A quantitative analysis of apolipoprotein binding to SR-BI: multiple binding sites for lipid-free and lipid-associated apolipoproteins

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Abstract Competitive binding experiments were performed using Y1-BS1 adrenal cells to provide information about the interaction of HDL apolipoproteins with scavenger receptor class B, type I (SR-BI). Exchangeable apolipoproteins apolipoprotein A-I (apoA-I), apoA-II, apoE-2, apoE-3, and apoE-4 as phospholipid complexes bind like HDL_3 to SR-BI via their multiple amphipathic α-helices; the concentrations required to reduce the binding of HDL_3 to SR-BI by 50% (IC_{50}) were similar and in the range of 35–50 μg protein/ml. In the case of apoA-I, peptides corresponding to segments 1–85, 44–65, 44–87, 149–243, and 209–241 all had the same IC_{50} as each other ($P = 0.86$), showing that a specific amino acid sequence in apoA-I is not responsible for the interaction with SR-BI. The distribution of charged residues in the amphipathic α-helix affects the interaction, with class A and Y helices binding better than class G* helices. Synthetic α-helical peptides composed of either L or D amino acids can bind equally to the receptor. Association with phospholipid increases the amount of apolipoprotein binding to SR-BI without altering the affinity of binding. Lipid-free apolipoproteins compete only partially with the binding of HDL to SR-BI, whereas lipidated apolipoproteins compete fully. These results are consistent with the existence of more than one type of apolipoprotein binding site on SR-BI.—Thuahnai, S. T., S. Lund-Katz, G. M. Anantharamaiah, D. L. Williams, and M. C. Phillips. **A quantitative analysis of apolipoprotein binding to SR-BI: multiple binding sites for lipid-free and lipid-associated apolipoproteins. *J. Lipid Res.* 2003.** 44: **1132–1142.**

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The role of scavenger receptor class B, type I (SR-BI) in lipoprotein metabolism has been studied widely (1). Initially identified for its ability to bind oxidized LDL (2), SR-BI was subsequently shown to be the first molecularly defined receptor that can mediate the selective uptake of cholesteryl ester (CE) from HDL (3) and LDL (4, 5) into cells. SR-BI resides in the plasma membrane of cells and is composed of a glycosylated extracellular domain and two membrane-spanning domains near the N- and C-terminal regions of the molecule (6, 7). Comparison of SR-BI to a closely related scavenger receptor, CD36, shows that while both receptors can mediate binding of HDL, only SR-BI is efficient at promoting selective CE uptake, and this function is attributable directly to the extracellular domain of SR-BI (8, 9). Also, mutational analysis of this region of the receptor revealed two arginine residues that may be important for efficient uptake of CE from HDL (10). More recently, it was shown that the unique ability of SR-BI to mediate selective lipid uptake does not require accessory proteins or structural components of the cell membrane (11), although a PDZ domain protein appears to be required for the receptor to properly translocate to the plasma membrane in hepatocytes in vivo (12).

The most widely studied ligands for SR-BI are the serum lipoproteins, in particular HDL, due to its participation in reverse cholesterol transport (13, 14). A range of ligands besides lipoproteins has been identified, including anionic phospholipids (15), advanced glycation end products (16), and apoptotic cells (17). Both the protein and lipid components of HDL can bind to SR-BI, but the selective uptake of lipids into the cell requires protein-protein

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Abbreviations: CE, cholesteryl ester; CNBr, cyanogen bromide; DMPC, 1,2-dimyristoyl phosphatidylcholine; GdnHCl, guanidine hydrochloride; MLV, multilamellar vesicle; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; rHDL, reconstituted HDL; SR-BI, scavenger receptor class B, type I; SUV, small unilamellar vesicle.

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interaction between the lipoprotein and the receptor since phospholipid vesicles and lipid emulsion particles devoid of apolipoproteins cannot mediate selective uptake (18). Instead, apolipoprotein-free lipid particles seem to fuse with the cell membrane after interaction with SR-BI (18). One model of SR-BI-mediated selective lipid uptake mechanism invokes formation of a hydrophobic channel between the bound lipoprotein particle and the cell membrane (19), while another invokes a hemifusion process (9). Regardless of the mechanism, it is clear that there is a high correlation between lipoprotein binding and the selective uptake of lipids consistent with SR-BI-mediated lipid uptake involving the two sequential steps of lipoprotein particle binding and lipid uptake (8, 9, 19, 20). With regard to efflux of cholesterol from cells, it is still controversial whether binding to the receptor is an absolute requirement for the process. There is a report that provides evidence that SR-BI-dependent efflux of cholesterol requires a productive binding of the extracellular lipid acceptor (21), while another study shows that binding of liposomal acceptors to SR-BI is not necessary (22).

