Peptide model of a highly conserved, N-terminal domain of apolipoprotein E is able to modulate lipoprotein binding to a member of the class A scavenger receptor family

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Abstract **Apolipoprotein E plays a critical role in plasma lipoprotein clearance. Peptide models of a highly conserved, N-terminal domain of this protein have been shown to increase the binding of low density lipoprotein (LDL) to fibroblast cell surfaces independently of the low density lipoprotein receptor. Here we provide data to show that these peptides not only increase the binding of LDL, but also of high density lipoprotein, though not acetylated LDL. We also have data suggesting that this novel activity is mediated, at least in part, by a member of the scavenger receptor family, SR-AI. Furthermore, we show that this activity is also prominent in macrophages, a cell relevant to atherogenesis. In addition, this current paper provides evidence suggesting that this complex binding activity is initiated by a peptide–receptor interaction, and that our peptides are able to induce activity at physiologically relevant concentrations. This study provides evidence for a possible novel receptor interaction and further anti-atherogenic properties of apolipoprotein E and raises the possibility of a therapeutic potential of our peptide models.—**Dominguez, S. R., H. Miller-Auer, C. A. Reardon, and S. C. Meredith. **Peptide model of a highly conserved, N-terminal domain of apolipoprotein E is able to modulate lipoprotein binding to a member of the class A scavenger receptor family.** ***J. Lipid Res.*** **1999.** 40: **753–763.**

Supplementary key words apolipoprotein E • peptide models • lipoproteins • LDL • HDL • scavenger receptors

Apolipoprotein E (apoE) plays a critical role in lipid metabolism, and its deletion in mice leads to the development of hypercholesterolemia and atherosclerotic plaques reminiscent of the human disease (1–3). The known receptors for apoE are members of the LDL receptor (LDL-R) family, notably the LDL-R and the LDL-R-related protein (LRP; 4–6). In the case of both these receptors, binding is mediated by a highly conserved cluster of cationic residues at positions 140–150 in human apoE. A second region of at least equally high conservation is an anionic region encompassing positions 40–60, but the role of this domain in apoE function is not known. We have shown previously that peptide models of the anionic N-terminal domain mediate specific, high affinity cell surface binding of LDL by a pathway independent of the LDL receptor. This binding is conformationally specific, and our previous studies have begun to elucidate the structure–function relationships governing models of this domain of apoE (7–9). Furthermore, binding isotherms and competition data indicate that the binding is likely to be mediated by a specific receptor.

In this paper, we will demonstrate that our peptides increase the cell surface binding not only of LDL, but also of HDL, though not of acetylated LDL. We will show that this novel activity is mediated, at least in part, through a member of the scavenger receptor family, SR-AI. Furthermore, we will show that this activity is not limited to fibroblasts, but is also prominent in macrophages, a cell more relevant to atherogenesis. Finally, we will provide evidence which suggests that this complex binding activity is initiated by a peptide–receptor interaction and that our peptides are able to induce activity at physiologically relevant concentrations, i.e., at molarities comparable to those of plasma apoE.

EXPERIMENTAL PROCEDURES

Peptide synthesis, purification, and characterization

Sequences of all peptides used in these studies are given in Results. Bioactive peptides used in these studies are designated peptides I, III, and V, and form part of a series of models of the conserved anionic N-terminal subdomain of apoE (7–9). Methods for synthesis and cleavage from resin of side-chain lactam

Abbreviations: apo, apolipoprotein; LDL, low density lipoprotein; LDL-R, LDL receptor; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline; Ac-LDL, acetylated LDL; DiI, 3,3′-dioctadecylindocarboxycyanine; MEF, murine embryonic fibroblasts; FBS, fetal bovine serum; LRP, LDL-receptor-related; DMEM, Dulbecco's modified Eagle's medium; BCA, bicinchoninic acid; CHO, Chinese hamster ovary cells.

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crosslinked peptides have been given in detail elsewhere (7, 8). Peptides were purified by FPLC using a Pharmacia FPLC GP-250 Plus (Pharmacia Biotech Inc., Piscataway, NJ) and a preparative RESOURCE Q anion exchange column (Pharmacia Biotech Inc., Piscataway, NJ). The solvent system consisted of a gradient from 0.05 to 1.0 M ammonium bicarbonate. Peptide purity was assessed by analytical reverse phase HPLC, mass spectrometry, amino acid analysis, and in some instances amino acid sequencing. For synthesis of ^{3}H-containing peptides, uniformly labeled [^{3}H]Gly from Amersham (5 mCi/mmole) was protected for tBOC synthesis by the method of Schnabel (10), and incorporated into the synthesis. A typical specific radioactivity of the final product was 931 cpm/nmole.

Lipoproteins and lipoprotein-deficient serum

Plasma was obtained from normolipidemic volunteers in good health. Prior to isolation of lipoproteins the following preservatives were added to the plasma: 1 mM PMSF and BHT, and 0.1% (w/v) EDTA. LDL ($1.019 < d < 1.050$ g/ml^3), HDL ($1.063 < d < 1.21$ g/ml^3), and the bottom fraction ($d > 1.21$ g/ml^3) were isolated by differential flotation as described previously (11). Lipoprotein-deficient serum (LPDS) was prepared by extensively dialyzing the bottom fraction against phosphate-buffered saline (PBS). After dialysis the lipoprotein-deficient plasma was converted into serum by incubation with 10 NIH units/ml thrombin at 4°C for 24 h and the resulting clot was removed by ultracentrifugation at 39,000 *g*. The resulting LPDS was then concentrated with an Amicon filtration cell using a YM2 filter to a final protein concentration of 50 mg/ml and stored frozen. Acetylated-LDL (Ac-LDL) was prepared by incremental additions of acetic anhydride to LDL in a saturated solution of sodium acetate as previously described by Basu et al. (12). 3,3′-Dioctadecylindocarboxycyanine (DiI)-labeled lipoproteins were prepared by a modification of the procedure of Reynolds and St. Clair (13). DiI was solubilized in DMSO at a concentration of 30 mg/ml. Lipoproteins were then incubated for 18 h at 37°C in LPDS with DiI at a ratio of 300 mg DiI/1 mg LDL/5 mg LPDS. The DiI-labeled proteins were purified by FPLC using a fast desalting column and PBS as a solvent. Iodinated lipoproteins were prepared using Iodobeads (Pierce, Rockford, IL) according to the manufacturer's instructions. ^{125}I-labeled lipoproteins were used within 3–5 days of labeling. All lipoproteins were stored under N_2 and used within 1 month of their isolation.

Cell culture

All cell culture incubations were at 37°C in a humidified 95% air 5% CO_2 atmosphere. All binding experiments were performed using murine embryonic fibroblasts (MEF), which were a kind gift from Drs. Joachim Herz and Thomas E. Willnow (University of Texas Southwestern Medical Center, Dallas). These included wild type (MEF1) and "double mutant," LDL receptor/LDL receptor-related protein (LRP)-deficient (MEF4) cells. Cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS), 1% l-glutamine, and l% penicillin streptomycin solution (medium A). J774A.1 cells (TIB67 macrophages) were obtained from American Type Culture Collection (ATCC) and also maintained in medium A.

