Conformationally Specific Enhancement of Receptor-Mediated LDL Binding and Internalization by Peptide Models of a Conserved Anionic N-Terminal Domain of Human Apolipoprotein E†

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ABSTRACT: In this paper, we test the hypothesis that peptide models of a highly conserved domain of apolipoprotein E (amino acids 41−60 in human apo E) modulate the binding and internalization of LDL to cell surface receptors in a conformationally specific manner. Three peptides were compared: peptide **I** containing the natural sequence of amino acids 41−60 of human apo E; peptide **III** containing side-chain lactam cross-links designed to enhance α-helical structure; and peptide **II** containing cross-links designed to prevent formation of α-helices. Peptide **III** was shown previously to consist of two short α-helical domains linked by a turn and to have more α-helical content than peptide **I**, while peptide **II** was shown to have less helical content than either peptide **III** or **I** (Luo et al., 1994). Peptide **III** induced a 30-fold increase in the specific binding of ^{125}I-LDL to normal human skin fibroblasts and a 60-fold increase in the binding to fibroblasts lacking the LDL-R. This same peptide also restored the binding to normal fibroblasts of ^{125}I-LDL from a patient with familial defective apolipoprotein B, the $R_{3500} \rightarrow Q$ mutation. Analysis of binding indicated an increase in the apparent number of binding sites, with little effect on the affinity of ^{125}I-LDL for the cell surface. Heparinase treatment of the cells did not abrogate this effect, suggesting that the increased binding is not mediated by cell surface glycans. LDL internalization but not degradation was also increased by peptide **III**. Similar but smaller effects were also induced by peptide **I**. Peptide **II** was much less active than peptide **I** or **III**. Thus, the order of biological activity was the same as the order of α-helical content, i.e., peptide **III** > peptide **I** > peptide **II**. These results suggest a hitherto unknown biological function for a highly conserved domain of apolipoprotein E, and this bioactivity was shown by peptide models to be specific to the α-helical conformation.

Apolipoprotein E is a ligand for the LDL receptor (LDL-R) and the LDL receptor related protein (LRP), to which it binds through a cationic domain at residues 140−150 in human apo E (Mahley, 1988; Innerarity et al., 1983). The most highly conserved domain, however, is an anionic region encompassing residues 41−60 of human apo E (Matsushima et al., 1988; Braddock et al., 1990). The structure and function of the latter domain are not known. It is possible that the anionic domain might interact with receptor and/or heparin-binding domains of apo E and/or apo B, since the latter are cationic. Through this interaction, the conserved amino-terminal domain of apo E might modulate binding of LDL, IDL, and other lipoproteins to their cell surface receptors.

The binding of apo E and apo B to receptors appears to be conformationally specific. Two examples from the literature are the following. First, while endogenous apo E in β-VLDL is inactive for binding to LRP, exogenous apo E is active, suggesting that a reversible conformational change in apo E may be required for receptor binding activity (Kowal et al., 1990). Second, the delipidated N-terminal thrombolytic fragment of apo E binds to the LDL-R with only $^{1}/_{500}$th of the affinity of HDL_c, despite the fact that apo E is virtually the only protein on HDL_c (Wilson et al., 1991; Innerarity et al., 1979). Since both delipidated apo E and lipidated apo E appear to bind to LDL-R via the same domain at amino acids 140−150, the protein may undergo a conformational change which activates it for binding to the receptor.

In this paper, we test the hypothesis that a peptide containing the conserved anionic domain (residues 41−60) and certain models of this domain activate apo E or apo B for binding to cell surface receptors in a conformationally specific manner. We have previously shown, by a detailed structural analysis using two-dimensional NMR spectroscopy, that a peptide model of the domain is constrained by two side-chain lactam cross-links to adopt an α-helical conformation. This peptide, termed peptide **III**, contains two short α-helical segments connected by a turn, a structure resembling that of the corresponding domain in apo E. In this paper, we demonstrate that a synthetic peptide containing the unmodified sequence of amino acids 41−60 of human apo E enhances specific binding to and internalization by cell surface lipoprotein receptors. To demonstrate that the biological activity is conformationally specific, we show that the α-helical peptide model, peptide **III**, is even more active

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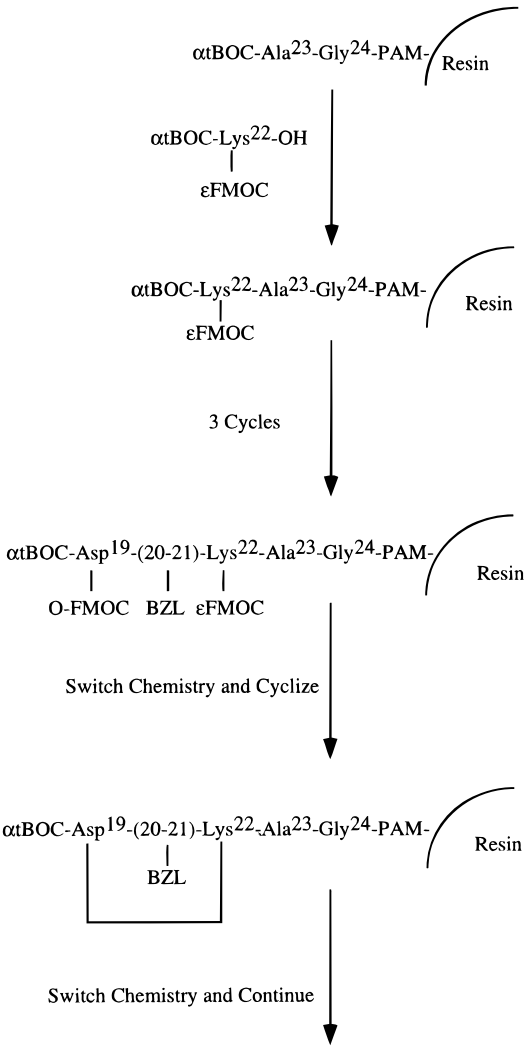

FIGURE 1: Synthetic scheme used in the the synthesis of peptides **II** and **III**. The figure shows the synthesis of the first lactam bridge in peptide **III**, using a synthetic scheme modeled after that of Felix et al. (1988).

than the un-crossed-linked peptide, while another model (peptide **II**), which is unable to adopt the α-helical conformation, is far less active.

