141−150]-18A (hE18A) and its end-protected analogue, Ac-hE18A-NH_2. The importance of positively charged residues and the role of the hydrophobic residues in the receptor binding domain were also studied using four analogues. Ac-LRRLRRRLLR-18A-NH_2 [Ac-hE(R)18A-NH_2] and Ac-LRKMRKRLMR-18A-NH_2 (Ac-mE18A-NH_2) contained an extended hydrophobic face, including the receptor binding region. Control peptides, Ac-LRLLRKLKRR-18A-NH_2 [Ac-hE(Sc)18A-NH_2], had the amino acid residues of the apo E receptor binding domain scrambled to disrupt the extended hydrophobic face, and Ac-RRRRRRRRRR-18A-NH_2 (Ac-R_{10}18A-NH_2) had only positively charged Arg residues as the receptor binding domain. The effect of the dual-domain peptides on the uptake and degradation of human LDL by fibroblasts was determined in murine embryonic fibroblasts (MEF1). LDL internalization was enhanced 3-, 5-, and 7-fold by Ac-mE18A-NH_2, Ac-hE18A-NH_2, and Ac-hE(R)18A-NH_2, respectively, whereas the control peptides had no significant biological activity. All three active peptides increased the level of degradation of LDL by 100%. The LDL binding and internalization to MEF1 cells in the presence of these peptides was not saturable over the LDL concentration range that was studied (1−10 μg/mL). Furthermore, a similar enhancement of LDL internalization was observed independent of the presence of the LDL receptor-related protein (LRP), LDLR, or both. Pretreatment of cells with heparinase and heparitinase abolished more than 80% of the enhanced peptide-mediated LDL uptake and degradation by cells. We conclude that the dual-domain peptides enhanced LDL uptake and degradation by fibroblasts via a heparan sulfate proteoglycan (HSPG)-mediated pathway.

Apolipoprotein E (apo E)[1] plays an important role in the metabolism of triglyceride-rich lipoproteins, such as very low-density lipoprotein (VLDL), chylomicrons, and chylomicron remnants (*1*). It mediates the high-affinity binding of apo E-containing lipoproteins to the low-density lipoprotein (LDL) receptor and members of its gene family, including LDL receptor-related protein (LRP), very low-density lipoprotein receptor (VLDLR), and the apo E2 receptor (apo E2R) (*2*). The putative and complex role of apo E in atherosclerosis has been emphasized by several observations. (i) Mice that overexpress human apo E have lower levels of total plasma cholesterol (*3*). (ii) Intravenous injection of human apo E into cholesterol-fed rabbits protects these animals from atherosclerosis (*4*). (iii) Loss of the apo E gene in mice produces spontaneous atherosclerosis (*5*, *6*) which is ameliorated when macrophage-specific apo E expression is reconstituted in apo E-deficient mice (*7*).

Apo E is a 299-amino acid residue protein with a molecular weight of 34 200. Thrombin cleavage studies with apo E indicated a two-domain structure which explained the observation that the C-terminal domain of apo E (residues 192−299) is essential for its binding to hypertriglyceridemic

† This work was supported in part by NIH Grants PO3HL30303 and HL44480.

* To whom correspondence should be addressed: Department of Medicine, UAB Medical Center, 1808 7th Ave. S., Birmingham, AL 35294. Phone: (205) 934-1884. Fax: (205) 975-8079. E-mail: ananth@uab.edu.

[1] Abbreviations: Ac, acetyl; apo B, apolipoprotein B; apo E, apolipoprotein E; DMPC, dimyristoylphosphatidylcholine; ICl, iodine monochloride; LDL, low-density lipoproteins; LDLR, low-density lipoprotein receptor; LRP, LDL receptor-related protein; LPDS, lipoprotein-deficient serum; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; PBS, phosphate-buffered saline; VLDL, very low-density lipoproteins; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TGRLP, triglyceride remnant lipoprotein.

10.1021/bi991209w CCC: $19.00
Published on Web 12/11/1999

VLDL and the N-terminal 22 kDa domain (residues 1−191) binds to the LDLR (*8*). Additional physicochemical characterization of the protein and its mutants has extended this concept and has shown that the region of residues 192−299 binds to phospholipid while the amino-terminal domain (residues 1−191) is a globular structure that contains the LDL receptor binding domain in the helix 4 (residues 130−166) region (*9*). Studies with synthetic peptides (*10*) and monoclonal antibodies pinpointed the LDL receptor binding domain of apo E between residues 129 and 169, a domain enriched with positively charged amino acids, Arg and Lys (*11*−*14*).

Studies with synthetic peptides characterized the structural features of the LDL receptor binding domain of apo E (*13*−*15*). Residues 141−155 of apo E, although containing the positively charged residues of the native binding domain, did not compete for binding of LDL in a human skin fibroblast assay, but did so only as tandem covalent repeats [i.e., $(141-155)_2$]. In contrast, when the $[141-155]_2$ peptide was N-terminally acetylated, enhanced LDL interaction with fibroblasts occurred (*16*). The N-acetylated $[141-155]_2$ analogue selectively associated with cholesterol-rich lipoproteins and mediated their acute clearance in vivo (*16*). Moreover, these studies indicated that the prerequisite for receptor binding is that the peptides be helical (*15*). A separate study using synthetic peptides modified to increase the extent of lipid association by *N*,*N*-distearyl derivatization of glycine at the N-terminus of the native sequence (residues 129−169) of apo E also showed enhanced uptake and degradation (*17*). Finally, although LDLR binding is mediated by the cationic sequence of residues 141−155 of human apo E, Braddock et al. (*18*) have demonstrated that model peptides of the highly conserved anionic domain (residues 41−60 of human apo E), while not thought to be involved in the binding of apo E to the LDLR, modulate the binding and internalization of LDL to cell surface receptors. However, these peptides do not enhance LDL degradation.