Lipoprotein binding to cell surface

Lipoprotein binding to cells was measured by modification of described procedures (14, 15). Two days prior to cell binding experiments, cells were trypsinized and plated into 12-well plates at a concentration of approximately 7.0×10^4 to 1.0×10^5 cells/well. For experiments measuring the effects of induction of LPDS on binding activity, the media were changed 24 h prior to the start of the experiment to DMEM + 10% LPDS; all other experiments left the media unchanged. One hour prior to the start of the experiment, peptides were dissolved in DMEM from which an aliquot was removed to determine the peptide concentration. The concentration was measured by the BCA method; the BCA method was calibrated using peptide standards the concentrations of which had been determined by amino acid analysis. LPDS was then added to a final concentration of 10%. To these solutions, labeled lipoproteins (either DiI or ^{125}I) were added to achieve a final concentration of 10 μg/ml. These solutions were then gently mixed and divided into two vials. One vial received an excess of unlabeled lipoprotein to achieve a final concentration of 500 μg/ml (a 50-fold excess). The latter solution was used to determine the nonspecific association of the lipoprotein with the cell surface. The vials were incubated at room temperature and the cells remained at 4°C for 1 h prior to the start of the experiment. At the start of the experiment, the cells were washed once briefly with PBS and then 0.5 ml of the appropriate ice-cold experimental solution was added. The cells were incubated for 3 h at 4°C in the dark while being gently agitated. At the end of the incubation period, the experimental medium was removed and each dish was washed three times rapidly, followed by two 10-min washes with 1 ml of ice-cold PBS containing 2 mg/ml bovine serum albumin (BSA), and one final rapid wash with PBS. In experiments using DiI as a label, each well then received 1 ml of isopropanol. The cells were incubated for 15 min after which the solutions were collected, centrifuged at approximately 800 *g* for 10 min, and the fluorescence was measured using a Hitachi F-2000 Fluorescence Spectrophotometer. The cell proteins were dissolved by incubation at room temperature for 15 min with 1 ml of 0.1 M NaOH and the protein content was determined by the BCA method. In experiments using ^{125}I as a label, the cells were incubated for 15 min at room temperature with 1 ml of 0.1 M NaOH; a 100-μl aliquot was removed on which the radioactivity was measured and the cell protein was determined by the BCA method. Specific lipoprotein binding was determined by subtracting nonspecifically bound lipoprotein from the total bound. Unless otherwise stated, each data point was the average of duplicate or triplicate samples and is one representative of multiple experiments.

Preliminary studies on binding of peptide V to LDL suggested that its affinity for the LDL surface was similar to that previously reported for peptide III, i.e., $K_d \approx 1$–10 μM. Data will be presented below (see Fig. 7) that this is indeed the case. As in previous studies, we sought experimental conditions to eliminate as far as possible any heterogeneity of the LDL with respect to bound peptide, i.e., conditions under which the surface of LDL would be saturated with peptide. If there are n sites on LDL to which free peptide, P_f, can bind, then:

$$LDL_n + nP_f \rightleftharpoons LDL \cdot (P_b)_n$$

where $LDL \cdot (P_b)_n$ is a peptide–LDL complex containing n molecules of peptide. The complexity of the system is reduced by driving the reaction to the right by adding a large excess of peptide, i.e., using peptide concentrations ⩾two orders of magnitude above K_d. Accordingly, as in our previous experiments, ^{125}I-labeled LDL at various concentrations was incubated at the start of a cell experiment with peptide V at a concentration of 2 mg/ml (0.8 mM). Under these conditions, therefore, $P_{total} \approx P_{free} >> K_d$. A similar argument can be made for peptide binding sites on the cell surface. If S_f = peptide binding sites on the cell surface, then:

$$S_f + P_f \underset{K_{d2}}{\rightleftharpoons} Cx$$

where Cx = peptide-site complex. The binding of LDL to the cell surface in the presence of peptide can be defined heuristically as the formation of a ternary complex of LDL, peptide and site on the cell surface, e.g., a receptor:

$$(\text{LDL} \cdot \text{P}_\text{b})_\text{f} + \text{S}_\text{f} \underset{K_{d3}}{\rightleftharpoons} \text{Ternary Complex}$$

$$K_{d3} = \frac{(\text{LDL} \cdot \text{P}_\text{b})_\text{f}(\text{S}_\text{f})}{\text{Ternary Complex}}$$

where S_f = free sites on the cell surface for binding LDL in the presence of peptide V, and $(LDL \cdot P_b)_f$ is free peptide–LDL complex, i.e., not bound to the cell surface. The term "heuristic" is used for the ternary complex as we do not assume the existence of a direct physical contact among lipoprotein, peptide, and receptor. The above equation can be put into experimental terms as Ternary Complex ≡ LDL bound to the cell surface, $(LDL \cdot P_b)_f = LDL_{total}$ − LDL bound to the cell surface, and $S_f = S_{total}$ − LDL bound to the cell surface, where S_{total} = total sites for the heuristic ternary complex, a parameter. The experimental data was then analyzed by non-linear least squares methods, to yield K_{d3} and S_{total}.

Effects of pretreating cells with heparinase, $NaClO_3$, LPDS, and EGTA on LDL binding to the cell surface

Heparinase, sodium chlorate, and EGTA were purchased from Sigma (St. Louis, MO). To test the role of cell surface glycans in the binding of LDL to MEF4 cells in the presence of peptides, cells were treated with 2.5 U/ml heparinase for 45 min at 37°C and washed prior to the start of the experiment as described by Ji et al. (16). In addition, cells were grown for 48 h in the presence of 25 mm $NaClO_3$, an inhibitor of enzymatic sulfation (17). To test the ability to induce this activity by cholesterol deprivation, cells were incubated in the presence and absence of LPDS for 24 h prior to the experiment. Furthermore, to determine the effect of calcium on binding activity, experiments were performed in the presence and absence of 5 mm EGTA. The binding of LDL to cells with these varying treatments was determined as described above.

Transfections

Plasmid expression vectors for the murine scavenger receptors AI (mSR-AI) and BI (mSR-BI) were a kind gift from Dr. Monty Krieger (Massachusetts Institute of Technology, Cambridge, MA). Tissue culture reagents were obtained from Life Technologies (GIBCO-BRL, Gaithersburg, MD) unless otherwise stated. CHO-K1 cells (61-CCL) were obtained from American Type Culture Collection (ATCC) and maintained in a 50/50 solution of low glucose DMEM and F12 medium supplemented with 25 mm HEPES, 10% FBS, 1% l-glutamine, and 1% penicillin streptomycin solution (medium B). CHO cells stably transfected with CD36 (CHO-CD36) were obtained from ATCC (CRL 2092) and were maintained in medium B + 400 mg/ml G418. For transient transfections, 2.5×10^5 CHO-K1 cells/well were plated in 6-well plates in medium B. After 24 h the cells were incubated with 1 μg plasmid DNA and 1 μl Lipofectamine (GIBCO-BRL) in 1 ml OPTI-MEM media without serum for 5 h according to the manufacturer's instructions. The cells were maintained in medium B for an additional 48 h at which time binding experiments were performed as described above, except that 1 ml of experimental solution was used per well and all washes were performed with 2 ml. The level of receptor expression was monitored by measuring the level of AcLDL binding. For transfection experiments, binding activity was evaluated as described above and compared to binding in mock transfected cells. As a control for the quantity of scavenger receptor expressed in transfected cells, binding of acetylated LDL was measured, using the methods described above.