Clearly, a molecular understanding of the lipid selective uptake process requires that the interactions of apolipoproteins with SR-BI be characterized thoroughly. The initial study (23) demonstrated that individual HDL apolipoproteins (apoA-I, apoA-II, and apoC-III), reconstituted into HDL particles, bind to SR-BI. Subsequent work with apoA-I has shown that its conformation is important for receptor interaction. Thus, conformational changes induced by adding different amounts of phospholipid to create HDL particles of different sizes alter the affinity of binding to SR-BI (24, 25). The apoA-I/SR-BI interaction is complex in that there is not a unique recognition site in the apolipoprotein because domains in both the N- and C-terminal regions of the molecule can mediate binding (26). Apparently, N- and C-terminal regions can both bind because they contain an amphipathic α-helix that is a recognition motif for SR-BI (26). The interactions of apoA-II and apoE with SR-BI are less well understood, and contradictory results have been published. Thus, it has been reported that the presence of apoA-II together with apoA-I in HDL particles can either increase (27) or decrease (20) binding to SR-BI. In the case of apoE, it has been claimed recently that apoE/phospholipid complexes can (18, 28) and cannot (29) bind to SR-BI. Furthermore, contrary to the situation with apoA-I (25), it is reported that lipidation of apoE reduces its ability to bind to the receptor (29).

The purpose of this study is to provide new quantitative information and to clarify some of the discrepancies concerning the binding of apolipoproteins to SR-BI. We find that apoA-I, apoA-II, and apoE in the lipid-free state lack the ability to fully compete for HDL binding to SR-BI. However, once these apolipoproteins are associated with phospholipids, they all demonstrate similar abilities to compete fully for the binding of HDL to SR-BI. Results using various domains of apoA-I and apoE and synthetic peptides show that the SR-BI/apolipoprotein interaction is not amino acid sequence specific, but that the class of amphipathic α-helix affects the binding.

EXPERIMENTAL PROCEDURES

Materials

1,2-Dimyristoyl phosphatidylcholine (DMPC) and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) were purchased from Avanti Polar Lipids (Albaster, AL). Carrier-free ^{125}I-Na (15 Ci/mg) was purchased from New England Nuclear (Boston, MA). Ham's F-10 and minimal essential media for tissue culture were purchased from Bio Whittaker (Walkersville, MD). Bovine serum albumin, horse serum, and fetal bovine serum were purchased from Sigma (St. Louis, MO). Cortrosyn, a synthetic analog of adrenocorticotropic hormone, was purchased from Organon (West Orange, NJ).

Human HDL was isolated from normolipidemic donors by sequential ultracentrifugation (30). ApoA-I and apoA-II were isolated from human HDL as described (31). Prior to use, the purified apolipoproteins were resolubilized in 6 M guanidine hydrochloride (GdnHCl) and dialyzed against Tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl, 0.1 mM EDTA; pH 7.4). Human apoE isoforms were expressed in *Escherichia coli* and purified according to the method of Morrow et al. (32); apoE samples were solubilized in 6 M GdnHCl and 1% β-mercaptoethanol, and dialyzed extensively against 100 mM ammonium bicarbonate buffer prior to use. Synthetic peptides of human apoA-I were synthesized using an automated solid-phase peptide synthesizer as described (33). The peptides were blocked at the N- and C-terminal and corresponded to amino acid residues 1–43, 44–65, 44–87, and 209–241 of apoA-I with molecular masses of 4,824, 2,438, 5,087, and 3,749 Da, respectively. The amphipathic α-helical peptides L-18A (DWLKAFYDKVAEKLKEAF), D-18A (18A sequence but containing D amino acids), L-37pA (18A-proline-18A), D-37pA, and DL-37pA (D-18A-proline-L-18A; the proline separating the two 18A segments was the L form) have been described before (34) and were prepared in a similar way; the molecular masses of the 18A peptides (which were N- and C-terminal blocked) and 37pA were 2,243 Da and 4,483 Da, respectively. Concentrations for the peptides were determined from the absorbance at 280 nm using the following molar absorbtivities: 8,250, 5,690, 11,380, 1,280, 13,940, and 6,970 M^{-1} cm^{-1} for apoA-I peptides 1–43, 44–65, 44–87, 209–241, the 18A peptide, and the 37pA peptide, respectively. As necessary, HDL and apolipoproteins were labeled with ^{125}I using the iodine monochloride method (35); the specific activities of the labeled products were in the range of 300 to 1,500 dpm/ng protein.