Most of the peptides described above used some form of the natural apo E receptor binding sequence. To design a peptide with the minimal structural features of apo E necessary for lipoprotein receptor binding, we incorporated the two minimum essential features of apo E suggested from concepts arising from these previous studies: (1) the receptor binding domain (residues 141−150 from the human apo E sequence) at the N-terminus and (2) a strong lipid binding domain at the C-terminus of the synthetic peptide. We hypothesized that since lipid binding is essential for surface localization of the peptide on lipoproteins and for the receptor binding domain of apo E to be appropriately accessible to bind to the LDL receptor, then joining a well-characterized, lipid-associating peptide such as the model class A amphipathic helix, 18A, with the sequence DWLKAFYDKVAEKLKEAF (*19*) to the peptide of residues 141−150 of apo E should be sufficient to confer LDLR binding activity. Thus, we designed the peptide LRKLRKRLLR-18A (hE-18A), in which LRKLRKRLLR is the region of residues 141−150 of human apo E and 18A is a class A amphipathic helical peptide that associates with phospholipids and lipoprotein surfaces (*20*). To characterize the role of individual amino acid residues in the peptides, several additional analogues were prepared. The importance of positively charged residues and the role of the hydrophobic residues in the receptor binding domain were also studied using four analogues. The putative receptor binding domain of apo E, LRKLRKRLLR, is well-conserved in several mammalian species (Table 1). A peptide with the mouse apo E sequence, LRKMRKRLMR-18A (mE-18A), where two conserved Leu residues in hE-18A were changed to Met, was also synthesized. To determine whether the receptor binding is sequence- or charge-specific, we also synthesized and studied the properties of an analogue, LRRLRRRLLR-18A [hE(R)-18A]. We also synthesized N- and C-protected two-domain peptides since this would better reflect the natural state domains and since Ac-18A-NH_2 was previously shown to be more helical with a higher lipid affinity for phospholipids than the unprotected peptide, 18A (*19*). The sequences of these peptides are shown in Table 2. Two additional analogues were designed as control peptides. One had the identical amino acid residues present in the native human apo E receptor binding domain, but scrambled Ac-LRLLRKLKRR-18A-NH_2 [Ac-hE(Sc)18A-NH_2] for disrupting the extended hydrophobic face. The other peptide had positively charged Arg residues in all 10 positions of the N-terminal domain, and thus has a higher positive charge density, like the receptor binding domain Ac-RRRRRRRRRR-18A-NH_2 (Ac-R_{10}18A-NH_2). We compared the properties of these N-

Table 1: Comparison of the Receptor Binding Domain of Apo E in Different Species[a]

Species	Starting Residue no.	Sequence
Human	141	LRKLR**KRLLR**
Rabbit	134	LRKLR**KRLLR**
Monkey	141	LRKLR**KRLLR**
Mouse	133	LRKMR**KRLMR**
Rat	133	LRKMR**KRLMR**
Bovine	140	LRKL***p*KRLLR**
Pig	140	L***R*n**VR**KRL**V**R**
Dog	133	MRKLR**KR**VL**R**

[a] The bold residues are the conserved residues. The underlined residues represent changes (from the human sequence) where the property of the amino acid is conserved. The bold italicized lowercase residues (n being Asn and p being Pro) indicate a substitution where the substituted residue has properties different from those of the residue in the human sequence at that position.

Table 2: Sequence of Dual-Domain Peptides Studied[a]

Lipid Binding Domain	
18A	DWLKAFYDKVAEKLKEAF
Peptides Synthesized	***Sequence***
	141 150
hE-18A	***LRKLRKRLLR***DWLKAFYDKVAEKLKEAF
mE-18A	***LRKMRKRLMR***DWLKAFYDKVAEKLKEAF
hE(R)-18A	***LRRLRRRLLR***DWLKAFYDKVAEKLKEAF
hE(Sc)-18A	***LRLLRKLKRR***DWLKAFYDKVAEKLKEAF
R_{10}-18A	***RRRRRRRRRR***DWLKAFYDKVAEKLKEAF

[a] The bold italic residues represent the human apo E receptor binding domain (corresponding to residues 141−150 in apo E). 18A (DWLKAFYDKVAEKLKEAF) represents the lipid-binding domain of the dual-domain peptides. mE-18A contains the mouse apo E receptor binding domain. hE(R)-18A contains the human apo E receptor binding domain with the Lys residues substituted with Arg residues. hE(Sc)-18A contains the human apo E receptor binding domain with its sequence scrambled. R_{10}-18A has 10 Arg residues in place of the apo E receptor binding domain. The peptides were synthesized with their N-terminus acetylated and the C-terminus amidated.

terminally protected peptides with those of Ac-18A-NH_2 as a control peptide (*20*, *21*). The study presented here describes the physicochemical properties and the effects of these peptides on human LDL binding, internalization, and degradation in mouse embryonic fibroblasts (MEF). Surprisingly, our studies demonstrate that these dual-domain peptides have the unique ability to bypass the LDLR and LRP receptor pathways yet enhance dramatically the rapid uptake of LDL by a cellular pathway that involves cell surface heparan sulfate proteoglycan (HSPG). This peptide-enhanced LDL uptake pathway suggests an alternate pharmacological route for LDL cholesterol lowering, independent of expression of LDLR family members that could be effective in LDLR-deficient subjects.

MATERIALS AND METHODS

Synthesis of the Peptides. Peptides were synthesized by Fmoc chemistry, using the automatic peptide synthesizer from Protein Technology, according to the procedure described by us previously (*22*). The peptide resin was subjected to HF cleavage to ensure the complete removal of the 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) group from Arg residues. For the cleavage of all the peptides, except mE-18A, from the resin, Trp, mercaptoethanol, and dimethyl sulfide were used as scavengers, and for the cleavage of mE-18A, Met, Trp, mercaptoethanol, and dimethyl sulfide were used as scavengers. The cleaved peptides were purified on a preparative C-4 reversed-phase HPLC column, and the purity was determined by C-18 analytical reversed-phase HPLC and confirmed by mass spectral analysis.

Preparation of LDL and Lipoprotein-Deficient Serum (LPDS). Plasma LDL was prepared from human plasma obtained from the Red Cross by sequential centrifugation using density ultracentrifugation (*23*). The LDL (1.006−1.063 g/mL) was washed with 150 mM NaCl by overlayering and recentrifuging and dialyzed exhaustively against 150 mM NaCl containing 0.24 mM EDTA. The purity of the LDL fraction was determined by Superose 6 (Pharmacia) column chromatography and by agarose electrophoresis. For preparation of LPDS, the density of the plasma was adjusted to 1.21 g/mL with potassium bromide and the plasma centrifuged at 50 000 rpm and 4 °C for 24 h. The top fraction consisting of lipoproteins was removed. The lower fraction, which is devoid of lipoproteins, was dialyzed extensively against 150 mM NaCl at 4 °C for 60 h. The LPDS was sterilized by filtration through a 0.22 μm Millipore filter.