MATERIALS AND METHODS

Peptide Synthesis and Purification. All amino acids were purchased from Applied Biosystems (ABI, Foster City, CA) except N^{α}-tBoc Asp O^{β}-Fmoc and N^{α}-Boc Lys N^{ϵ}-Fmoc, which were from Bachem (Torrance, CA) or Peninsula Laboratories Inc. (Belmont, CA), and [^{3}H]Gly was from Amersham Corp. (Arlington Heights, IL). For synthesis of radiolabeled peptides, the tBoc group was attached to [^{3}H]Gly by the method of Schnabel (1967). All peptides were synthesized on the 0.5 mmol scale using an ABI 431A peptide synthesizer equipped with system 1.12 software with minor modifications to account for the lower solubility of the Fmoc derivatives and in order to solvate a larger volume of resin when in Fmoc chemistry. In order to make peptides containing intramolecular amide cross-links, a synthetic plan similar to that of Felix et al. (1988) was used, as represented in Figure 1 for peptide **III**. To aid in the eventual removal of peptide chains in which a lactam bridge had failed to form, any unreacted Lys ϵ-amino side-chain groups were coupled to N^{α}-tBoc-*S*-(methylbenzyl)-Cys. After completion of the peptide synthesis, and cleavage of peptide from resin, cysteine-containing peptides (i.e., un-cross-linked peptides) were removed from the mixture by reaction with PAM−resin−cysteine beads. The progress of the synthesis was monitored using the quantitative ninhydrin procedure (Sarin et al., 1981). Peptides were cleaved from the resin with HF in an Immuno-Dynamics (La Jolla, CA) HF apparatus for 1 h at −3 to −5 °C, using HF/DMS/anisole/*p*-thiocresol/resin = 10/1/1/0.2/1 (v/v/v/v/w). Peptide was extracted multiple times from the resin in 2.5% (w/v) ammonium bicarbonate in water and ether, and the aqueous phases from the extractions (≈1 L) were pooled and lyophilized. The peptides were desalted by gel filtration chromatography, using Sephadex G-25 (0.5% w/v ammonium bicarbonate as eluent), and purified by preparative reverse-phase HPLC using a Waters 600 E HPLC (Waters Chromatography Division, Millipore, Inc., Milford, MA) and a preparative C-4 Dynamax column (Rainin Instrument Co., Inc., Woburn MA; 5 μm bead size, 300 Å pore size, 21.4 mm diameter, 250 mm length). The solvent system consisted of a gradient of 0.05 M trimethylamine phosphate (pH 3.00) and acetonitrile. Peptides eluted at approximately 28−32% acetonitrile.

Peptide Characterization. Peptides were assessed by amino acid analysis, analytical HPLC, and fast atom bombardment mass spectroscopy in order to assess purity and to ascertain whether the cross-links had formed and to check for uncyclized impurities. Occasionally, the peptides were sequenced for an additional check of purity, using an ABI 473A sequencer with an on-line ABI 120 PTH-amino acid analyzer with pulsed liquid configuration using Edman degradation chemistry. Amino acid analysis was performed on a Hewlett Packard 1090 HPLC (Palo Alto, CA) as previously described (Heinrikson & Meredith, 1984). Fast atom bombardment mass spectrometry was performed on a Finnigan MAT 90 mass spectrometer. The lyophilized sample was dispersed in glycerin, and the sample was ionized with xenon gas at a voltage of 8 kV. The criteria for purity were that the peptides possessed the correct molecular weight as determined by mass spectroscopy, that the amino acid composition and sequence were as expected, and that only one peak be present on a C-18 column in an analytical reverse-phase HPLC separation. Specific radioactivity of ^{3}H-labeled peptides was calculated by triplicate measurements of radioactivity of stock solutions of which the concentration had been previously determined in triplicate by amino acid analysis.

LDL and Lipoprotein-Deficient Plasma Isolation. Plasma was obtained from the University of Chicago Hospitals blood bank plasmapheresis program from normolipidemic patients undergoing therapeutic plasmapheresis for myasthenia gravis or from normolipidemic volunteers in good health. Prior to isolation the following preservatives were added to the plasma: 1 mM/mL PMSF and BHT, 0.02% w/v NaN_3, and 0.1% w/v EDTA. LDL ($1.019 \leq d \leq 1.063$ g/cm^3) and the bottom fraction ($d > 1.21$ g/cm^3) were isolated by differential flotation as described previously (Havel et al., 1955). Following the isolation of either fraction, the concentrations of PMSF and BHT were readjusted to 1 mM and NaN_3 and EDTA to 0.2% and 0.1% w/v, respectively.

Familial Defective Apolipoprotein B (FDB) and Glutathione S-Transferase/Receptor Associated Protein (GST−RAP) Fusion Protein. Plasma from a patient who was

heterozygous for the $R_{3500} \rightarrow Q$ mutation of apolipoprotein B (FDB) (Innerarity et al., 1987, 1990) was the kind gift of Thomas Innerarity at the Gladstone Institute, San Francisco, CA. A fusion protein of 39 kDa α_2-macroglobulin receptor associated protein with glutathione *S*-transferase (GST−RAP) (Herz et al., 1991; Williams et al., 1992) was the generous gift of Dudley Strickland, American Red Cross, Rockville, MD.

Effects of Peptides on Cell Surface Binding, Internalization, and *Degradation of ^{125}I-LDL.* Preparation of lipoprotein-deficient serum and iodination of lipoproteins were as described by Goldstein et al. (1983). The protein concentration was measured by the Lowry method (Lowry et al., 1951). The radioiodinated LDL was used on the third day after iodination. Lipoprotein binding to cells was measured by described procedures (Goldstein et al., 1983). The following HSF cell lines were obtained from American Type Culture Collection (ATCC): CRL 1475 (normal) and GM 486 (Kaneko et al., 1978) (cells deficient in the LDL-R from a mutation in the promoter resulting in a type 1 LDL-R mutation) and 701B (cells with an uncharacterized defect in the LDL-R, the Khatchadurian mutation (Katchadurian & Uthman, 1973). The cells were grown in modifided Eagle's medium supplemented with 25 mM HEPES, 10% fetal bovine serum, 1% L-glutamine, and 0.5% penicillin streptomycin solution. The cells were maintained at a temperature of 37 °C with an ambient CO_2 concentration of 5% in T-75 flasks until needed. Cells were trypsinized and plated into 35 mm diameter six-well tissue culture dishes containing 2 mL of medium. When two-thirds confluence had been reached, the growth medium was removed and replaced with medium containing modified Eagle's medium supplemented with 25 mM HEPES, 10% lipoprotein-deficient serum (LPDS), 1% L-glutamine, and 0.5% penicillin/streptomycin solution (DMEM + LPDS). The cell monolayers were incubated for 20−24 h prior to the start of the experiment in order to increase the expression of LDL receptors on those cells possessing them. One hour prior to the beginning of the experiment, ^{125}I-LDL was pipetted into a sterile vial containing DMEM + LPDS to achieve a final concentration of 10 μg of ^{125}I-LDL/mL of solution. The solution was gently mixed and the contents of the vial were divided. Depending on the experiment, one of the replicates received the indicated concentration of one of the following: the *i* to *i* + 3 dicyclic peptide, the *i* to *i* + 5 dicyclic peptides, or the linear analog, and one replicate received no peptide. The resulting experimental groups were then gently mixed and split into two equal parts. One part remained as is and one part received LDL to achieve a concentration of cold LDL of 50 times the concentration of radiolabeled LDL. This latter experimental group was used to determine the nonspecific association of ^{125}I-LDL with the cells. The experimental groups were then incubated for 1 h before the beginning of the experiment at room temperature; then 1 h prior to the start of the experiment the cells were placed on an orbital shaker at 4 °C (one rotation/s). At the start of the experiment, the DMEM + LPDS medium was removed from the cells and replaced with 1 mL of the appropriate ice-cold experimental solutions. The cells were incubated for 3 h at 4 °C while being gently agitated (one rotation/s). At the conclusion of the incubation period, the experimental medium was removed from the cells, and each dish was washed three times rapidly with 2 mL of ice-cold PBS containing 2 mg/mL bovine serum albumin (BSA). The rapid washes were followed by two 10 min washes of the same solution and then a final quick wash with ice-cold PBS (with no added BSA). Each of the cell dishes then received 2 mL of ice-cold PBS containing heparin (10 mg/mL). The cells were incubated with agitation for 30 min at 4 °C after which the solution was collected and ^{125}I measured. The cells were dissolved by incubation at room temperature for 15 min with 1 mL of 0.1 N NaOH. The cell solution was removed quantitatively from the dish, and the protein content was determined by the Lowry method. Specific binding was determined by subtracting nonspecifically bound LDL from the total LDL bound. Unless otherwise specified, each data point was the average of triplicates.