Binding of peptide V to HDL and LDL

The binding of peptide V to HDL and LDL was measured using the method of Hümmel and Dreyer (18) and Cann and Hinman (19). Briefly, a Sephadex G-25 fine column was equilibrated in PBS containing various concentrations of [^{3}H]Gly–peptide V. A small solution of lipoprotein at a known concentration was injected onto the column. The absorbance at 280 nm was measured to assess lipoprotein mass, and the radioactivity of each eluting fraction was measured to calculate peptide bound to the lipoprotein, which eluted at the void volume of the column. The concentration of peptide V bound to the lipoprotein was calculated from the amount of radioactivity associated with this peak minus the concentration of the free peptide, which was the concentration of the peptide in the equilibration buffer. Utilizing the different concentrations of free and bound peptide a binding isotherm was formulated and analyzed by a nonlinear least square fit (20), using the equation $P_b = (S_t P_f)/(K_d + P_f)$, where P_b = concentration of peptide bound to the lipoprotein, S_t = total sites, K_d = dissociation constant, and P_f = concentration of free peptide in solution.

Inhibitor studies

Lipoprotein binding was measured essentially as described above, but in the presence of putative inhibitors. For these experiments, labeled lipoprotein concentration (10 μg/ml) and peptide concentration (2 mg/ml) were kept constant while concentration of the inhibitor was varied. In all these experiments peptide and lipoproteins were incubated together on MEF4 cells.

In these experiments, either ^{125}I-labeled LDL or ^{125}I-labeled HDL was used as the labeled amino acid, and the results are quantitated as labeled lipoprotein specifically bound to the cell in the presence of unlabeled lipoprotein inhibitors. Specifically bound is defined as in the previous section. Theoretical curves for binding of competitors (^{125}I-LDL or ^{125}I-HDL) were calculated by nonlinear least squares analysis using the equation:

$$\%\ \text{Binding} = \left(\frac{S_T - I_{B,D}}{S_T}\right)100\%$$

where % Binding refers to the percentage of labeled lipoprotein (^{125}I-LDL or ^{125}I-HDL) binding to the cell at various concentrations of inhibitor, S_T = total sites on cell surface for binding labeled lipoprotein, and $I_{B,D}$ = bound inhibitor, displacing labeled lipoprotein. In turn, $I_{B,D}$ is given by the equation:

$$I_{B,D} = \frac{S_T I_f}{K_{d,D} + I_f}$$

where I_f = concentration of free inhibitor = $(I_{total} - I_{B,D})$, and $K_{d,D}$ = apparent dissociation constant for inhibitor in the presence of labeled lipoprotein. Combining the above two equations yields:

$$\%\ \text{Binding} = \left(\frac{S_T - \frac{S_T I_f}{K_{d,D} + I_f}}{S_T}\right)100\%$$

which is of the general form y = 100 + ((x*a)/(x + b)).

RESULTS

Peptide design

The sequences of peptides I, III, IV, and V are given below.

	Peptide	Crosslink Periodicity
I.	H_2N–GQTLSEQVQEELLSSQVTQELRAG-COOH	none
III.	H_2N–GDTLKEQVQEELLSEQVKDELKAG-COOH	i to i + 3
IV.	H_2N–GQDLSEKVQEELLESQVKDELLKAG-COOH	i to i + 4
V.	H_2N–GDTLKEQVQEELLEQVKDELKAG-COOH	i to i + 3

Peptide I contains amino acids 41–62 of human apoE, plus a Gly spacer at each end. Peptide III is a bioactive peptide which we previously reported to increase the binding of LDL to LDL-R/LRP negative cells in a conformationally specific manner. Structurally, peptide III contains two α helical segments separated by a central non-α helical, flexible loop (7, 8). We have proposed that these peptides may model a conformational switch domain in apoE (8). Peptides III and V promote LDL binding to MEF4 cells (lacking LDL receptor and LRP), whereas peptide IV does not. Thus, peptide IV, though not further analyzed in this paper, is included in the figure above to illustrate the conformational specificity of this activity. Peptide IV lacks the biological activity of the other peptides despite obvious sequence homology, and despite a high degree of structural order (nearly 100% α helical by circular dichroism and two-dimensional NMR) (9). Our previous studies of this biological activity centered on peptide III. During the course of these studies, we discovered fortuitously that deleting Ser14 from peptide III yielded peptide V, which had a 3-fold higher biological activity than peptide III (**Fig. 1**). The studies described in this paper mainly utilized peptide V.

Effects of pretreating cells with heparinase or sodium chlorate

The binding of β-VLDL and other lipoproteins to the LRP is mediated by moderate affinity interactions with cell surface glycans (10). To assess whether cell surface glycans play a role in the peptide V-mediated binding of LDL to MEF4 (LDL-R/LRP-deficient) cells, two approaches were taken. First, MEF4 cells were pretreated with heparinase, which abrogates the binding of β-VLDL to the LRP (18). Second, MEF4 cells were grown in the presence of $NaClO_3$, an inhibitor of enzymatic sulfation and cell surface glycan synthesis (19). The binding of DiI-LDL was then measured in the presence and absence of peptide V. As shown in **Fig. 2A**, peptide V increased the binding of LDL to MEF4 cells whether or not cells were pretreated with heparinase. Similarly, growing cells in the presence of $NaClO_3$ also had no effect on the observed activity (Fig. 2B). These results suggest that the increase in LDL binding induced by peptide V is not mediated via sulfated or heparinase sensitive glycosaminoglycans.

Effects of LPDS and EGTA on peptide V-mediated LDL binding

The apparent lack of effect of cell surface glycans on the activity of peptide V suggested that peptide V-mediated LDL binding might not be mediated by a member of the LDL-R family. Indeed, the activity was present on

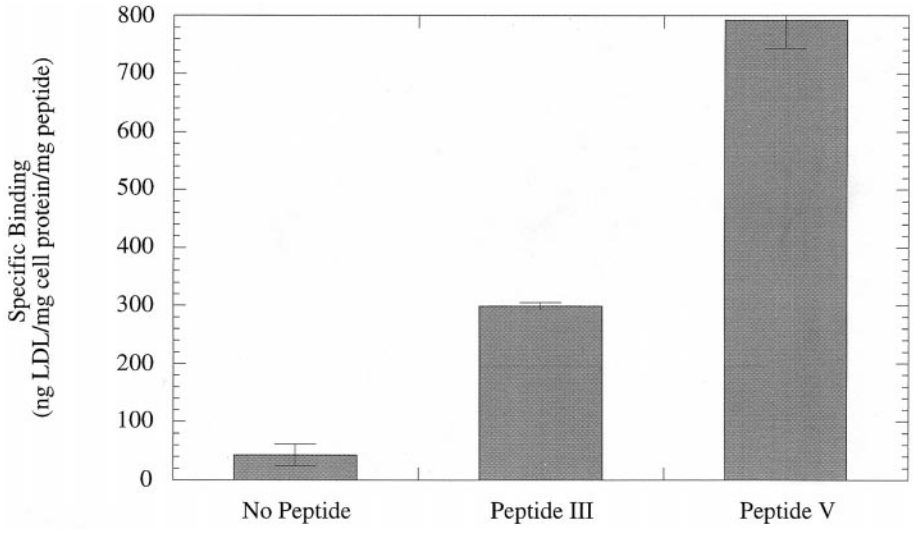

Fig. 1. Comparison of the activities of peptides III and V. MEF4 cells were incubated with DiI-LDL at a concentration of 10 μg/ml in the presence or absence of peptides at a concentration of 2 mg/ml, and in the presence or absence of a 50-fold excess unlabeled LDL. Binding activity was measured as described in experimental procedures. Level of activity refers to the specific binding, i.e., ng LDL/mg cell protein/mg peptide bound, minus the amount of nonspecific binding. Nonspecific binding is the labeled LDL bound in the presence of a 50-fold excess of unlabeled LDL. Data points represent the means of duplicate samples ± standard deviation and are one representative of multiple experiments.