^{125}I Labeling of LDL and Peptides. LDL or peptides were labeled with ^{125}I using the method of Bilheimer et al. (*24*). Briefly, LDL or peptide solution was mixed with glycine buffer [1 M glycine (pH 10)] at a concentration of 200 μL of glycine buffer/mL of LDL or peptide solution. ICl solution (2 M NaCl and 0.5% ICl) was added at a concentration of 3.2 μL/mg of protein. ^{125}I (Amersham; carrier-free in NaOH solution) was then added and the mixture immediately applied to a desalting column (Bio-Rad; Econopak 10DG) which had been pre-equilibrated with the desired buffer. The ^{125}I-labeled material was separated from free ^{125}I in 1 mL aliquots which were then counted to identify the labeled material. Peak fractions were pooled, and the specific activity was determined.

LDL−Peptide Complex Formation. The addition of the peptide was studied in two ways. (a) The peptide and LDL were added simultaneously to the cell medium. (b) The peptide was preincubated with LDL for 1 h at room temperature prior to addition to the cells. The peptide enhanced the uptake of [^{125}I]LDL more when preincubated with LDL than when added to the cells simultaneously with LDL (results not shown).

Agarose Gel Electrophoresis. Agarose gel electrophoresis was carried out according to the procedure of Asztalos et al. (*25*). LDL and the LDL−peptide mixtures were electrophoresed on a 0.7% agarose gel. Tris-Tricine buffer (25 mM, pH 8.6) was used for both gel and electrode buffers. Since the peptides are positively charged, the wells were made in the center of the gel to allow for movement in both directions. Samples (2 μL) of LDL and various ratios of LDL−peptide complexes were diluted with 2 μL of Tris-Tricine buffer containing 10% glycerol and bromophenol blue. Samples were electrophoresed at a constant voltage of 250 V for about 2 h or until the dye reached the top of the gel. After the run, the gel was stained with Coomassie blue.

Binding, Internalization, and Degradation of LDL by Mouse Embryonic Fibroblasts. The binding, internalization, and degradation of LDL in fibroblasts [obtained from American Type Culture Collection (ATCC), Manassas, VA] were assessed using the method of Goldstein et al. (*23*). All cells were grown in DMEM in six-well plates and used at 75−90% confluence. The seeding density of the cells that were used was $1.5-3.0 \times 10^5$ cells/mL of medium. Cells were incubated with DMEM containing LPDS 24 h prior to use to upregulate LDL receptors. LDL was sterilized by filtering through a 0.22 μm filter. For binding experiments, the cells were then incubated with the indicated concentrations (0−50 μg) of [^{125}I]LDL at 4 °C for 2 h in the presence or absence of peptides. Nonspecific binding was assessed in the presence of a 50-fold excess of unlabeled LDL with or without peptides. After being washed with ice-cold PBS (containing 2 mg/mL BSA) to remove excess labeled lipoprotein, the cells were incubated with dextran sulfate (4 mg/mL, Pharmacia, M_r of 500 000) or heparin (Sigma Chemical Co., 10 mg/mL) for 1 h to release specifically bound [^{125}I]LDL. The counts in the dextran sulfate wash reflect the amount of LDL bound to cells. The presence of the peptide did not change the ability of heparin/dextran sulfate to release LDL from the cells.

The cells were washed with cold PBS and then dissolved in 0.1 N NaOH, and a 0.5 mL aliquot of the cell suspension was counted. These counts reflect the amount of LDL that is internalized. The amount of protein was estimated by the method of Lowry (*26*). Degradation of LDL was assessed using the protocol described above for 4 °C, except that the cells were incubated at 37 °C for 5 h. Degradation was assessed by precipitating [^{125}I]LDL from the medium with 17% trichloroacetic acid (TCA) (0.5 mL of 50% TCA was added to 1 mL of medium) and incubating at 4 °C for 30 min (*23*). The precipitate was removed by centrifugation. The supernatant was treated with 10 μL of 40% potassium iodide and 40 μL of 30% hydrogen peroxide. The free ^{125}I was extracted with 2 mL of chloroform. The upper aqueous layer (0.5 mL) was then counted. This represents the amount of [^{125}I]monoiodotyrosine produced by the degradation of apo B in LDL.

Table 3: Percent Helicity of E-18A Analogues

	peptide[a]	peptide−DMPC (1:20)[a]
18A	6.0[b]	49.0[b]
hE-18A	36.0	46.0
mE-18A	36.3	44.0
hE-(R)18A	51.2	70.8
Ac-18A-NH_2	55.0[c]	72.0[c]
Ac-hE18A-NH_2	69.3	74.3
Ac-mE18A-NH_2	67.8	77.3
Ac-hE(R)18A-NH_2	64.6	66.4
Ac-hE(Sc)18A-NH_2	26.0	33.0
Ac-R_{10}18A-NH_2	20.0	27.0

[a] The peptide concentration was 100 μM. The peptide−lipid complexes were made by mixing appropriate volumes of peptide and DMPC (100 μM peptide and 2 mM DMPC) and incubating overnight at 4 °C. [b] From ref *19*. [c] From ref *21*.

The cell surface HSPG was removed by treating the cells with heparinase and heparitinase (Sigma Chemical Co.) at a concentration of 3 units/mL of medium for 2 h at 37 °C. Mouse embryonic fibroblasts (MEF1), the LRP-deficient mutant (PEA13), and the LRP/LDL double mutant (MEF4) (*27*) were used to determine the role of these receptors (LDLR and LRP) in these experiments. In all the cell experiments, the average value of triplicate determinations was used.

Circular Dichroic Spectrometry. CD spectra were recorded on a signal-averaging AVIV 62DS spectropolarimeter as described previously (*21*). Briefly, CD spectra were obtained at 25 °C by signal averaging of four scans recorded every nanometer from 260 to 190 nm using a cell with a 0.01 cm path length. Peptide concentrations were 100 μM in PBS (pH 7.4). Peptide−DMPC complexes (1:20 molar ratio) were prepared as described previously (*21*), and the change in peptide helicity upon lipid association was measured. The helical content of the peptides was estimated from the mean residue ellipticity, $[\theta]_{MRE}$ (deg cm^2 dmol^{-1}), at 222 nm using the equations described in detail by Morrisett et al. (*28*).