In more recent experiments, mouse embryonic fibroblast cell lines were used, obtained from Drs. Joachim Herz and Thomas E. Willnow (University of Texas Southwestern Medical Center, Dallas). These included wild-type and LDL-receptor/LDL-receptor related protein (LRP) deficient cells ("double mutants"). In the experiments concerning the mouse embryonic fibroblasts, two different assays for LDL binding were used: first, the above cited method of Goldstein et al. (1973); and second, the method of Stephan and Yurachek (1993), which measures binding of 3,3′-dioctadecylindocarboxycyanine-labeled LDL (DiI-LDL) by fluorescence spectroscopy.

To measure LDL internalization and degradation, two sets of experiments were conducted. First, internalized ^{125}I-LDL was measured as described (Goldstein et al., 1983) as the ^{125}I radioactivity within cells at 37 °C resistant to release by heparin. To measure proteolytic degradation of ^{125}I-LDL, the retained medium is treated with trichloroacetic acid as described (Goldstein et al., 1983). Second, incorporation of [^{14}C]oleate into cholesteryl esters was measured as described (Goldstein et al., 1983) using LDL-receptor negative cells.

Effects of Pretreating Cells with Heparinase on LDL Binding to the Cell Surface. To test the role of cell surface glycans in the binding of LDL to LDL-receptor negative cells, these cells were treated with heparinase (Sigma, heparinase I, catalog no. 2519) and washed as described by Ji et al. (1993). The binding of ^{125}I-LDL to these cells, in the presence and absence of peptides, was then measured as described above.

Binding Isotherm of the Peptides to LDL. To measure the binding of peptide **I** to LDL, an Amicon filtration system was utilized as described previously (Yokoyama et al., 1980). Because of nonspecific adsorption of peptide **III** to the Amicon filter, the binding of peptide **III** to LDL was measured using the chromatographic method of Hümmel and Dreyer (1962). Bound peptide was calculated by subtracting the (free + bound) − (free) peptide, and the data were analyzed by a nonlinear least squares fit (Yamaoka et al., 1981), using the equation $P_b = (S_t P_f)/(K_{d1} + P_f)$, where P_b = concentration of peptide bound to LDL, S_t = total sites, K_{d1} = dissociation constant, and P_f = concentration of free peptide in solution.

Binding Isotherms for the Peptide−LDL Mixtures to Human Skin Fibroblasts. To obtain a more detailed analysis of the binding of LDL and LDL−peptide mixtures to the cell surface, human skin fibroblasts were grown, and human LDL was prepared and isolated in the same manner as for the above LDL binding studies. ^{125}I-LDL was incubated at increasing concentrations alone or in the presence of 1 mg/

mL of peptides **I**, **II**, or **III** at room temperature for 1 h prior to the start of the experiment. The binding of a range of concentrations of ^{125}I-LDL with or without peptides to the surface of human skin fibroblast cells was then measured as described above. In order to analyze these binding isotherms mathematically, experimental conditions were chosen so that peptide III adsorption to the cell would not significantly lower the concentration of peptide **III** in the aqueous phase and so that the LDL surface would be saturated by peptide. To satisfy both of these requirements, experimental conditions were chosen so that [peptide] >>> [LDL] and so that [peptide] >>> K_{d1}, the dissociation constant for the binding of peptide to the LDL particle. We show below that

$$\text{peptide} + \text{LDL} \underset{K_{d1}}{\rightleftharpoons} \text{complex} \quad (1)$$

and that the K_d in the above equation for peptide **III** is in the micromolar range. Peptide concentrations were chosen so that the LDL surface would be saturated with peptide, i.e., approximately 2 orders of magnitude above the K_d. Thus, in these experiments, virtually all of the LDL is present as saturated peptide **III**−LDL complexes. Furthermore, under these conditions, the concentration of free peptide is approximately equal to the total concentration of peptide ($P_{total} \approx P_{free}$) throughout the concentration range of LDL used in the experiment:

$$P_{free} + \text{LDL}_{free} \underset{K_{d1}}{\rightleftharpoons} \text{peptide}-\text{LDL} + S_{free} \underset{K_{d2}}{\rightleftharpoons} \text{ternary complex} \quad (2)$$

where S_{free} = binding sites on the cell surface for the LDL−peptide **III** complex and K_{d2} = the dissociation constant for the ternary complex of these sites and LDL−peptide **III**. A proportionality constant, α, was included to account for nonspecific binding of peptide−LDL complexes to the cell surface. The binding parameters were determined using a nonlinear least squares fit as follows. Since P_{total} >>> LDL, $P_{total} \approx P_{free}$ and total LDL ≈ LDL−peptide complex, i.e., (LDL_t) ≈ ($LDL-peptide_{total}$), the equation for the reaction scheme shown as eq 2 can be approximated as

$$(\text{LDL}-\text{peptide})_{free} + S_{free} \underset{K_{d2}}{\rightleftharpoons} \text{ternary complex} \quad (3)$$

where $(LDL-peptide)_{free}$ = LDL−peptide complex not bound to the cell surface (free in solution), S_{free} = unoccupied binding sites for the LDL−peptide complex on the cell surface, and ternary complex = ternary complex of LDL−peptide−site. Thus

$$K_{d2} = \frac{(\text{LDL}-\text{peptide})_{free}(S_{free})}{(\text{ternary complex})} \quad (4)$$

Since ternary complex <<< $(LDL-peptide)_{total}$, it follows that $(LDL-peptide)_{free} \approx (LDL-peptide)_{total}$. Since $(LDL-peptide)_{total} \approx LDL_{total}$ and $S_{free} = S_{total}$ − ternary complex, where S_{total} = total sites on the cell surface, eq 4 becomes

$$K_{d2} = \frac{\text{LDL}_{total}(S_{total} - \text{ternary complex})}{(\text{ternary complex})} \quad (5)$$

which yields

$$\text{ternary complex} = \frac{\text{LDL}_{total}S_t}{K_{d2} + \text{LDL}_{total}} \quad (6)$$

Accounting for nonspecific binding using the proportionality constant α, one obtains

$$\text{ternary complex} = \frac{\text{LDL}_{total}S_{total}}{K_{d2} + \text{LDL}_{total}} + \text{LDL}_{free}\alpha \quad (7)$$

This final equation is in experimentally measurable terms: the variables are ternary complex and $(LDL)_{total}$, and the remaining terms are parameters which can be calculated using nonlinear least squares methods.