Cyanogen bromide fragments of apoA-I

Human apoA-I was subjected to fragmentation at three internal methionine residues using cyanogen bromide (CNBr), as described by Morrison et al. (36); the peptides were purified by HPLC. Briefly, 30 mg of apoA-I was digested with 3 ml of CNBr in 70% trifluoroacetic acid (TFA) for 24 h. The reaction was terminated by the addition of deionized water, after which the reaction mixture was lyophilized and subjected to reversed-phase HPLC (VYDAC 22 mm inner diameter × 25 cm, particle size 10 μm) using an acetonitrile-water (containing 0.1% TFA) solvent system with a gradient of 0% to 60% acetonitrile for 90 min at a rate of 5 ml per min. Fractions at retention times 55.3 min, 57 min, 62 min, 65 min, and 68 min corresponding to fragments 1, 2, 3, 4, and 5, respectively, were collected. The samples were lyophilized and characterized using an analytical HPLC and mass spectral analysis using a PE-Sciox APT-III triple-quadrupole ion spray mass spectrometer (MS core facility at UAB-Birmingham). The fragments had retention times of 55.3 min, 57 min, 62 min, 65 min, and 68 min and corresponded to masses of 3,190 Da, 4,350 Da, 7,427 Da, 9,880 Da, and 10,700 Da, respectively. The

samples of interest were the 9,880 Da fragment corresponding to the N-terminus and the 10,700 Da fragment corresponding to the C-terminus of apoA-I. The lyophilized samples were dissolved in TBS and the concentrations determined from the absorbance at 280 nm using the extinction coefficients 2.04 and 0.45 for 1 mg/ml solutions of the N- and C-terminal peptides, respectively.

Preparation of small unilamellar vesicles

The method of Barenholz et al. (37) was adapted to prepare small unilamellar vesicles (SUVs). POPC was dissolved in chloroform-methanol (1:1, v/v) and dried under nitrogen onto the wall of a glass tube and placed in a vacuum oven to completely remove any remaining solvent. To form multilamellar vesicles (MLVs), the lipid was then rehydrated in TBS. This dispersion was sonicated on ice under nitrogen using a tapered titanium tip (Branson Sonifier 350) for 5 min followed by 1 min of cooling. The initially cloudy lipid mixture became translucent after this cycle was repeated 10 times. Thereafter, the sample was centrifuged in a Beckman 50 Ti rotor for 2 h at 4°C at 145,000 *g* to remove the titanium debris that was produced during the sonication, and to separate any remaining MLV from the small SUV layer. The top SUV layer was removed and used in competitive binding experiments.

Discoidal reconstituted HDL preparation and characterization

Discoidal reconstituted HDL (rHDL) complexes containing DMPC and apoA-I (2:1, w/w) were prepared by incubating the lipid-protein solution at the phase transition temperature of DMPC, 24°C. The appropriate amount of phospholipid dissolved in chloroform-methanol (1:1, v/v) was dried on the wall of a glass tube with nitrogen and allowed to dry completely in a vacuum oven. The dried lipid was then dissolved in TBS to form MLV. After equilibration in a 24°C water bath, the lipid and apoA-I solutions were mixed and further incubated in the same water bath for 30 min, at which time the initially turbid lipid-protein solution became clear. Under the experimental conditions used, there was essentially quantitative incorporation of protein into the lipid-protein complexes; formation of the complexes was assessed by analysis using nondenaturing 8–25% gradient electrophoresis gels (Amersham-Pharmacia). Similar discoidal complexes of apoA-II, apoE isoforms and the various peptides (38) were prepared in the same fashion. Additionally, some rHDL particles were prepared by using DMPC-SUV and cycling the lipid-protein mixture across the phase transition temperature of DMPC. This was accomplished by cycling the lipid-protein solution at 10°C for 10 min followed by 10 min at 37°C several times. Results of experiments using rHDL prepared by direct incubation of apolipoprotein with either MLV or SUV were similar.