RESULTS AND DISCUSSION

Purity and Secondary Structure of the Peptides. The peptide purity was determined by HPLC analysis and confirmed by mass spectral analyses. Each peptide is helical in PBS, and the helicity increases in the presence of DMPC (Table 3). The helicity of the lipid binding domain, 18A, in aqueous buffer is 6%. The addition of 10 amino acids, reflecting the apo E LDL receptor binding domain, to 18A increased its helicity to 36%. Substituting the lysine residues in the LDL receptor binding domain with arginine further increased the helicity to 51%. Protecting both ends of 18A increased the helicity greatly, from 6 to 55%, while those of hE-18A and mE-18A increased from 36 to 69%. The hE-(R)-18A itself has a high helical content (51%); its helicity increased upon N- and C-terminal protection to 65%. When they bind to DMPC, the helicities of all unprotected peptides except hE(R)-18A are in the same range (44−49%), indicating the known influence of phospholipid on the stability of the helical characteristics of the amphipathic peptide. Lipid-bound hE(R)-18A was 71% helical which is comparable to the helicity of the end-protected lipid-bound peptides. The helicity of the lipid-bound dual-domain peptides is similar to that of Ac-18A-NH_2 (Table 3), indicating that the addition of LDL receptor binding domain does not disrupt the helicity of Ac-18A-NH_2, and in fact, it increases the helicity in both the lipid-free and lipid-bound form. However, the helicities of Ac-R_{10}18A-NH_2 and Ac-hE(Sc)18A-NH_2 in aqueous solutions were 20 and 26%, respectively. Their helicities in the presence of lipid did not increase significantly (27 and 33%, respectively) and were lower than that of Ac-18A-NH_2.

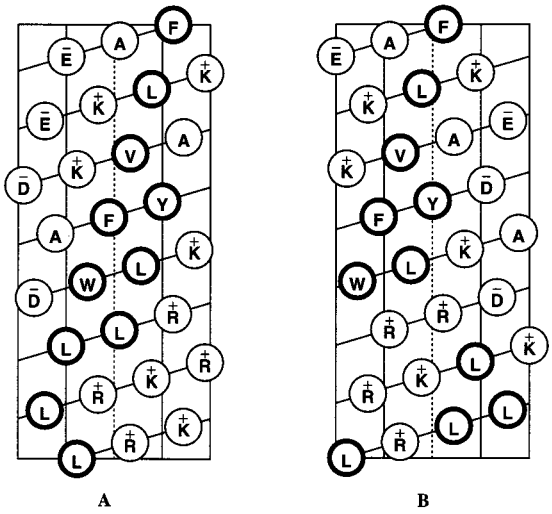

FIGURE 1: Helical net analysis of (A) hE18A and (B) the control peptide hE(Sc)18A. The amino acids are shown in one-letter code. The hydrophobic residues are in bold circles, and the charges of the amino acids are also shown. The hydrophobic residues in the apo E receptor binding domain (LRKLRKRLLR) of hE18A appear to extend the hydrophobic face of the 18A part of the molecule. However, in hE(Sc)18A, this sequence is scrambled. Although the amino acids are the same, the scrambling of the sequence alters the structure of hE(Sc)18A and the apparent extension of the hydrophobic face is lost.

The helical net analyses suggest an explanation for the helicity changes observed in our model peptide studies. For example, the hE-18A sequence (Figure 1A) shows a continuous nonpolar face with a 180° twist. The hydrophobic amino acids present in the additional receptor binding domain can extend the nonpolar face of the amphipathic lipid-associating peptide 18A domain, inducing an elongation of the peptide helix. All the positively charged residues from the receptor binding domain and one from the 18A domain are clustered at the lower right-hand corner of the helical net diagram (Figure 1A). A similar cluster is also seen in apo E itself, suggesting that this structure may be important for the LDL receptor binding of apo E (*29*). The negatively charged residues are clustered in the upper left-hand corner with adjacent positively charged residues potentially neutralizing the charge in this domain. This analysis suggests that hE18A has a localized net positive charge (arginine- and lysine-rich) that enables it to interact with negative charges. As stated above, hE18A also has an extended hydrophobic face that enhances its binding to lipids. The increase in helicity of the peptides upon lipid binding may stabilize the positive charge domain and enhance the ability of the peptide to interact with negatively charged molecules. On the other hand, the helical net analysis of Ac-hE(Sc)18A-NH_2 (Figure 1B) indicates the absence of the extended hydrophobic face in the apo E receptor binding domain, which may explain the observed decreased helicity in both the absence and presence of DMPC.

Binding of the Peptides to LDL. Peptides were incubated with LDL at the weight ratios indicated in the legend of

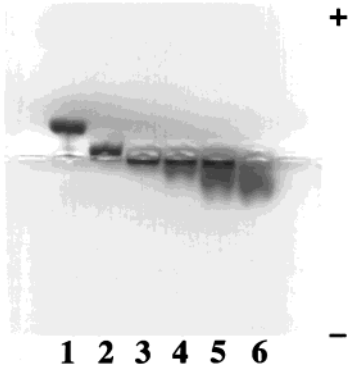

FIGURE 2: Agarose gel (0.7%) of LDL (10 μg) and LDL (10 μg) incubated with different concentrations of hE18A. The gel was stained with Coomassie Blue: lane 1, LDL (10 μg); lane 2, LDL and hE18A (1 μg) (1:14 molar ratio); lane 3, LDL and hE18A (2.5 μg) (1:35 molar ratio); lane 4, LDL and hE18A (5 μg) (1:70 molar ratio); lane 5, LDL and hE18A (10 μg) (1:140 molar ratio); and lane 6, hE18A (10 μg).

Figure 2 for 1 h at room temperature; these mixtures were analyzed by agarose gel electrophoresis (Figure 2). LDL alone migrated toward the anode as a single, homogeneous band (lane 1), while the hE-18A peptide alone (10 μg) appeared as a diffuse band migrating toward the cathode (lane 6). The anodic mobility of the LDL incubated with peptide was retarded, and the degree of retardation of LDL mobility was dependent upon the ratio of peptide to LDL. At a peptide:LDL molar ratio of 35:1, no free peptide was detected (Figure 2, lane 3). However, at higher peptide concentrations (70:1 and 140:1 molar ratios, lanes 4 and 5), free peptide was detected (stained material moving toward the cathode, Figure 2, lane 6). These results indicate that the peptide associates with LDL. Similar results were observed with all other peptide analogues used in this study. Thus, these two-domain peptides bind to LDL and alter its net surface charge. The control peptide, Ac-18A-NH_2, bound to the LDL, but it did not change the mobility of LDL on agarose gels, reflecting the peptide's net zero charge (data not shown).