Ligand Blots. Fresh rat liver membranes were isolated and electrophoresed under nonreducing conditions for ligand blotting according to the procedure of Hui et al. (1980). Proteins were transferred to nitrocellulose paper and ligand blots obtained according to the methods of Hui et al. (1980). They were then dried on paper and exposed to Kodak X-OMAT AR film using a DuPont Cronex Lightning Plus intensifying screen for 44 h at −70 °C. The LDL alone blot was subsequently reexposed to film under the same conditions for 3 weeks.

RESULTS

Design, Synthesis, and Characterization of Peptides. The following three peptides were synthesized:

I. H_2N-GQTLSEQVQEELLSSQVTQELRAG-CO_2H

II. H_2N-GDTLSEKVKEELLESQVKQELLDA-CO_2H

III. H_2N-GDTLKEQVQEELLSEQVKDELKAG-CO_2H

Peptide **I** contains the conserved domain (amino acids 41−60) and amino acids 61 and 62 of human apo E, plus a Gly spacer at each end. The two models, peptides **II** and **III**, contain side-chain lactam cross-links between the *i*th and *I* + 5th or *i*th and *I* + 3rd residues, respectively. Amino acids 41−60 are arrayed as two potential structures, the π- and α-helices, in the helical net diagrams shown in Figure 2. Peptide **III** was previously shown to contain two short α-helical domains separated by a turn (Luo et al., 1994). The hydrophobic side chains of the two α-helical domains are oriented toward each other to allow for the formation of a hydrophobic "pocket". The goal of synthesizing peptide **II** was to disrupt the α-helices; in addition, it is compatible with the hypothetical structure, the amphiphilic π-helix. We have previously shown by circular dichroic spectroscopy that peptide **II** has a far lower α-helical content than peptides **I** or **III** (Braddock et al., 1990; Luo et al., 1994). As shown in the figure, this domain appears amphiphilic only when arrayed as a π-helix. The positions of all lipophilic and acidic residues of peptide **I** have been conserved in peptides **II** and **III**. The placement of cross-links (solid lines) and salt bridges (broken lines) was chosen, wherever possible to replace Gln or Ser, since Gln and Ser resemble the cross-links and salt bridges in being hydrophilic but having no net charge; in addition, Gln has a side-chain amide like that of the cross-link.

By the criteria of analytical HPLC, amino acid analysis, fast atom bombardment mass spectrometry, and amino acid

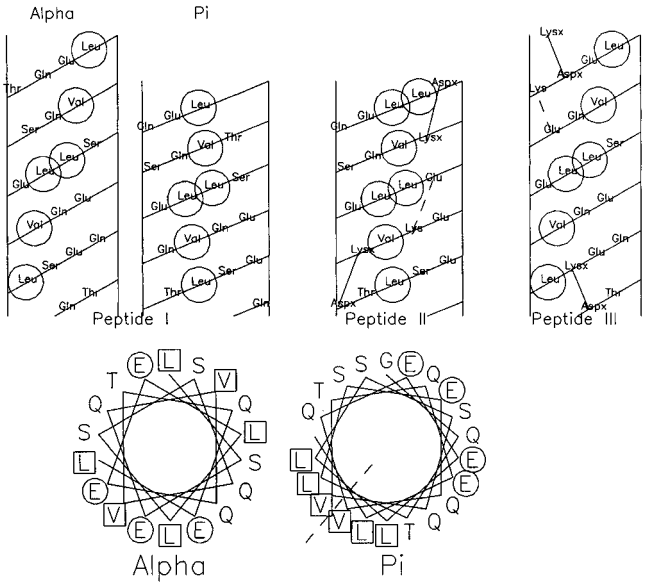

FIGURE 2: Helical net and wheel drawings for peptides **I**, **II**, and **III**. Top row, from left to right: helical net diagrams of peptide **I** arrayed as an α- and a π-helix, peptide **II** arrayed as a π-helix, and peptide **III** arrayed as an α-helix. Lys and Asp residues incorporated into lactam bridges are represented as Lysx and Aspx, respectively, and are connected by solid lines. Hypothetical salt bridges are shown as dotted lines. Lipophilic residues are circled. Bottom row, from left to right: α- and π-helical wheel representations of portions of peptide **I**. Acidic residues (in this case, all Glu) are boxed; lipophilic amino acids are circled. A plane of symmetry in the hypothetical π-helix is represented by a dotted line.

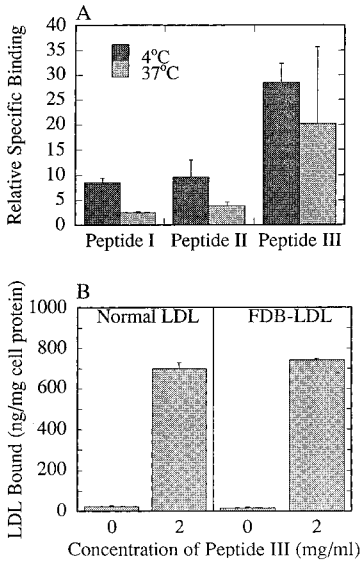

FIGURE 3: (A) Relative binding of ^{125}I-LDL to normal HSF at 4 and 37 °C, in the presence of peptides **I**, **II**, and **III**. Binding of LDL alone (no peptides) is given a value of 1. Peptides (2 mg/mL; 0.78 mM) + ^{125}I-LDL or ^{125}I-LDL alone were incubated with human skin fibroblasts, and the specific binding was measured as described in Materials and Methods. Nonspecific binding was measured as that not inhibitable by a 50-fold excess of unlabeled LDL, and subtracted from total binding, as described in Materials and Methods. Data are the mean (±standard deviation) of quadruplicate measurements. (B) Binding of normal and familial defective apo B-containing ^{125}I-LDL to normal HSF at 4 °C. Experimental conditions and data analysis are as described for data shown in panel A.

sequencing, the peptide products were judged to be >99% pure and of the desired sequence. Details of the structure of these peptides were reported separately (Luo et al., 1994).

*Effect of Peptides **I**, **II**, and **III** on the Binding of ^{125}I-LDL to Normal HSF Surfaces.* Figure 3A shows the relative specific binding of human LDL to the surface of normal human skin fibroblast cells in the presence and absence of the various peptides. Binding of LDL alone has the relative value of 1. All three peptides increased LDL binding at 4 and 37 °C, with the greatest effect induced by peptide **III** (30-fold increase at 4 °C). The effects of peptide **III** were more than twice that observed for the other peptides, indicating that its conformation is the most active. Although the relative increase in binding of peptide **III**−LDL at 4 °C is greater than at 37 °C, the absolute bindings are similar (369 and 368 ng of ^{125}I-LDL/mg of cell proteins, respectively, compared with 12.9 and 18.2 ng/mg of cell protein in the absence of peptide **III**). At 4 °C, the effects of the peptides were dependent upon the peptide concentration in LDL-R-positive HSF (figure not shown). While peptide-induced increases in ^{125}I-LDL binding dropped off rapidly for peptides **I** and **II**, with little or no effect at 200 μg/mL, peptide **III**−LDL binding persisted at the lowest concentration tested, 20 μg/mL. Therefore, peptide **III** increased LDL binding to HSF at concentrations where the effects of the other peptide analogues were not apparent.