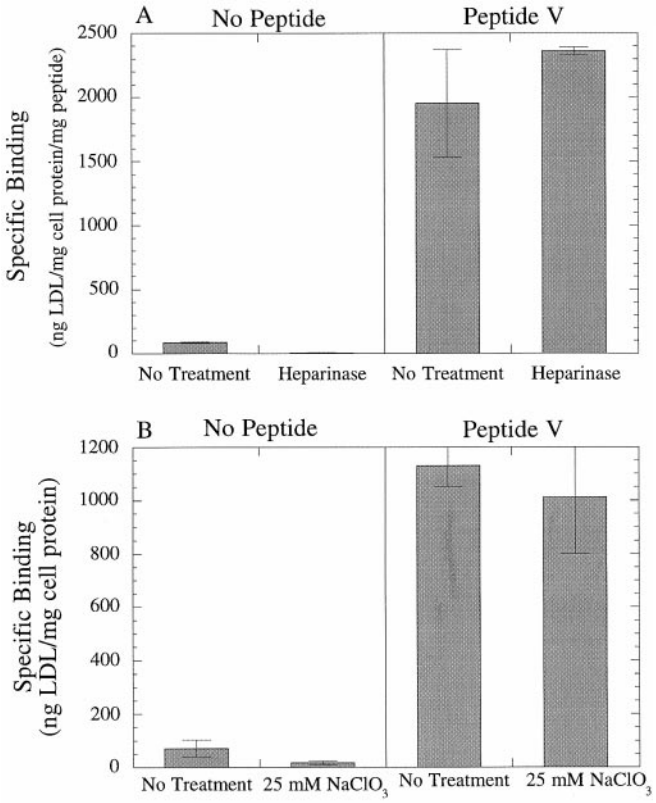

Fig. 2. (A) Effects of pretreating cells with heparinase. MEF4 cells were treated with 2.5 U/ml heparinase for 45 min at 37°C and washed once quickly with PBS. Binding activity was then measured as described in Fig. 1. Data points represent the means of triplicate samples ± standard deviation and are one representative of several experiments. (B) Effects of incubating cells in the presence of $NaClO_3$. MEF4 cells were incubated in the presence of 25 mm $NaClO_3$ for 48 h prior to the start of the experiment; binding activity was measured as described in Fig. 1. Data points represent the means of duplicate samples ± standard deviation and are one representative of several experiments.

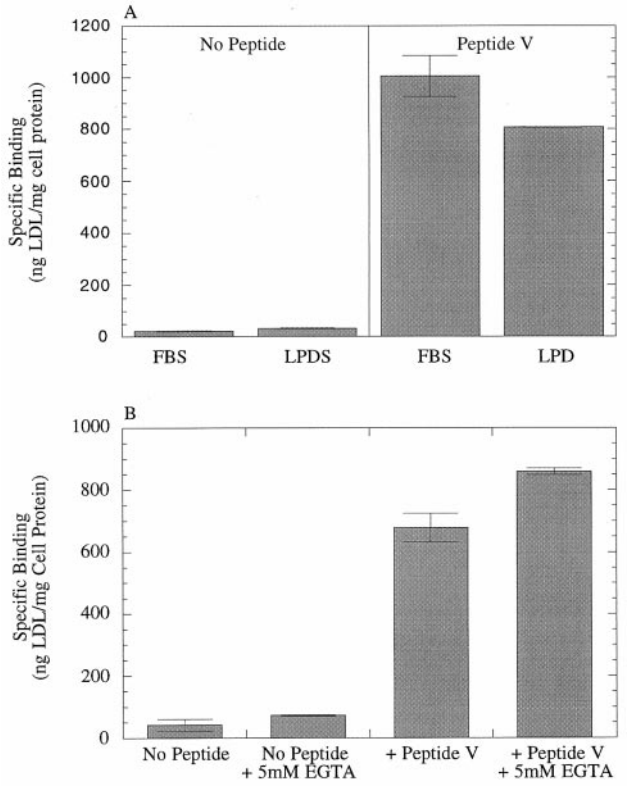

Fig. 3. (A) Effects of cholesterol deprivation on binding activity. MEF4 cells were grown in either normal FBS or in LPDS for at least 24 h prior to the start of the experiment and binding activity was measured as described in Fig. 1. Data points represent the means of triplicate samples ± standard deviation and are one representative of several experiments. (B) Effects of calcium on binding activity. Binding assays were performed either using MEF1 or MEF4 cells in the absence or presence of 5 mm EGTA and activity was measured as described in Fig. 1. Data points represent the means of duplicate samples ± standard deviations and are one representative of several experiments.

MEF4 cells which lack both LDL-R and LRP. As further assessment of the possible role of members of the LDL-R family, we investigated two other characteristics sometimes displayed by members of the LDL-R family: up-regulation by lipoprotein deprivation (for LDL-R itself (14)) and calcium dependency of ligand binding (for all known members of the LDL-R family (21–23)). To test the effects of lipoprotein-deficient serum (LPDS) on the activity of peptide V, MEF4 cells were grown for either 48 h in normal serum or for 24 h in normal serum followed by 24 h in LPDS prior to the start of the experiment. As shown in **Fig. 3A**, both sets of cells had similar levels of activity indicating that preincubating with LPDS was unable to induce, and was not necessary for, this activity. To test whether the peptide V-mediated LDL binding depends on calcium, LDL binding to MEF4 cells was measured in the presence or absence of EGTA, which chelates Ca^{2+} but does not interfere with cell adherence. As shown in Fig. 3B, the addition of EGTA did not inhibit the binding activity; if anything, there was a slight increase in activity suggesting that Ca^{2+} might even inhibit this binding activity. The above results suggest that the increased binding of LDL in the presence of peptide V might not be mediated by a member of the lipoprotein receptor family.