Effect of the Dual-Domain Peptides on LDL Uptake in Mouse Fibroblasts. Our peptides were designed to mimic apo E containing both the "minimal" LDL receptor binding domain and an efficient lipid binding domain. Since the data presented above demonstrated that these peptides associate with LDL, we next tested whether they interact with the LDL receptor of mouse fibroblasts (MEF1). At a constant LDL concentration, specific internalization of LDL was dependent on the concentration of peptide, hE-18A, or Ac-hE18A-NH_2 that was used (Figure 3A). At the highest peptide:LDL ratio, a 7-fold enhancement of LDL internalization was observed relative to that with LDL alone. The enhanced internalization of the LDL−peptide complex was seen at each ratio tested but to a lesser degree than at a 1:1 (w/w) ratio. Furthermore, at the highest peptide concentration (10 μg) that was used, Ac-hE18A-NH_2 was 2 times more effective than hE18A. This was observed for all the peptides. Neither the control lipid-associating peptide, Ac-18A-NH_2, nor the receptor binding region, LRKLRKRLLR, without the lipid binding domain, was able to enhance LDL uptake (results not shown). The other two control peptides [Ac-R_{10}18A-NH_2 and Ac-hE(Sc)-18A-NH_2] exhibited significantly weaker ablity to enhance LDL uptake ($\leq$2-fold). Ac-R_{10}18A-NH_2 enhanced LDL uptake 2-fold, while the Ac-hE(Sc)18A-NH_2 was 1.5 times

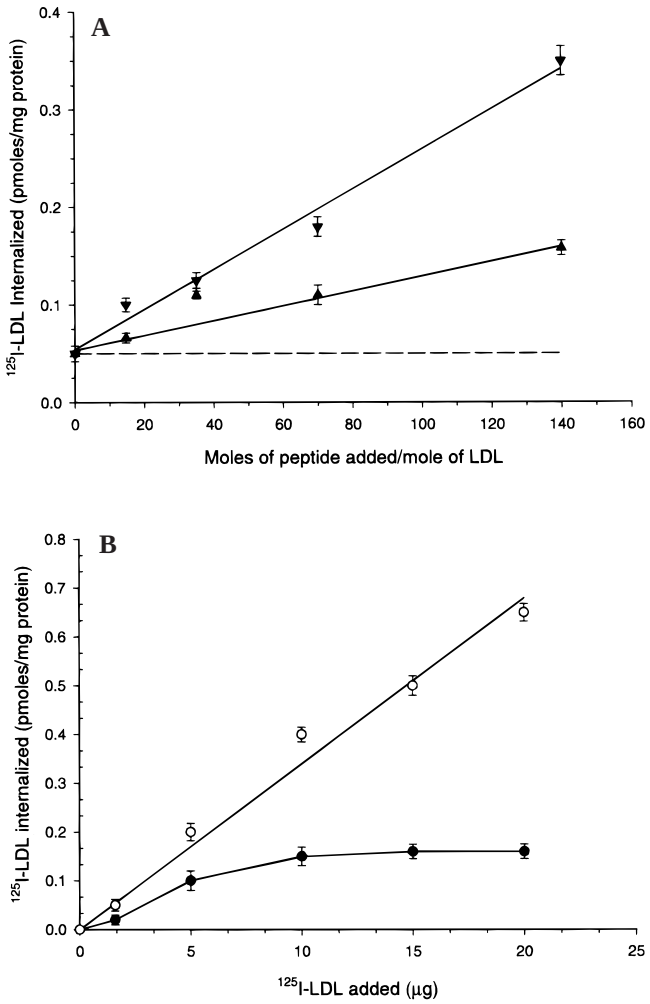

FIGURE 3: (A) Effect of different concentrations of hE18A and Ac-hE18A-NH_2 on the specific internalization of [^{125}I]LDL in MEF1 cells. Cells were grown in DMEM in a monolayer in six-well plates. Details are given in Materials and Methods. [^{125}I]LDL (10 μg) was incubated with different concentrations of peptides (1−10 μg) at room temperature for 1 h. This mixture was then incubated with the cells for 2 h at 37 °C, and the effect of the peptide on internalization of LDL was measured after incubation for 2 h at 37 °C as described in detail in Material and Methods: (− − −) LDL alone, (▲) LDL and hE18A, and (▼) LDL and Ac-hE18A-NH_2. (B) Extent of internalization of [^{125}I]LDL by MEF1 cells as a function of LDL concentration. Black circles represent data for LDL internalized without peptide, and white circles represent data for LDL internalized in the presence of the peptide. The saturation in LDL binding that is observed in the absence of the peptide is abolished by the peptide.

as effective as the control LDL. This result suggests a spatial specificity of the charged residues for enhanced LDL uptake perhaps conferred on the peptide by the extended α-helical hydrophobic face as diagrammatically shown in Figure 1A. Sparrow et al. (*30*) identified the region of residues 130−150, which includes the domain that we have used, as a lipid binding domain since it formed spontaneous complexes with DMPC and increased its α-helicity at a molar ratio of 125:1 (PC:peptide), providing additional support for the extended hydrophobic face and lipid binding capacity of the domain.

Since protected peptides resemble an internal peptide sequence where terminal charges are eliminated and an increased helicity is observed (Table 3), the enhanced uptake compared to that of unprotected equivalent peptides may be

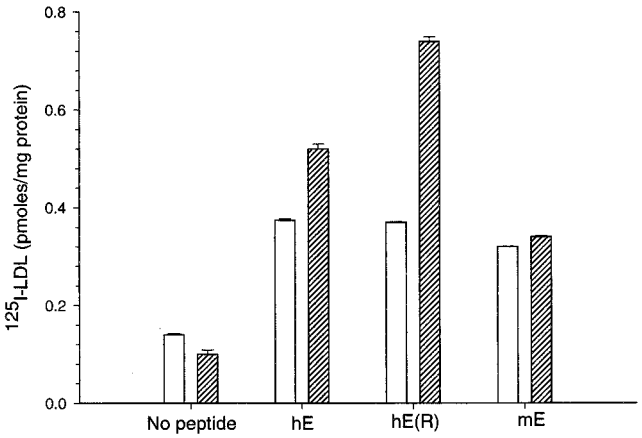

FIGURE 4: Internalization and degradation of [^{125}I]LDL (10 μg) in the presence of 10 μg of Ac-hE18A-NH_2 (hE), Ac-hE(R)18A-NH_2 [hE(R)], and Ac-mE18A-NH_2 (mE). The MEF1 cells were grown and treated with the [^{125}I]LDL−peptide complex as mentioned in Materials and Methods. The extent of internalization was detremined after incubation of the cells for 2 h at 37 °C, while the extent of degradation was measured after incubation of the cells for 5 h at 37 °C. The white bars represent degradation data, and the bars with diagonal lines represent internalization data.