*Effect of Peptide **III** on the Binding of ^{125}I-FDB to the Normal HSF Surface.* The ability of peptide **III** to increase the binding of a mutant form of LDL (the $R_{3500} \rightarrow Q$ mutation of human apolipoprotein B, FDB) to HSF surfaces was examined. The binding of ^{125}I-LDL to normal HSF from a patient heterozygous for this disorder was approximately half that of the binding of ^{125}I−LDL containing normal apo B (16.5 ± 3.9 versus 24.5 ± 3.1 ng of LDL/mg of cell protein, respectively), which agrees with findings of other investigators (Innerarity et al., 1987, 1990). The presence of 2 mg/mL peptide **III**, however, increased ^{125}I-FDB−LDL binding 63-fold (Figure 3B). A control group incubated with normal ^{125}I-LDL−peptide **III** bound 29-fold more than ^{125}I-LDL alone. The absolute magnitudes of the binding for both normal and FDB ^{125}I-LDL in the presence of peptide **III** were similar, indicating that peptide **III** might be able to reverse the effects of the $R_{3500} \rightarrow Q$ mutation on the binding of LDL to the cell surface.

Peptide Binding to the LDL Surface. To test the hypothesis that the above synthetic peptides form a complex with LDL, the binding of peptides **I** and **III** to LDL was measured. The binding isotherm for peptide **I** (Figure 4A, K_d and number of sites: 11.5 μM and 23 sites per LDL, respectively) indicates saturable, reversible binding of peptide **I** to LDL with a moderate affinity. The binding of peptide **III** to LDL was probed using the chromatographic method of Hümmel and Dreyer (1992) because of nonspecific adsorption of peptide **III** by the Amicon filter. The isotherm obtained using this method (Figure 4B) yielded $K_d = 1.1$ μM, and 2.0 binding sites per LDL particle. Thus, peptide **III**, constrained to form an α-helix, has about 10-fold greater affinity for LDL than does peptide **I**. In addition, peptide **I** binds to more than 10 times as many sites as does peptide **III**, suggesting that while peptide **I** may bind to LDL through relatively nonspecific interactions with lipids, peptide **III** may bind more specifically, perhaps through protein−protein interactions with apo B.

Binding of the Peptide−LDL Mixtures to HSF. Binding isotherms of human LDL to LDL-R-positive HSF were performed in the presence and absence of peptides. In these

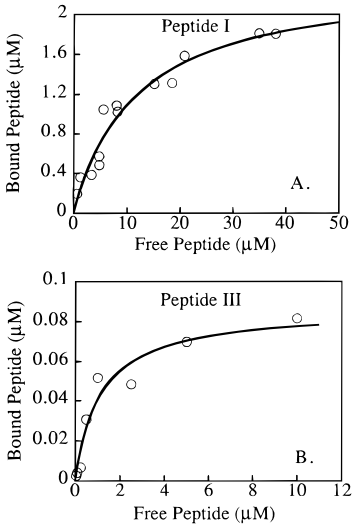

FIGURE 4: (A) Binding of peptide **I** to normal human LDL. LDL (112 mg of protein 5 mL of PBS) was incubated with various concentrations of [^{3}H]Gly-containing peptide **I** in an Amicon filtration cell. After 1 h, aliquots of 100 μL were filtered, the radioactivity was measured, and the concentration of free and bound peptide was calculated from this value, as described in Materials and Methods. Data points are the mean of four determinations. Nonlinear least squares analysis yielded $K_d = 11$ μM, and 23 sites per LDL particle. (B) Binding of peptide **III** to normal human LDL. For the chromatographic method of Hümmel and Dreyer (1962), a Sephadex G-25 column was equilibrated with a buffer containing various concentrations of peptide **III**, ranging from 5×10^{-8} to 5×10^{-5} M. LDL (2×10^{-10} M) was added to the top of the column, and the free and bound peptide concentrations were determined as described in Materials and Methods. Nonlinear least squares analysis yielded $K_d = 1.1$ μM and 2.0 sites per LDL particle.

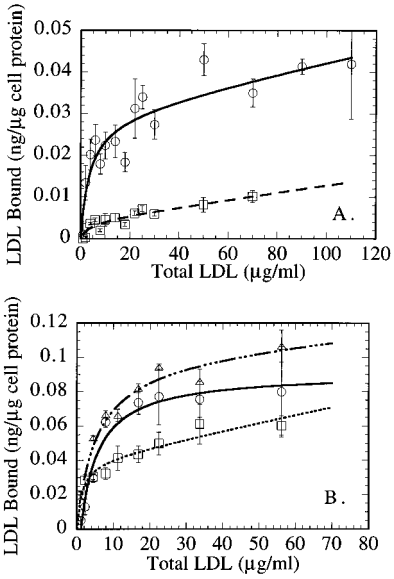

FIGURE 5: In these experiments, the results shown in panel A (top) were performed using a single batch of cells; the experiments shown in panel B (bottom) were performed using another, similar batch of cells. In each case, the control experiments (LDL alone, no peptides) were performed at the same time and using the same batch of cells and other reagents as in the experiments using LDL + peptide. (A) Binding of ^{125}I-LDL in the presence or absence of peptide **III** to normal HSF at 4 °C. The ^{125}I-LDL concentrations were varied, while the peptide concentration was 1 mg/mL through the experiments. Bound LDL was measured and nonspecific binding subtracted as described in Materials and Methods. The dotted line is LDL alone (no peptide); the solid line is LDL + peptide **III**. (B) Binding of ^{125}I-LDL in the presence or absence of peptides **I** and **II** to normal HSF at 4 °C. The ^{125}I-LDL concentrations were varied, while the peptide concentration was 1 mg/mL through the experiments. Bound LDL was measured and nonspecific binding subtracted as described in Materials and Methods. The dotted line is LDL alone (no peptide); the dot-dash line is LDL + peptide **I**; the solid line is LDL + peptide **II**.

experiments, peptide concentrations were kept constant approximately 2 orders of magnitude above the K_d for the binding of peptide to LDL, and the LDL concentration was varied. These conditions were chosen (1) so that free peptide concentration would be essentially constant, (2) so that peptide adsorption to the cell would also be essentially constant, and (3) so that LDL would be saturated with peptide over the entire range of LDL concentrations.

The results of these experiments are shown in Figure 5, and values obtained for K_d and number of sites are given in Table 1. Peptide **III** increased the total number of binding sites for LDL on the cell surface by a factor of 5 but had little or no effect on the affinity (K_d) of the LDL for the cell surface. Thus, the LDL−peptide **III** complex and LDL possess similar affinities for the cell surface (K_d of 5.1 and 7.9 nM, respectively), but the complex has a greater number of binding sites per cell than that of LDL alone (25.0 and 5.2×10^9 sites/mg of cell protein, respectively). The high affinity of LDL−peptide **III** for the cell surface suggests that a specific cell surface receptor may be involved.