Effects of peptide V on the binding of different classes of lipoproteins to the cell surface

Experiments were conducted to determine whether peptide V was able to modulate the cell surface binding of lipoproteins other than LDL. Accordingly, we measured the binding of DiI-labeled LDL, HDL, and AcLDL to MEF4 cells in the presence and absence of peptide V. As shown in **Fig. 4**, peptide V not only increased the binding of LDL, but greatly increased the binding of HDL. In fact, though the number of ng of HDL bound/mg of cell protein is less than the corresponding quantity for LDL, both the fold-increase relative to the amount of binding in the absence of peptide and the molarity of particle binding was greater for HDL than for LDL. In contrast, peptide V caused only a slight increase in binding of AcLDL. To confirm that the effects of peptide V were not attributable to the use of DiI, a lipophilic dye,

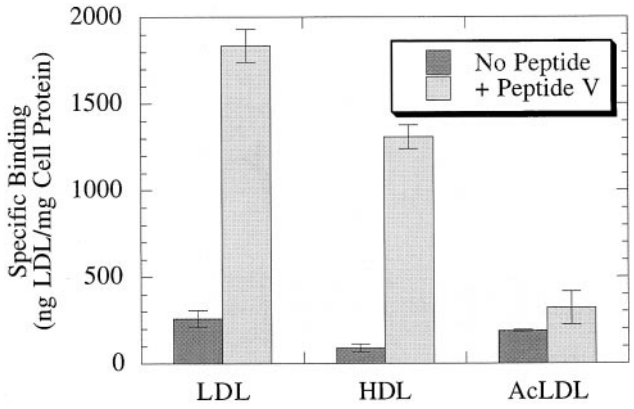

Fig. 4. Effects of peptide V on the binding of different classes of lipoproteins to the surface of MEF4 cells. MEF4 cells were incubated with DiI-labeled LDL, HDL, or Ac-LDL at a concentration of 10 μg/ml in the presence or absence of 2 mg/ml peptide V. Nonspecific binding was determined by performing experiments in the presence of a 50-fold excess (500 μg/ml) of unlabeled lipoproteins. Labeled lipoproteins were prepared and binding experiments performed as described in Fig. 1. These experiments were also repeated in the same manner except utilizing ^{125}I-labeled LDL, HDL, and Ac-LDL which yielded the same results. Data points shown represent the means of duplicate samples ± standard deviation and are one representative of numerous repeated experiments.

these experiments were also repeated using ^{125}I-labeled lipoproteins. Essentially the same results were obtained using ^{125}I-labeled lipoproteins as with the DiI label (data not shown).

Effects of peptide V on LDL binding to members of the scavenger receptor family

Several lines of evidence suggested that the effects of peptide V might be mediated by a member of the scavenger receptor family. First, the known ligands of the classical scavenger receptors are all polyanionic macromolecules, consistent with the highly anionic nature of peptide V. Furthermore, the combination of HDL or LDL with peptide V could resemble anionic modified lipoproteins, similar to AcLDL or OxLDL, both ligands of the scavenger receptor family. To test this possibility, transient transfections of Chinese hamster ovary (CHO) cells were performed using plasmid DNA from the murine scavenger receptors mSR-AI and mSR-BI, and the binding of LDL to these cells in the presence and absence of peptide V was tested. CHO cells were chosen for transfection because they do not appear to express SR-AI and SR-AII (24). As illustrated in **Fig. 5A**, in cells transfected with mSR-AI there was about a 100–200 ng increase in LDL binding (per mg cell protein) over control mock transfected cells depending on the level of receptor expression (as monitored by Ac-LDL binding). In contrast, as shown in Fig. 5B, no increase in binding was seen in cells transfected with mSR-BI. In addition, the specific LDL binding to control CHO cells and CHO cells stably transfected with CD36 (another member of the class B scavenger receptor family) was similar (Fig. 5C). These results suggest that peptide V mediates the binding of LDL to scavenger receptor AI. In addition, however, there is some portion of the binding that occurs even in mock transfected cells, indicating the involvement of another receptor in addition to scavenger receptor AI.

Effects of peptide V on lipoprotein binding to macrophages

Based upon the previous results, we tested the ability of peptide V to mediate the binding of both LDL and HDL to macrophages, cells which are known to express the scavenger receptor AI and which are involved in atherosclerotic plaque formation. Thus, it was hypothesized that peptide V should increase binding of HDL and LDL to macrophages. Binding experiments were performed using J774 cells (a macrophage cell line) in the same manner as for those utilizing MEF4 cells. **Figure 6** illustrates that, as predicted, peptide V greatly increases the binding of both LDL and HDL to J774 cells. As found by other investigators (25), these macrophage-like cells express the LDL-R and thus bind small amounts of LDL in the absence of peptide V. They do not, however, normally bind significant amounts of HDL. Thus, there is a much greater increase in HDL than LDL binding to the macrophage cell surface.

Peptide V binding to the surface of LDL and HDL

In considering the possible mechanism by which peptide V increases lipoprotein binding to cell surface via scavenger receptor AI and/or other receptors, two heuristic possibilities presented themselves. First, it is possible that the peptide and lipoprotein form a binary complex, which then is competent to bind to a cell surface receptor:

$$\text{LDL} + \text{Peptide} \rightleftharpoons \text{LDL} - \text{Peptide} + \text{R} \rightleftharpoons \text{Ternary Complex}$$

where R = receptor binding sites for the LDL–peptide binary complex. Alternatively, the peptide could interact with the cell surface receptor first, thereby facilitating the binding of the lipoprotein particle:

$$\text{Peptide} + \text{R} \rightleftharpoons \text{Peptide} - \text{R} + \text{LDL} \rightleftharpoons \text{Ternary Complex}$$

It is also possible that both mechanisms are operative, perhaps on different receptors. Both mechanisms are unified in that lipoprotein binds to the receptor only in the presence of peptide. The first mechanism requires that peptide be able to bind to lipoproteins in the absence of receptor, while the second hypothesis posits that peptide binds to receptor independently of lipoprotein. Thus, to assess whether the first hypothesis is plausible, binding of [^{3}H]Gly–peptide V to HDL and LDL was measured using the chromatographic method of Hümmel and Dreyer (18). The isotherm obtained using this method (**Fig. 7**) for peptide V binding to LDL yielded a $K_d = 2.3 \times 10^{-6}$ m, and approximately 120 binding sites per LDL particle. Thus, the binding of peptide V to LDL was of modest affinity and nonspecific. Even more strikingly, there was no measurable binding of peptide V to HDL. These results suggest that peptide V does not form a stable binary complex with lipoprotein before binding to the cognate receptor.