Cell culture experiments

Y1-BS1 murine adrenal cells were grown in Ham's F-10 medium (supplemented with 12.5% horse serum, 5% fetal bovine serum, and 50 μg/ml of gentamycin) in a humidified 5% CO_2 incubator at 37°C as described before (19). For experiments, Y1-BS1 adrenal cells were seeded in 12 or 24 well tissue culture flasks and allowed to grow for 2 to 3 days until ~75% to 80% confluency was reached. The cells were then stimulated for 24 h with 100 nM cortrosyn (dissolved as a 100 mM stock solution in PBS) in serum-free Ham's F-10 medium to increase the expression of SR-BI protein. Immunoblot analysis confirmed the increased expression of SR-BI upon stimulation with hormone (data not shown). For competitive binding experiments, the monolayer was washed twice with serum-free Ham's F-10 medium (at 4°C) and then equilibrated at 4°C. Subsequently, 10 μg protein/ml of ^{125}I-HDL_3 plus increasing amounts of the unlabeled competitor in serum-free medium were incubated with the cell monolayer for 2 h at 4°C. Control wells were also included that contained only ^{125}I-HDL_3 and no competitor. After the incubation, the cell monolayer was washed three times with 2 ml of ice-cold PBS containing 0.1% BSA plus one additional wash with 2 ml of PBS alone, then 0.1 N NaOH was used to solubilize the cell monolayer. Aliquots of this lysate were taken to determine the cell-associated ^{125}I in a γ counter, and the protein content was measured using a modified Lowry method (39). HDL binding and selective CE uptake were measured as described previously (19).

Control experiments were conducted to establish that incubation of ^{125}I-HDL_3 with unlabeled competitor did not lead to displacement of ^{125}I-apolipoprotein from the HDL particle. We incubated ^{125}I-HDL_3 with a 10-fold excess of unlabeled apoA-I for 2 h at 4°C, and passed the mixture over a gel-filtration column (Pharmacia Superdex 200, 1.6 × 60 cm). Displaced ^{125}I-apolipoprotein was not detected in the fractions that contained lipid-free apoA-I, and the specific activity of the ^{125}I-HDL_3 did not decrease by more than 5% (data not shown). To examine further the ability of apolipoproteins to exchange from human HDL_3, the apolipoproteins on the lipoprotein particle were labeled by reductive methylation using [^{14}C]formaldehyde. HDL_3 labeled in this fashion was incubated with excess human VLDL (the VLDL-HDL phospholipid ratio was 10:1, v/v) at 37°C, and the VLDL was rapidly precipitated at different times by the addition of manganese phosphate. About 10% of the radiolabeled apolipoprotein transferred to VLDL within 1 min, but there was no further detectable transfer over a 24 h period. It follows that <10% of the labeled apoA-I on HDL_3 particles is available for transfer to excess lipoprotein particles under the conditions of the competitive binding assay used in this study. Consequently, the reductions in the amount of ^{125}I-HDL_3 associated with cells when ^{125}I-HDL_3 was presented together with an unlabeled competitor were due to competition for binding to SR-BI and are not due to displacement of ^{125}I-apolipoprotein from HDL_3 in the extracellular medium. In the above experiments, care was taken to ensure that the effective concentration of the ligands in the incubation medium was accurate by preventing any loss of sample through binding to the polypropylene tube used for mixing the ^{125}I-HDL_3 and competitors. This was accomplished by prerinsing the polypropylene tubes with a 10% BSA solution, washing with deionized water, and completely removing any trace of liquid from the tubes prior to use. In addition, freshly dialyzed samples (stored in 6M GdnHCl) of lipid-free apolipoproteins were used to ensure that the proteins were not significantly self-associated and were available to compete for binding to SR-BI. To confirm this, control experiments were performed by cross-linking the apolipoproteins at various concentrations using bis(sulfosuccinimidyl)suberate (40). At least up to 0.5 mg/ml lipid-free apoA-I, apoA-II, and the apoE3-22 kDa fragments were monomeric; apoE-3 existed mostly as monomers, but some tetramers were present under these conditions. For apoE isoforms, the storage buffer also contained 1% β-mercaptoethanol to prevent inter- and intramolecular disulfide bonds from forming.