Ligand Blotting of LDL to Rat Liver Cell Membranes. To assess whether the LDL-R was involved in the effect described above, ligand blotting of ^{125}I-LDL to rat hepatocytes in the presence and absence of peptide **III** was performed. In this experiment, rat hepatocyte cell membrane proteins were electrophoresed and then electrotransferred to nitrocellulose paper. While these experiments represented a change in cell type, the high surface density of LDL-receptor molecules on rat hepatocyte membranes permitted an assessment of the role of this receptor in the effect of peptide **III** on cell surface binding. A band of M_r 1.4×10^5, the approximate M_r of the LDL receptor, is seen in the LDL−peptide **III** mixture after an exposure time of 44 h (Figure 6), which is absent with LDL alone at this exposure and is only faintly visible after 3 weeks of exposure. These results indicate that peptide **III** causes increased binding of ^{125}I-LDL to the LDL receptor.

Table 1

Binding of Peptide−LDL Mixtures to Cell Surfaces		
experimental group	K_d (nM)	sites (particles of LDL/mg of cell protein)
LDL alone	1.89 ± 0.90	$3.36 \pm 0.45 \times 10^{10}$
LDL + peptide **I**	5.20 ± 2.50	$9.80 \pm 1.20 \times 10^{10}$
LDL + peptide **II**	7.10 ± 2.50	$8.50 \pm 1.40 \times 10^{10}$
LDL alone	7.90 ± 5.80	$5.20 \pm 1.80 \times 10^{9}$
LDL + peptide **III**	5.10 ± 3.00	$2.50 \pm 0.05 \times 10^{10}$

Binding of Peptides to LDL		
experimental group	K_d (μM)	sites (peptide molecules/LDL particle)
peptide **I**	11.50 ± 2.50	23.00 ± 5.00
peptide **III**	1.14 ± 0.40	1.95 ± 0.20

*Effect of Peptides I and III on the Binding of ^{125}I-LDL to Receptor-Deficient HSF and Effect of Peptides **I** and **II** on Binding of DiI−LDL to MEF Cell Surfaces.* To test whether there were binding sites involved other than the LDL-R, we performed binding experiments on mutant human skin fibroblasts lacking the LDL-R. Both Katchadurian and GM

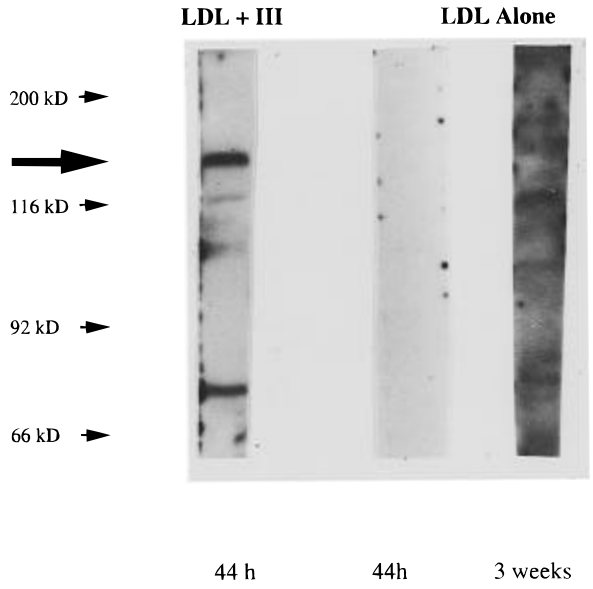

FIGURE 6: Ligand blotting of ^{125}I-LDL in the presence or absence of peptide **III** to rat liver cell membranes. Each lane received 100 μg of crude liver cell membranes. After electrophoresis, lanes were cut, electrotransferred onto nitrocellulose, and incubated with ^{125}I-LDL with or without 1 mg/mL peptide **III**, as described in the text. Times of exposure are shown in the figure.

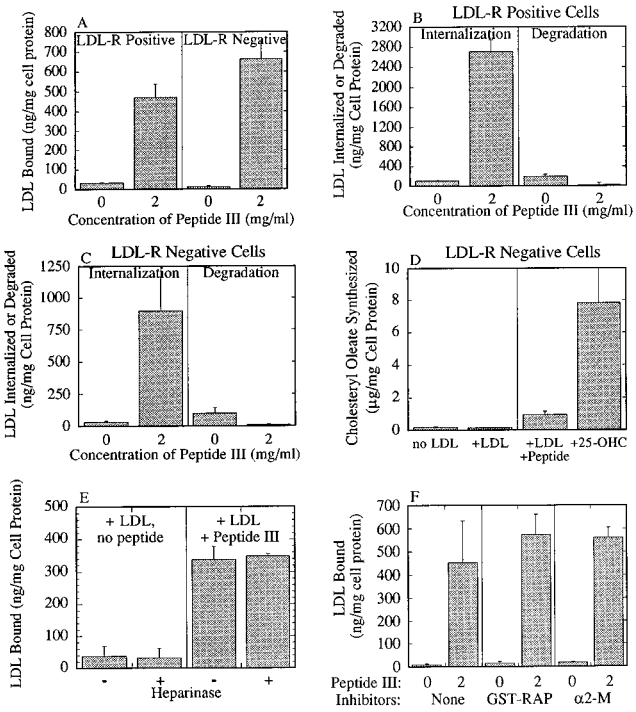

FIGURE 7: (A) Comparison of the effects of peptide **III** (2 mg/mL) on the binding of ^{125}I-LDL to normal and LDL-receptor (LDL-R) deficient HSF. The figure shows results obtained using cells with the Katchadurian (1973) mutation; essentially identical results were obtained with GM 486 cells having a mutation in the promoter region of the LDL-R gene and expressing no LDL-R. Experimental conditions are as described in Figure 3A. (B and C) Effect of peptide **III** on binding and internalization of ^{125}I-LDL in LDL-receptor positive and negative human skin fibroblasts, respectively. The experimental conditions used for this experiment were essentially the same as for the previous experiments except that the cells were incubated at 37 °C and allowed to internalize ^{125}I-LDL. Specific LDL internalization and proteolysis are measured as described in Materials and Methods. (D) Effect of peptide **III** on the incorporation of [^{14}C]oleate into cholesteryl oleate by LDL-receptor negative cells. LDL-receptor negative cells were incubated for 6 h at 37 °C with 150 μg/mL LDL, with or without 2 mg/mL peptide **III** in DMEM containing 10% (v/v) LPDS. The cells were then treated with a sodium [1-^{14}C]oleate−albumin complex (oleate at 0.1 mM) for 2 h at 37 °C as described (Goldstein et al., 1983); the cells were then analyzed for ^{14}C-containing cholesteryl oleate by thin-layer chromatography. The positive control was 25-hydroxycholesterol. (E) Effects of pretreating cells with heparinase on LDL binding to the cell surface. Cells were treated with heparinase (Sigma, heparinase I, catalog no. 2519) and washed as described by Ji et al. (1993). The binding of ^{125}I-LDL to these cells, in the presence and absence of peptides, was then measured as described above. (F) Competition between ^{125}I-LDL−peptide **III** and GST−RAP or α_2-macroglobulin. ^{125}I-LDL was incubated with or without peptide **III** and then binding of LDL was measured in the presence or absence of two potential inhibitors, GST-RAP and α_2-macroglobulin. Experimental conditions and data analysis are essentially as described for Figure 3A.