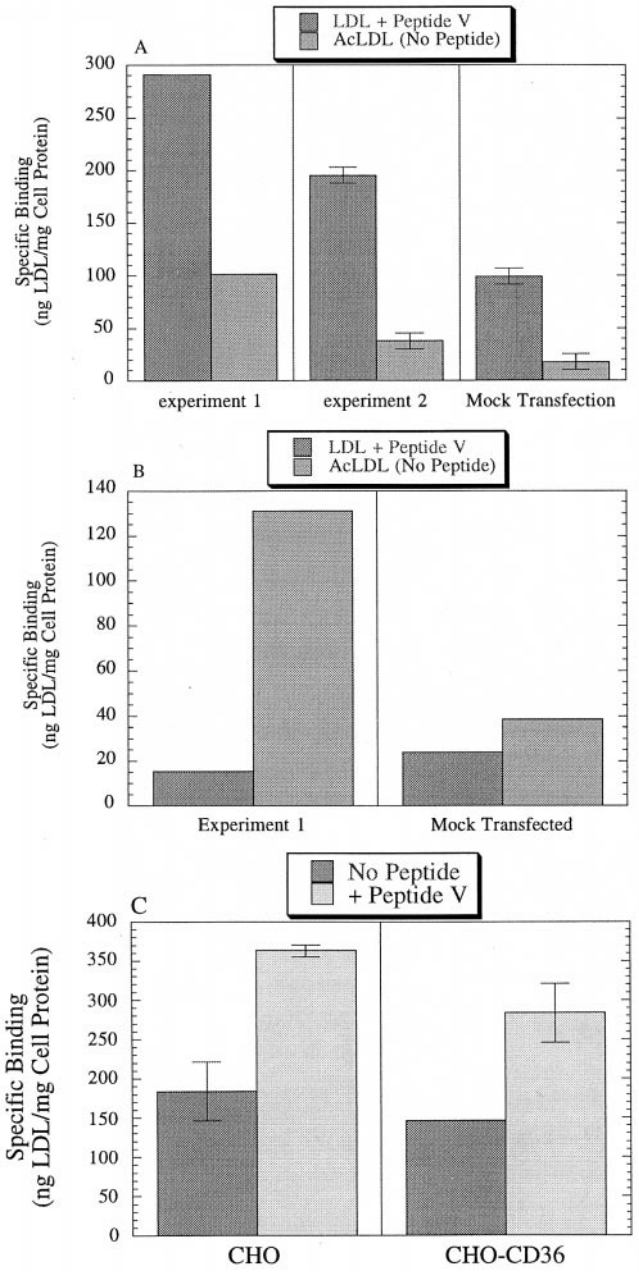

Fig. 5. (A) Net specific binding activity of LDL to mSR-AI mediated by peptide V. CHO cells were transiently transfected with the murine scavenger receptor mSR-AI as detailed in experimental procedures and binding experiments were performed on these cells and mock transfected CHO cells as described. The data are representative examples of two independent transfections and the activity is expressed as the specific binding of LDL to the cell in the presence of peptide V; also shown are similar experiments in mock transfected cells. As stated in Methods, the level of functional receptor expression was monitored by the amount of Ac-LDL binding. The level of Ac-LDL binding is expressed as the specific binding of Ac-LDL (ng/mg cell protein) to transfected cells, and is compared with Ac-LDL binding in mock transfected cells. The first experiment represents one sample and the second was performed in duplicate. (B) Net specific binding activity of LDL to mSR-BI mediated by peptide V. CHO cells were transiently transfected with the murine scavenger receptor mSR-BI as detailed and binding experiments were performed on these cells and mock transfected CHO cells as described. The data represent one transfection experiment and the activity is expressed as the specific binding to transfected cells of LDL in the presence of peptide V, as described for (A). The level of functional receptor expression was monitored by Ac-LDL binding and also expressed as previously described for (A). (C) Effects of peptide V on the binding of LDL to CHO-CD36 cells. CHO cells stably transfected with the class B scavenger receptor CD36 and untransfected CHO cells were purchased from ATCC and binding experiments were performed as described. Activity here is expressed as ng LDL/mg cell protein bound minus the amount of nonspecific binding (labeled LDL bound in the presence of a 50-fold excess of unlabeled LDL). Data points are the means ± standard deviation of duplicate sample and are one representative experiment of several.

Effect of binding of LDL and HDL on MEF4 cells preincubated with peptide V

The above results suggest that peptide V most likely activates a receptor (or receptors) for HDL and LDL binding, which are not its (their) canonical ligands. If this is true then the effects of peptide V should be maintained if cells are first incubated with the peptide, and then washed several times to remove any unbound peptide. To determine the level of peptide remaining on the cell surface after a series of washes, [^{3}H]Gly–peptide V was incubated with the cells for 1 h and then five successive washes were performed, and the radioactivity of each wash was measured. **Figure 8A** shows that by the third wash, no further peptide is removed by additional washes, and approximately 0.1–0.2% of the original peptide in solution remains bound to the cell surface, i.e., a nominal concentration of approximately 1.2 μm. Despite this fact, as illustrated in Fig. 8B, when binding experiments are performed on these cells, there is still about 50% retention of binding activity for LDL after one or four washes of the cell surface after peptide incubation. Similar results were also obtained with HDL and when macrophages were used instead of MEF4 cells (data not shown). These data are consistent with the proposal that part of the activity is due to formation of a peptide–receptor complex followed by the formation of a ternary lipoprotein–peptide–receptor complex. Furthermore, these data illustrate that peptide V is able to induce its activity at μm concentrations, which parallels the concentration of apoE found in the plasma.

Inhibition studies

To characterize this binding interaction further, inhibition studies of LDL and HDL binding were conducted. For these experiments, a constant concentration of ^{125}I-labeled LDL or ^{125}I-labeled HDL with peptide V was incubated with increasing concentrations of unlabeled LDL, HDL, or AcLDL, and the amount of bound radioactivity was measured. The results of these experiments are shown in **Figs. 9A** and **B**. As can be seen for HDL, both HDL and LDL are able to compete effectively for HDL binding. AcLDL, however, is able to inhibit HDL binding by only about 40%. In contrast, LDL is able to compete effectively for LDL binding, as expected, and HDL is able to compete for LDL binding but not as effectively as LDL (only about 60% inhibition). It appears that HDL and LDL do not exhibit complete reciprocal competition of each other. In a similar

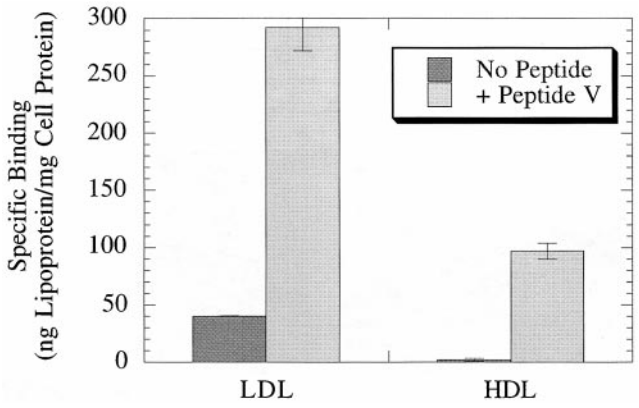

Fig. 6. Effects of peptide V on the binding of LDL and HDL to J774 macrophages. Experiments were conducted with DiI-labeled LDL and HDL at a concentration of 10 μg/ml as described previously for MEF4 cells utilizing the macrophage cell line, J774, instead of fibroblasts. Data points represent the means ± standard deviation of duplicate samples and are one representative experiment of several.

vein, AcLDL is only a very moderate inhibitor of LDL binding (about 20% inhibition). This cumulative data support the idea that another receptor besides SR-AI might be involved in this observed activity, and that these multiple binding sites have differential binding affinities for HDL, LDL, and AcLDL in the presence of peptide V.