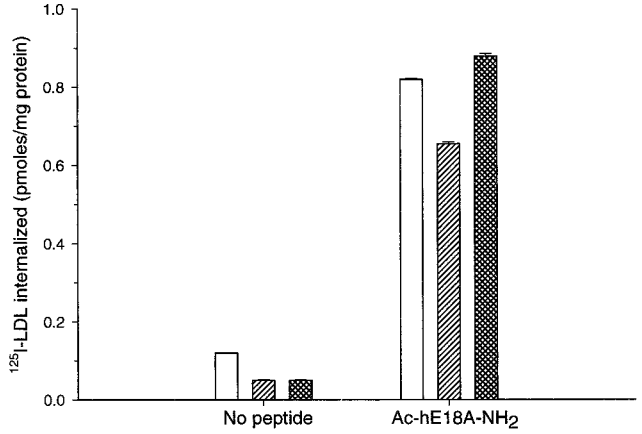

FIGURE 5: Comparison of the specific internalization of [^{125}I]LDL (10 μg) after coincubation with 10 μg of Ac-hE18A-NH_2 for 1 h at room temperature in wild-type and LDLR(−/−)/LRP(−/−) (MEF 4) and LRP(−/−) (PEA13) mouse embryonic fibroblasts. [^{125}I]LDL and peptide were coincubated for 1 h at room temperature, then filtered with a 0.22 μm filter, and incubated with the cells for 2 h at 37 °C. Details are given in Materials and Methods. The white bars represent data from wild-type cells (MEF1); the bars with diagonal lines represent data from LRP(−/−) cells, and the bars with the crosshatches represent data from the LDLR(−/−)/LRP-(−/−)cells.

due to an increased charge density due to an extension of the helix and clustering of positive charges. Although hE-(R)-18A is as helical as the protected peptide, it did not enhance LDL uptake to the same extent as the end-protected peptide (Figure 3A). Since the end-protected peptides were more active in enhancing LDL uptake, all further experiments were carried out using the modified peptides.

The ability of the Ac-hE18A-NH_2 peptide to enhance LDL uptake was observed over a broad range of LDL concentrations (1−25 μg/mL) below and above its K_d (≈4 μg/mL) (*31*). The rate of internalization of [^{125}I]LDL increased with increasing concentrations of LDL (Figure 3B) with the peptide concentration constant at 10 μg/mL of medium. In the absence of peptide, as expected in the LDLR upregulated cells, curvilinear, saturable internalization of LDL is observed (Figure 3B, lower curve, ●). However, in the presence of 10 μg of peptide, the uptake is nonsaturable, and linear over this concentration range, indicating that additional non-LDL receptor pathways are the major route(s) involved in the binding and uptake of peptide−LDL complexes.

We next tested whether amino acid substitutions in the apo E ligand binding domain alter the LDL−peptide complex uptake by the fibroblasts. When all the lysine residues are changed to arginine, internalization of the Ac-hE(R)18A-NH_2−LDL complex was enhanced 7-fold relative to that of LDL alone but was similar to that of the parent molecule, Ac-hE18A-NH_2 (Figure 4). Substituting two hydrophobic Leu residues (Ac-hE18A-NH_2) with hydrophobic Met residues (Ac-mE18A-NH_2) mimicks the mouse apo E receptor binding domain. Ac-mE18A-NH_2 enhanced internalization relative to that of LDL alone (3.5-fold) (Figure 4), although to a lesser extent than Ac-hE18A-NH_2 (5-fold). Substitution of the two Lys residues of Ac-hE18A-NH_2 with Arg [Ac-hE(R)18A-NH_2] enhanced the internalization by the peptide even though the charge density was maintained. Neither the cationic domain nor the 18A by itself was able to enhance LDL binding and internalization (data not shown). Changes in the cationic domain of the two-domain peptides by substitution of Lys with Arg and/or Met did affect the extent of LDL binding or peptide cellular interaction. These data suggest that subtle changes in charge or hydrophobicity of the binding domain amino acids are important and modulate the enhanced uptake of the LDL−peptide complex, probably due to subtle changes in the structure (helicity or charge density) of the peptide.

The peptide−LDL complex also exhibited enhanced cellular degradation relative to that of LDL alone (Figure 4). Even though the degree to which internalization of the complex is enhanced is peptide-dependent, all complexes are degraded to the same extent, approximately 2-fold more than LDL alone. This suggests the possibility that the degradation reflects the fact that some LDL−peptide complex is being internalized through a receptor-dependent pathway, albeit a small proportion (since relative to internalization, the degradation appears retarded) or that degradation is at its maximum for these cells, and does not change even with enhanced LDL internalization.

Apo E is a ligand for the LDLR and is particularly important for members of this gene family (*2*, *32*, *33*). We used this information in our initial peptide design. However, our data indicate the enhanced LDL uptake in the presence of the dual-domain peptide was not saturable in the LDL concentration range that was studied, suggesting that the LDL−peptide complex was taken up by an alternate pathway. In support of this hypothesis, additional lines of evidence indicate that neither the LDLR nor the LRP is a major route of the observed enhanced peptide-mediated uptake of LDL by fibroblasts. Internalization of the LDL−peptide complex in LRP-deficient [LRP(−/−)] cells and in LDLR- and LRP-deficient cells [LDLR(−/−) and LRP(−/−)] was assessed. The rate of cellular uptake of human LDL in the absence of the peptides was low in MEF1 cells, but it was diminished by about 65% in both LRP(−/−) (PEA13 cells) and LDLR(−/−) and LRP(−/−) (MEF4 cells) cell types, as would be expected if receptor mechanisms are operable for LDL uptake (Figure 5). However, in the