486 cells were used, and both gave similar results. Figure 7A shows a comparison of the binding of LDL and the LDL−peptide **III** complex to the surface of receptor-positive and receptor-negative (Katchadurian) cells at 4 °C. As expected, native LDL binding to receptor negative cells was approximately 30% of that to receptor positive (10 vs 33 ng/mg of cell protein). However, the LDL-peptide **III** complex binding to both receptor-positive and receptor-negative cells was greatly enhanced (to 500 and 630 mg/ng of cell protein, respectively). This represents a 13- and 52-fold increase of the LDL−peptide **III** complex to the surface of receptor-positive and receptor-negative cells, respectively. These data indicated that there were binding sites other than the LDL-R on the cell surface for the LDL−peptide **III** complex.

To assess the effects of peptides on the binding of LDL to cells lacking two of the important potential receptors for LDL, mouse embryonic fibroblasts deficient in both the LDL receptor and the LRP were tested. Essentially the same effects were seen for these cells as with wild-type and LDL-receptor-deficient human skin fibroblasts. Peptide **I** induced a 9.5-fold increase in specific LDL binding (i.e., over LDL alone, without added peptide), and peptide **II** induced a 2.0-fold increase in specific LDL binding (i.e., over LDL alone) for wild-type cells. For double mutant cells, peptides **I** and **II** induced 9.0- and 1.6-fold increases in specific LDL binding over LDL alone, respectively.

Effect of Peptides on Binding, Internalization, and Degradation of ^{125}I-LDL by Human Skin Fibroblasts. As shown in Figure 7A−C, peptide **III** increased binding and internalization of ^{125}I-LDL in both LDL-receptor positive and negative human skin fibroblasts. In addition, as shown in Figure 7D, peptide **III** increased the incorporation of [^{14}C]oleate into cholesteryl oleate by LDL-receptor-negative cells by about 6-fold. This, however, is a modest change compared to the increases in binding and internalization. Furthermore, this 6-fold increase (in LDL-receptor-negative cells) corresponds to a level of LDL degradation which is only about 10% of that measured in LDL-receptor-positive human skin fibroblasts; further, the level of stimulation is modest compared to that induced by 25-hydroxycholesterol. The conclusion that peptide **III** stimulates LDL binding and internalization but not degradation is further supported by measurements of lysosomal proteolytic degradation of ^{125}I-LDL, as shown in Figure 7B,C. Compared with the dramatic effect of peptide **III** on ^{125}I-LDL binding and internalization, the effect of peptide on proteolysis of ^{125}I-LDL was minor for both LDL-receptor-positive and -negative cells.

*Pretreatment of Cells with Heparinase Does Not Abrogate the Effect of Peptide **III** on Binding of ^{125}I-LDL.* The binding

of some lipoproteins to cell surfaces (e.g., apo E-enriched remnant lipoproteins by cultured HepG2 cells) is known to be mediated by cell surface glycosaminoglycans, and this effect is abrogated by pretreating the cells with heparinase (Ji et al., 1993). To assess the role of cell surface glycosaminoglycans in the above effects of peptide **III**, LDL-receptor-negative human skin fibroblasts were pretreated with heparinase, and the binding of ^{125}I-LDL was then measured in the presence or absence of peptide **III**. As shown in Figure 7E, pretreating cells with heparinase did not inhibit the effects of peptide **III** on the binding of ^{125}I-LDL to the cell surface. These results suggest that the peptide **III**-induced increase in LDL binding is not mediated by glycosaminoglycans. They are also consistent with the high-affinity constants observed for binding of ^{125}I-LDL, in LDL−peptide **III** mixtures, to the cell surface.

Competition Experiments for the LRP on Receptor-Deficient HSF Cells. A potential site for binding of LDL−peptide **III** is the LRP, which has recently been shown to bind apo E-enriched β-VLDL (Kowal et al., 1990; Mukono et al., 1991; Hussain et al., 1991). The binding of LDL or the LDL−peptide **III** complex to receptor−deficient HSF cells was compared in the presence of two competitors for the LRP: α_2-macroglobulin and GST−RAP. As shown in Figure 7F, neither GST−RAP nor α_2-macroglobulin decreased the binding of LDL to the surface of receptor-deficient cells, indicating that the LRP is not involved in binding the LDL−peptide **III** complex. These results are also in agreement with those above showing that LDL-receptor/LRP-deficient mouse embryonic fibroblasts exhibit the same effects of peptides on LDL binding as wild-type cells.

DISCUSSION

In this paper we have used model peptides to show that a biological activity of a domain of apolipoprotein E is conformationally specific. A peptide containing the conserved amino acids 41−60 of human apolipoprotein E increases the specific binding of LDL to cell surface receptors. Peptide **III**, constrained by side-chain lactam cross-links to adopt an α-helical structure (Luo et al., 1994), displays more of this activity than even the naturally occurring sequence, while peptide **II**, which cannot and does not form an α-helix, is less active than peptide **I**. Thus, the order of activity is the same as the order of α-helical content, i.e., peptide **III** > peptide **I** > peptide **II**. Peptides **I**, **II**, and **III** all had the same chain length, net charge, hydrophile−lipophile balance, and placement of conserved acidic and lipophilic amino acids. Peptides **II** and **III** had the same number of salt bridges and covalent cross-links. Despite these similarities, peptide **III** was by far most active in its effects on LDL binding to cells. The moderate affinity of the peptides for LDL is not surprising in view of the recent finding that the main lipid-binding portion of apo E is residues 244−266 (Westerlund & Weisgraber, 1993). The present studies suggest that the α-helical form of this domain of apo E may be the bioactive structure. Although the extreme N-terminus of apo E is not known as a receptor-binding domain, these studies suggest that it may play a role in modulating the association of apo E-containing lipoproteins, such as chylomicron remnants, β-VLDL or IDL, with cell surface receptors. A mutant form of apo E, apo E-5, further supports this contention: the affinity of apo E for the LDL-R was doubled by a Glu to Lys mutation at residue 3, suggesting that the extreme N-terminus, contrary to previous beliefs, was involved in LDL−receptor interactions (Dong et al., 1990).