DISCUSSION

In this paper, we have shown that peptide V, a model for the highly conserved N-terminal anionic domain of apoE, increases the binding of not only LDL but also HDL to a cell surface receptor that is probably not a member of the LDL-R family. We have also presented evidence that a part of this activity may be mediated by scavenger receptor AI, but not by other scavenger receptors, SR-BI and CD36. Consonant with that proposal is the fact that the activity is native to macrophages as well as MEF4 cells. The effect is not global for all lipoproteins, as the effect on AcLDL is modest at best. Finally, our data suggest peptide V increases HDL and LDL binding by the initial formation of a peptide–receptor complex, which is then rendered competent to bind non-canonical ligands. From the data contained in this paper, we propose that peptide V and its congeners act by first binding to a cell surface receptor; the binary complex between receptor and peptide is then rendered competent to bind HDL and LDL, not normal ligands for this receptor. The mechanism of action of peptide V might be to serve as a bridge between receptor and novel ligands. Alternatively, the bound peptide could induce a conformational change in the receptor, such that its ligand specificity is altered, i.e., without a direct contact between peptide and ligand.

In this work we have focussed on peptide V, a more potent congener of our previous model, peptide III, to characterize the biological activity of peptide models of the most highly conserved domain of apoE, including amino acids 41–60 of human apoE. The activity of these model peptides is conformationally specific. Whereas peptides III and V promote LDL binding to MEF4 cells (no LDL receptor or LRP), peptide IV, a congener of ≈100% α-helicity by CD and two-dimensional NMR, is completely inactive. We have proposed elsewhere that this domain of apoE may represent a switch domain, i.e., one capable of adopting more than one stable secondary or tertiary structure (7–9). If this proposal is correct, then the switch could render apoE competent for mediating binding to a scavenger receptor and/or another as yet unidentified receptor for which it is not normally a ligand.

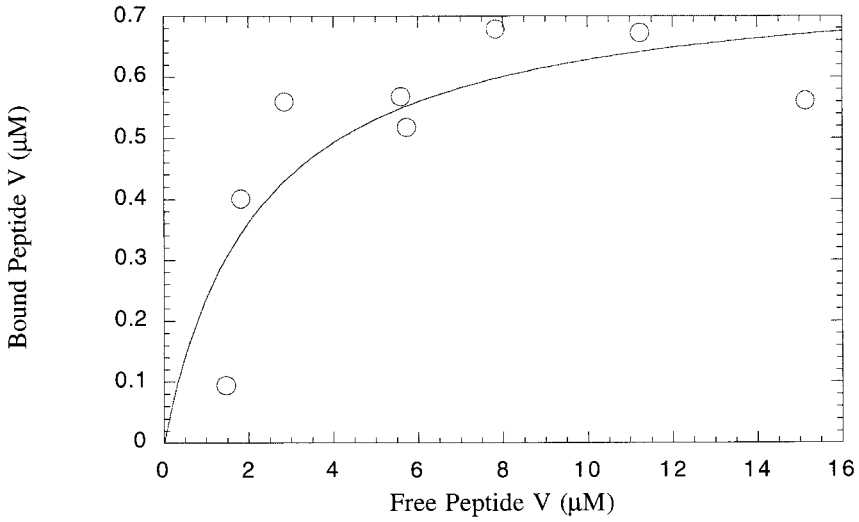

Fig. 7. Binding of peptide V to normal human LDL. The chromatographic method of Hümmel and Dreyer (18) was followed using a Sephadex G-25 column equilibrated in PBS containing various concentrations of peptide V, ranging from 1.2×10^{-6} to 2.3×10^{-5} m. LDL (1.9×10^{-8} m) was added to the top of the column, and the free and bound peptide concentrations were determined as described in Experimental Procedures. Nonlinear least squares analysis yielded $K_d = 2.3$ mm and 120 sites per LDL particle.

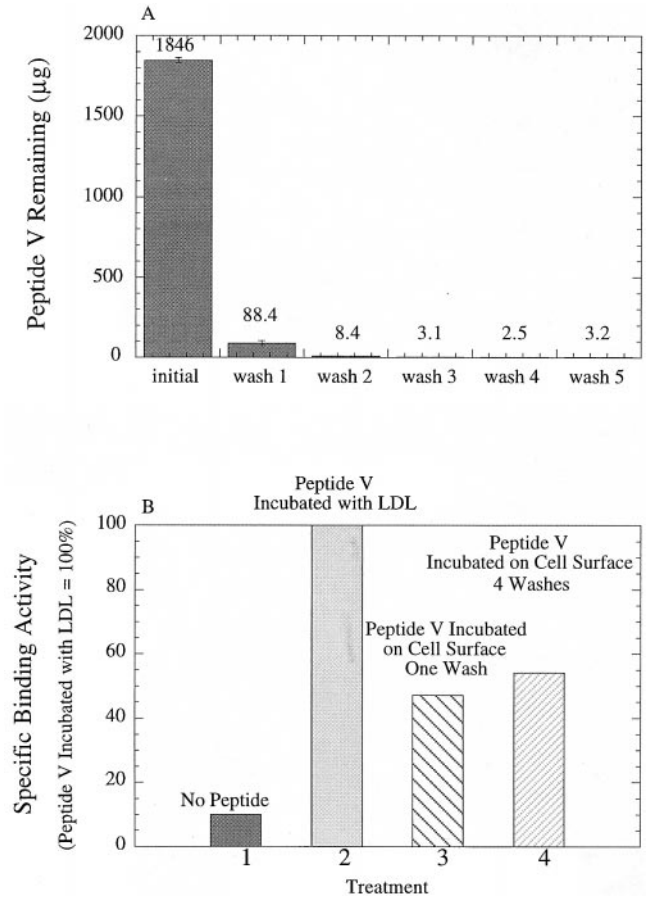

Fig. 8. (A) Amount of peptide V binding to the cell surface after a series of washes. [^{3}H]Gly-peptide V was dissolved in DMEM high glucose media at a concentration of 1846 μg/ml and 500 ml of this sample was incubated on the surface of MEF4 cells at 4°C for 1 h. This solution was then removed and the cells were washed up to five times with 1 ml of PBS. The initial solution and each wash solution were saved for analysis. An aliquot of each wash solution was counted and the amount of peptide remaining bound to the cell surface was determined. Actual numbers are given above each bar. Data point are the means ± standard deviation of triplicate samples. (B) Effects of binding of LDL to MEF4 cells preincubated with peptide V. MEF4 cells were incubated for 1 h at 4°C in DMEM high glucose without peptide (1) or with peptide V at a concentration of 2 mg/ml (3 and 4). The cells were then washed quickly either once (3) or four (1 and 4) times with PBS and then incubated for 3 h with a solution of 10 μg/ml DiI-LDL ± 500 μg/ml unlabeled LDL and binding activity was measured as in previous experiments. As a control (2), cells were incubated with DiI-LDL and peptide V together and binding activity was measured as described in Fig. 1. The binding activity obtained when peptide V and DiI-LDL were incubated together (2) was set = 100% and the activity of the other treatments was expressed relative to 2.