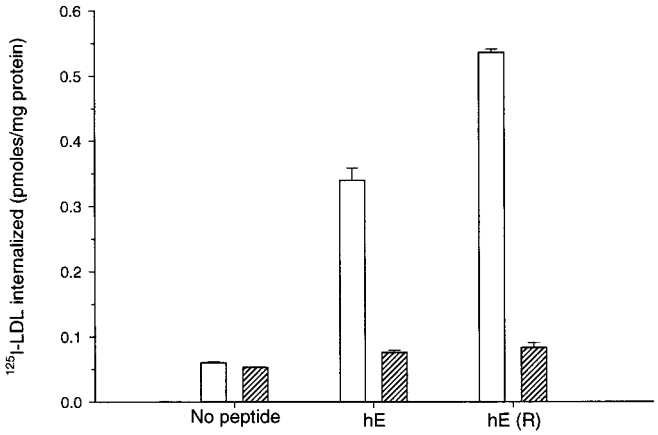

FIGURE 6: Effect of heparinase/heparitinase on the internalization of [^{125}I]LDL (10 μg) after incubation with 10 μg of Ac-hE18A-NH_2 (hE) and Ac-hE(R)18A-NH_2 [hE(R)] for 1 h at room temperature. MEF1 cells were pretreated with heparinase/heparitinase (3 units/mL) for 2 h at 37 °C and then incubated with the [^{125}I]LDL−peptide complex for 2 h at 37 °C. Experimental details are given in Materials and Methods. The white bars represent data for internalization without heparinase/heparitinase treatment, while the bars with diagonal lines represent data for internalization after pretreatment with heparinase/heparitinase.

presence of Ac-hE18A-NH_2, we observed a 7-fold (MEF1), 13-fold (PEA13), and 19-fold (MEF4) enhancement of LDL−peptide complex uptake relative to that of LDL alone in these cell lines.

Since the LDLR and LRP receptor pathways did not seem to be involved in LDL−peptide complex uptake, we tested an alternate pathway which binds lipoproteins through a positively charged domain [the HSPG pathway, a previously described (*34*, *35*) binding site for residues 141−150 of apo E]. Fibroblasts were incubated with heparinase and heparitinase (3 units each/mL) to remove surface HSPG (*34*, *36*). Treatment of MEF1 cells with heparinase/heparitinase did not affect the internalization of LDL alone (Figure 6), but the extent of internalization of peptide−LDL complexes was reduced by almost 75% for the Ac-hE18A-NH_2−LDL complex and by about 85% for the Ac-hE(R)18A-NH_2−LDL complex (Figure 6), i.e., to levels seen for LDL alone. The almost complete loss of enhanced LDL−peptide complex uptake after heparinase/heparitinase treatment indicates that the HSPG pathway is the major route for the peptide-mediated internalization of LDL. The α-helical cationic structure of these dual-domain peptides appears to direct the internalization of LDL via the HSPG pathway. Both the charge distribution and the presence of the hydrophobic face, however, seem to contribute to the enhanced LDL uptake. Ac-hE(Sc)18A-NH_2 contains the same number of positive charges within the E receptor binding domain, yet this is insufficient for enhanced uptake. Increasing the positive charge density with the Ac-R_{10}18A-NH_2 also is not effective, indicating that this is not a simple ionic interaction between LDL−peptide complex and the cell surface. It appears that a specific spatial cationic domain must be maintained that ensures the amphipathic helical character of the peptide. Small changes in the design like R for K or M for L can be tolerated, but the scrambled domain is an ineffective enhancer.

A tandem repeat of the apo E receptor domain $[141-155]_2$ inhibited LDL binding to the LDL receptor, and when N-acetylated, it enhanced the uptake of LDL (*16*). An anionic peptide used by Braddock et al. (*18*) associated with LDL and increased the extent of LDL binding by 6−7-fold in LDL receptor negative fibroblasts. However, the extent of LDL degradation was only 10% of that in LDL receptor positive cells. Moreover, the anionic peptide−LDL complex-enhanced activity was not diminished by treatment with heparinase, implicating a pathway different from that observed for the apo E analogues described in this paper. These two studies with two totally different peptides, one anionic and one cationic, indicate that each type causes an enhancement of LDL uptake by fibroblasts; however, there is not a concomitant increase in the extent of degradation, suggesting that the pathways responsible for internalization in all cases are different. The peptides described here, in contrast, enhance both internalization and degradation by fibroblasts. The 18A-incorporated lipid binding region may modulate this enhanced internalization and degradation. Apo E binds preferentially to larger lipoproteins, Sf > 12, and subpopulations of HDL. We predict that by altering the lipid binding domain (i.e., either the hydrophobic nature and or the length) of apo E mimetic peptides, they could be targeted to other lipoproteins and preferentially enhance their internalization by distinct pathways.

In summary, we demonstrated that a putative apo E receptor binding domain (residues 141−150) attached to an idealized lipid binding domain associates with LDL and enhances the LDL binding to murine fibroblasts. These peptides enhance both internalization and degradation, but the extent of degradation is lower than expected through receptor-mediated processes under our experimental conditions. The enhanced uptake and degradation occur primarily through the HSPG pathway, an outcome that has mechanistic implications since the E receptor binding domain and its putative heparin binding domain overlap (*36*). The observed enhancement could be due either to a receptor-independent HSPG pathway or to a HSPG-mediated, receptor-dependent pathway of yet undescribed origin which could direct LDL to peripheral sites (*36*) or to hepatic sites (*37*). Since enhanced LDL uptake should decrease the level of LDL plasma cholesterol, our studies suggest the feasibility of using apo E mimetics as described here to decrease plasma LDL levels in vivo.

We intend to express peptides with these properties in an apo E-knockout mouse model and study the effect of the expressed peptide on regression of spontaneously developed atherosclerosis (*4*). We have recently shown that a transgenic mouse model that expresses a synthetic peptide can be produced (*38*). These studies are important in designing apo E-mimicking peptides that can be readily synthesized and in demonstrating their potential use in therapeutic intervention of atherosclerosis.

ACKNOWLEDGMENT

We thank Dr. Steve Barnes and Marion Kirk for the mass spectral analyses of peptides and Dr. Don Muccio for allowing us to use the CD spectrometer.