The enhancement of lipoprotein binding and internalization to the cell surface is highly specific and is most compatible with receptor-mediated endocytosis. The binding and internalization of ^{125}I-LDL were inhibited by excess unlabeled LDL, showing the specificity of the binding and internalization. It is clear, however, that any effect of peptide **III** on receptor-mediated endocytosis cannot be confined to the LDL receptor, since similar enhancement of binding and internalization was apparent using both LDL-receptor-negative fibroblasts and LDL from a patient with familial defective apolipoprotein B which cannot bind to the LDL receptor. Recently, it has been demonstrated that apo E-enriched β-VLDL particles are capable of binding to both the LDL-R in normal cultured fibroblasts and the LDL-receptor-related protein (LRP) in mutant fibroblasts lacking the LDL-R (Kowal et al., 1990; Mukono et al., 1991; Hussain et al., 1991). In addition, Watanabe rabbits given intravenous injections of apo E (Wernette-Hammond et al., 1989) and transgenic mice which overexpress apo E (Shimano et al., 1992a,b) both showed marked reductions in the plasma lipoprotein levels and resistance against diet-induced hypercholesterolemia. These experiments demonstrate the existence of pathways other than the LDL receptor for the binding and internalization of LDL. Although one of these alternate pathways may be receptor-mediated endocytosis via the LRP, it seems unlikely that the LRP is responsible for the effects of peptide **III**, however, since two competitive inhibitors of ligand binding to LRP, α_2-macroglobulin and GST-RAP (Herz et al., 1991; Williams et al., 1992), did not inhibit the binding of LDL−peptide **III**. Two additional possible interpretations of this result are the following: (a) the LDL−peptide **III** complex bound to the LRP, but to a site that was not inhibited by either RAP or α_2-macroglobulin, or (b) peptide **III** interfered with the binding of RAP and α_2-macroglobulin to the LRP, and therefore these ligands were ineffective inhibitors.

One possible explanation of the increased number of binding sites for LDL in the presence of peptide **III** is that peptide **III** enhances LDL binding to cell surface glycosaminoglycans. Beisiegel et al. (1991) demonstrated that lipoprotein lipase enhances by 30-fold the cross-linking of chylomicrons to the LRP in HepG2 cells, an effect that could be abrogated by pretreating the cells with heparinase (Ji et al., 1993; Mulder et al., 1993). These observations are compatible with a two-step process, in which the LPL−chylomicron complex first binds to cell surface glycosaminoglycans, which then facilitates binding to the LRP. A similar mechanism has been proposed for the binding and internalization of protease−antiprotease complexes (Orth et al., 1992; Nykjaer et al., 1992). In our experiments, however, treating the cells with heparinase did not decrease the binding. Thus, it is unlikely that the effects of peptide **III** are mediated by heparinase-sensitive glycosaminoglycans. In addition, peptide **III** bears a net charge of −3; if its effect is in any way mediated by cell surface glycans, it would not likely be a direct effect, though it could possibly act by altering the confirmation of a cationic region of apo B. Another possibility was supported by preliminary experiments using *in situ* cross-linking according to the methods

of Beisiegel et al. (1991) in which peptide **III** caused enhanced cross-linking of LDL to a rat liver membrane protein with an apparent molecular weight of approximately 500 000 (figure not shown). Preliminary Western blot studies using anti-LRP antibodies (from Dudley S. Strickland, American Red Cross Laboratories) indicate that this protein is not the LRP. Additional work is necessary to identify this protein.

In contrast to the increased binding and internalization, peptide **III** does not increase degradation of ^{125}I-LDL, and the stimulation of cholesteryl ester synthesis by peptide **III** is modest. It is tempting to speculate as to the possible cause of this discrepancy between effects on binding or internalization versus degradation. Davies et al. (1981) have shown that modification of horseradish peroxidase to make it either more cationic or more anionic changes the rate of lysosomal degradation of the protein—increasing or decreasing the rate, respectively. The inhibition of this process by the anionic HRP appears to occur at the level of endosome−lysosome fusion or inhibition of lysosomal proteases. By analogy, peptide **III**—being highly anionic—may enhance binding and internalization of ^{125}I-LDL, while inhibiting endosome−lysosome fusion and lysosomal degradation.

The sites on LDL to which peptide **III** binds are not yet known. These three peptides conserve the positions of the acidic and lipophilic residues, all of which are highly conserved among species. Both apo E and apo B contain multiple cationic domains involved in binding cell surface receptors and glycosaminoglycans; residues 41−60 of apo E might interact with these cationic sites. The peptides all bind to LDL with moderate affinity, but of these three, peptide **III** had the highest affinity (K_d = 1.1 μM) and specificity (two sites per LDL) and the most potent effect upon LDL cell surface interactions. Since peptide **III** enhances the binding of ^{125}I-FDB−LDL to normal HSF, it is likely that the cationic domain around amino acid 3500, where FDB is mutated, may not be one of these sites. In addition, peptide **III** probably does not bind to the LDL-R-binding domain(s) of apo B: the magnitude of the effect of peptide **III** on ^{125}I-LDL binding decreased rapidly as the ^{125}I-LDL aged, while during the same time we observe the binding of LDL to the LDL receptor in normal fibroblasts to be unchanged. This effect appears to be due to radiation damage to apo B as observed by Khouw et al. (1993); if so, the damaged part of apo B, relevant to the effect of peptide III, does not appear to be the LDL-receptor binding domain of apo B.

These studies suggest a potential and hitherto unknown function of a highly conserved domain of apo E that may point toward a possible new avenue of therapeutic intervention in hyperlipemia. On the side of caution, it is possible that a peptide which increases binding and internalization of LDL might have a deleterious effect and even promote atherosclerosis. On the other hand, it is also possible that peptide **III** might allow for improved plasma clearance of LDL in patients with heterozygous familial hypercholesterolemia, since it might provide an alternate pathway for LDL clearance in patients lacking the normal number of LDL receptor molecules. These issues require further testing and verification.

In conclusion, we have shown that a biological effect of sequence within apo E is conformationally specific. Most monomeric oligopeptides in solution show little secondary structure. Secondary structure is generally induced in amphiphilic peptides by either self-association or adsorption onto an amphiphilic interface. The synthetic peptides used in this study demonstrate that particular secondary structures can be induced or prevented through the constraining effects of side-chain cross-links. With small alterations in amino acid sequence, peptides can be designed which, by dint of their specific secondary structure, will potentiate or inhibit biological effects. The high degree of secondary structure demonstrated by peptide **III** allowed us to examine the structure and function of a highly conserved but poorly understood domain of apo E. This powerful approach can be extended to a wide variety of systems both related to lipoproteins and outside of this field. For example, the existence of switch domains in viral coat proteins (Reed & Kinzel, 1991, 1993, 1994; Graf von Stosch et al., 1995), amyloidogenic proteins (McCubbin et al., 1988; Terzi et al., 1994; Soto et al., 1995; Soto & Frangione, 1995; Yamada et al., 1995a,b), and the serpin family of protease inhibitors (Carrell et al., 1991; Gettins et al., 1992) can be examined by restraining peptides to adopt one of two stable secondary structures. These techniques can be exploited to aid in drug design, as well as the study of ligand−receptor and other protein−protein interactions. Finally, advances in modular assembly of chemically synthesized protein molecules could allow the incorporation of constrained peptide domains in the context of a complete protein, thereby facilitating the systematic study of structure−function relationships in proteins.

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