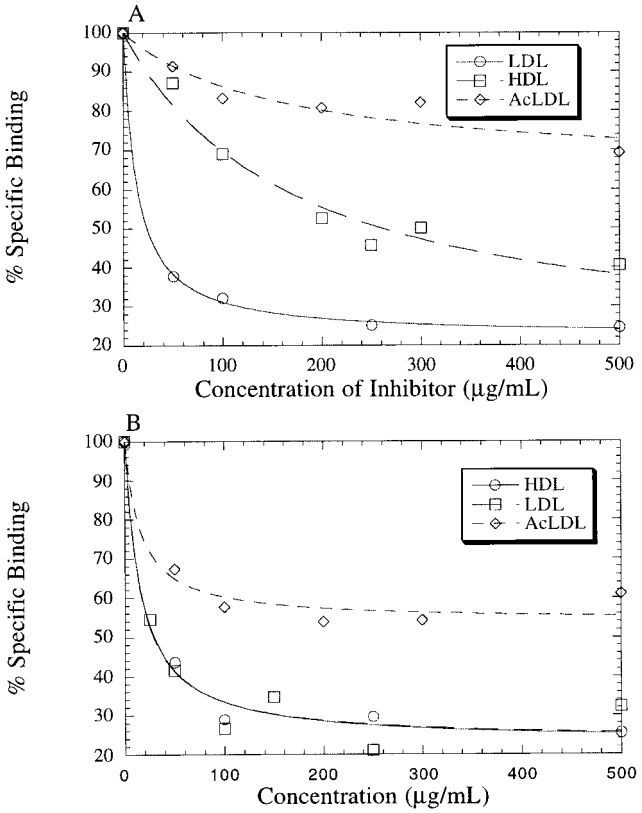

Fig. 9. (A) Inhibition of peptide V-mediated binding of LDL by LDL, HDL, and AcLDL. MEF4 cells were incubated with 10 μg/ml ^{125}I-labeled LDL, 1 mg/ml peptide V, and the indicated amount of unlabeled LDL, HDL, or AcLDL for 3 h at 4°C. The concentration of LDL bound was measured as described in Experimental Procedures. Theoretical curves were calculated using nonlinear least squares analysis utilizing the equation:

$$\% \text{ Binding} = \left(\frac{S_T - I_{B,D}}{S_T}\right) 100\%$$

where % Binding refers to the percentage of labeled lipoprotein (^{125}I-LDL in A, ^{125}I-HDL in B); the terms in the equation are described in Experimental Procedures. (B) Inhibition of peptide V-mediated binding of HDL by HDL, LDL, and AcLDL. MEF4 cells were incubated with 10 μg/ml ^{125}I-labeled LDL, 1 mg/ml peptide V, and the indicated amounts of unlabeled HDL, LDL, or AcLDL for 3 h at 4°C. The concentration of HDL bound was measured as described in Experimental Procedures. Theoretical curves were calculated as for Fig. 9A.

SR-AI may not account for all of the observed peptide V-mediated lipoprotein binding. Peptide V was able to increase the binding of LDL to control, nontransfected CHO cells, even though these cells are reported not to express scavenger receptor activity (24), though they do express the LDL-R and some of the observed LDL binding in mock transfected cells may be attributable to this receptor. Our inhibition data using MEF4 cells, however, also support the idea of the involvement of an additional receptor. AcLDL, one of the defining ligands for SR-AI, was able to inhibit only half of the peptide V-mediated HDL binding and even less, only about 25%, of peptide V-mediated LDL binding. The inhibition data also shed light on the possibility that HDL and LDL may be interacting at different sites on the cell surface, as evidenced by HDL's inability to inhibit completely peptide V-mediated LDL binding. Furthermore, it is not entirely clear whether our results in transfected CHO cells can be fully applied to MEF4 cells. Additional work is needed to shed light on the degree of scavenger receptor expression in these cells and to determine to what extent they are involved in the observed

binding activity. The work presented in this paper tested only a few of the scavenger receptors; additional work is necessary to characterize effects of peptide V on other members of this family (24–28). Of particular note are SR-AII and MARCO, which display similar ligand recognition to SR-AI and are predominantly expressed in macrophages. Similarly, two other macrophage scavenger receptors, CD68 and FcγRII-B2 (29, 30), are also potential candidates. At present, the list of known scavenger receptors is growing, and it is possible that the remainder of our activity is occurring through other novel, as of yet, unidentified members of the scavenger receptor family.

ApoE functions as a ligand for both the LDL-R, the LRP, and other members of the LDL-R family, to which it binds through a cationic domain at amino acids 140–150 in human apoE (4–6). The binding of apoE to cell surface receptors is conformationally specific, as shown by differences between exogenous and endogenous apoE in mediating the binding of β-VLDL to the LRP (23), and differences in the binding of lipidated (in HDL_c) and lipid free apoE to the LDL-R (31, 32). Here we have presented data consistent with the hypothesis that under some circumstances, apoE could serve as a ligand for another class of lipoprotein receptors, the scavenger receptors. Such a hypothesis would require that the highly anionic region of apoE would, under some circumstances, have a conformation similar to that of peptide V.

The proposal that apoE could, under some circumstances, function as a ligand for a scavenger receptor has implications for atherogenesis. In the artery wall, the majority of lipoprotein cholesterol taken up by macrophages is from modified LDL, particularly oxidized LDL, via a scavenger receptor mediated pathway (26). Peptide V promotes LDL and HDL, but not AcLDL binding to at least one member of the scavenger receptor family. It is possible then, that peptide V could oppose the binding of modified LDL by macrophages, by promoting the binding of LDL or HDL to scavenger receptor sites. To the extent that peptide V models a conformational state of the conserved anionic domain of apoE, the secretion of apoE by macrophages could therefore serve to modulate the binding of oxidized or other modified LDL by the macrophage. Data presented in this paper indicate that the effect of peptide V occurs even at micromolar concentrations, i.e., comparable to concentrations of apoE in the plasma. In addition, effects of apoE are often mediated by higher *local* concentrations. For example, lipid-free (or -poor) apoE is secreted by hepatocytes into the space of Dissé, and there mediates the binding of chylomicron remnants to LRP and the VLDL receptors on hepatocytes (33–37). As macrophages also secrete apoE, the local concentration of apoE near the macrophage cell surface could be even higher than that in plasma. In fact, some of the apoE secreted by macrophages remains on the cell surface, bound to proteoglycans. Indeed, the expression of apoE is enhanced by increasing cellular cholesterol content (38–40). Furthermore it has been shown in vitro that apoE secretion by lipid laden macrophages is able to facilitate the net efflux of cholesterol from macrophages into the media in the presence of the cholesterol acceptor, HDL_3 (41). Peptide V could potentially act in a fashion similar to apoE in this situation, encouraging the net efflux of cholesterol out of lipid-laden foam cells to HDL and also possibly LDL.

Scavenger receptors have been widely implicated in the uptake and deposition of cholesterol in the arterial wall during atherogenesis and for that reason have been under intense study (26). Recently, using double apoE/SR-A knockout mice, Suzuki et al. (42), have shown that these receptors clearly play a critical role in vivo in the development of atherosclerosis by demonstrating that SR-A deficiency results in a 60% reduction in atherosclerosis development in apoE-deficient mice. The role of apoE as a modulator of scavenger receptor function requires direct testing which would go beyond the suggestive data presented in this paper. It is possible, for example, that even if peptide V were a model for some conformational state of apoE, its ability to promote LDL binding would have a pro- rather than anti-atherogenic effect by promoting cholesterol entry into the macrophage. Nevertheless, if it can be shown that peptide V does facilitate the net efflux of cholesterol from macrophages, it then would be plausible that apoE modulates this critical scavenger receptor function, and that peptide V or its congeners would model this aspect of atherogenesis or even serve a potential therapeutic function as an anti-atherogenic agent.

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