REFERENCES

1. Cooper, A. D. (1998) *J. Lipid Res. 38*, 2173−2192.
2. Mahley, R. W. (1988) *Science 240*, 622−630.

3. Shimano, H., Yamada, N., Katsuki, M., Yamamoto, K., Gotoda, T., Harada, K, Shimada, M., and Yazaki, Y. (1992) *J. Clin. Invest. 90*, 2084−2991.
4. Yamada, N., Shimono, H., Mokuno, H., Ishibashi, S., Goutohda, M., Kawakami, M., Watanabe, Y., Akanuma, Y., Murase, T., and Takaku, F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 665−669.
5. Zhang, S. H., Reddick, R. L., Piedrahita, J. A., and Maeda, N. (1992) *Science 258*, 468−471.
6. Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setala, K., Walsh, A., Verstuyft, J. G., and Rubin, E. M. (1992) *Cell 71*, 343−353.
7. Spangenberg, J., and Curtis, L. K. (1997) *Biochim. Biophys. Acta 1349*, 109−121.
8. Bradley, W. A., and Gianturco, S. H. (1986) *J. Lipid Res. 27*, 40−48.
9. Wilson, C., Wardell, M. R., Weisgraber, K. H., Mahley, R. W., and Agard, D. A. (1991) *Science 252*, 1817−1822.
10. Sparrow, J. T., Sparrow, D. A., Fernando, G., Culwell, A. R., Kovar, M., and Gotto, A. M., Jr. (1992) *Biochemistry 31*, 1065−1068.
11. Rall, S. C., Jr., Weisgraber, K. H., Innerarity, T. L., and Mahley, R. W. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4696−4700.
12. Lalazar, A., Weisgraber, K. H., Rall, S., Giladi, H., Innerarity, T. L., Levanon, A. Z., Boyles, J. K., Amit, B., Gorecki, M., Mahley, R. W., and Vogel, T. (1988) *J. Biol. Chem. 263*, 3542−3545.
13. Dyer, C. A., and Curtis, L. K. (1991) *J. Biol. Chem. 296*, 22803−22806.
14. Dyer, C. A., Smith, R. S., and Curtis, L. K. (1991) *J. Biol. Chem. 266*, 15009−15015.
15. Dyer, C. A., Cistola, D. P., Parry, G. C., and Curtis, L. K. (1995) *J. Lipid Res. 36*, 80−88.
16. Nicoulin, I. R., and Curtis, L. K. (1998) *J. Clin. Invest. 101*, 223−234.
17. Mims, M. P., Darnule, A. T., Tover, R. W., Pownall, H. J., Sparrow, D. A., Sparrow, J. T., Vial, D. P., and Smith, L. C. (1994) *J. Biol. Chem. 269*, 20539−20647.
18. Braddock, D. T., Mercurius, K. O., Subramanian, R. M., Dominguez, S. R., Davies, P. F., and Meredith, S. C. (1996) *Biochemistry 35*, 13975−13984.
19. Venkatachalapathi, Y. V., Phillips, M. C., Epand, R. M., Epand, R. F., Tytler, E. M., Segrest, J. P., and Anantharamaiah, G. M. (1993) *Proteins: Struct., Funct., Genet. 15*, 349−359.
20. Chung, B. H., Palgunachari, M. N., Mishra, V. K., Chang, C.-H., Segrest, J. P., and Anantharamaiah, G. M. (1996) *J. Lipid Res. 37*, 1099−1112.
21. Mishra, V. K., Palgunachari, M. N., Segrest, J. P., and Anantharamaiah, G. M. (1994) *J. Biol. Chem. 269*, 7185−7191.
22. Palgunachari, M. N., Mishra, V. K., Lund-Katz, S., Phillips, M. C., Adeyeye, S. O., Alluri, S., Anantharamaiah, G. M., and Segrest, J. P. (1996) *Arterioscler. Thromb. Vasc. Biol. 16*, 328−338.
23. Goldstein, J. L., Basu, S. K., and Brown, M. S. (1983) *Methods Enzymol. 98*, 241−260.
24. Bilheimer, D. W., Eisenberg, S., and Levy, R. I. (1972) *Biochim. Biophys. Acta 260*, 212−221.
25. Asztalos, B. F., Sloop, C. H., Wong, L., and Roheim, P. S. (1993) *Biochim. Biophys. Acta 1169*, 291−300.
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
27. Willnow, T. E., and Herz, J (1994) *J. Cell Sci. 107*, 719−726.
28. Morrisett, J. D., David, J. S. K., Pownall, H. J., and Gotto, A. M., Jr. (1973) *Biochemistry 12*, 1290−1299.
29. Dong, L. M., Parkin, S., Trakhanov, S. D., Rupp, B., Simmons, T., Arnold, K. S., Newhouse, Y. M., Innerarity, T. L., and Weisgraber, K. H. (1996) *Nat. Struct. Biol. 3*, 718−722.
30. Sparrow, J. T., Sparrow, D. A., Culwell, A. R., and Gotto, A. M., Jr. (1985) *Biochemistry 24*, 6984−6988.
31. Basu, S. K., Goldstein, J. L., and Brown, M. S. (1978) *J. Biol. Chem. 253*, 3852−3856.
32. Guilaume, D., Bertrand, P., Dea, D., Davignon, J., and Poirer, J. (1996) *J. Neurochem. 66*, 2410−2418.
33. Ji, Z. S., Fazio, S., Lee, Y. L., and Mahley, R. W. (1994) *J. Biol. Chem. 269*, 2764−2772.
34. Ji, Z. S., Brecht, W. J., Miranda, R. D., Hussain, M. M., Innerarity, T. L., and Mahley, R. W. (1993) *J. Biol. Chem. 268*, 10160−10167.
35. Mahley, R. W., Weisgraber, K. H., and Innerarity, T. L. (1979) *Biochim. Biophys. Acta 575*, 81−89.
36. Al-Haideri, M., Goldberg, I. J., Galeano, N. F., Gleeson, A., Vogel, T., Gorecki, M., Sturley, S. L., and Deckelbaum, R. J. (1997) *Biochemistry 36*, 12766−12772.
37. Mahley, R. W., and Ji, Z. S. (1999) *J. Lipid Res. 40*, 1−16.
38. Garber, D. W., Datta, G., Kulkarni, K. R., Anderson, P., Yamanaka, S., Innerarity, T. L., and Anantharamaiah, G. M. (1997) *Circulation 96*, I490.

BI991209W