Data analysis

Direct binding of ligand to cells was analyzed using a nonlinear regression fitting to Eq. 1

$$B = \frac{[\text{ligand}]\, B_{max}}{[\text{ligand}] + K_d} + C\,[\text{ligand}] \qquad \textit{(Eq. 1)}$$

on Graph Pad Prism as described before (19). The results of the competitive binding experiments were analyzed using the logit

transformation (41). The parameter of interest is the IC_{50}, which is the concentration of the competitor ligand required to reduce the binding of ^{125}I-HDL_3 by 50%. To obtain the IC_{50} values most accurately, the competitor concentration was transformed to the logarithmic scale, and the amount of ^{125}I-HDL_3 bound to the SR-BI was expressed as logit (B) = ln B/(100-B) where B is the percent ^{125}I-HDL_3 bound to the cell. The IC_{50} is the concentration of competitor when B = 50 or logit B = 0; this value was determined using linear regression analysis (Graph Pad Prism). Differences between the various competitors of ^{125}I-HDL_3 binding were tested for statistical significance by using a nonparametric *t*-test (significance was set at an α value of 0.05). The IC_{50} values listed in **Table 1** were obtained by averaging multiple competition experiments. The errors in the IC_{50} values were derived from the linear regression analysis and were within no more than 10% of the mean in each case. The Hill slope for ^{125}I-HDL_3 versus HDL binding was obtained using the sigmoidal dose-response variable slope equation (Eq. 2) on Graph Pad Prism:

$$Y = \text{Bottom} + (\text{Top-Bottom})/(1+10\text{^}((\text{LogEC}_{50}\text{-X})*\text{HillSlope})) \quad \textit{(Eq. 2)}$$

RESULTS

Competitive binding of HDL

Our goal in this study was to provide more information regarding the first step of the SR-BI-mediated uptake of HDL lipids into cells. This two-step process involves the reversible binding of the lipid donor particle to SR-BI and the subsequent movement of the lipid to the cell membrane (see Appendix). Direct binding of HDL to Y1-BS1 cells involves both high-affinity, low-capacity (SR-BI) and low-affinity, high-capacity components (nonspecific binding) (19, 42). The high-affinity HDL binding to SR-BI and the associated CE selective uptake obtained by nonlinear regression analysis (see Experimental Procedures) are shown in **Fig. 1**. It is evident from the figure that the binding of a lipid donor particle, in this case HDL, is tightly correlated (r^2 = 0.98) with the movement of lipids into cells via SR-BI, providing support for the link between reversible binding and lipid movement. Analogous to the Scatchard treatment of receptor binding and the Michaelis-Menten treatment of enzyme kinetics (see Appendix), the isotherms for HDL binding to SR-BI and CE selective uptake are hyperbolic. This fit indicates that HDL binds to independent sites and that allosteric effects are not involved. In agreement with this, the binding of HDL to SR-BI in COS-7 cells transiently transfected with SR-BI shows only a high-affinity, saturable component [unpublished information, S. Thuahnai et al. and ref. (8)] consistent with there being a single class of binding site for HDL on SR-BI.

To compare the relative affinities of the various exchangeable apolipoproteins of HDL, we employed competitive binding experiments, with HDL as the labeled ligand. Experiments were conducted at 4°C to minimize cellular uptake and degradation of ligands through endocytosis. **Figure 2** shows the competitive binding of ^{125}I-HDL_3 versus unlabeled HDL_3. As expected, HDL_3 competes effectively with ^{125}I-HDL_3; note that 80–85% of ^{125}I-HDL_3 binding is inhibited at concentrations of unlabeled HDL_3 > 300 μg protein/ml. The lack of complete inhibition is likely due to a combination of nonsaturable nonspecific binding of the radiolabeled ligand to the cell and to the plastic tissue culture plates. In Fig. 2B, the competitive binding data from Fig. 2A was transformed by taking the log of the competitor

TABLE 1. Binding parameters for interaction of SR-BI with apolipoproteins

Competitor	IC_{50}[a]	IC_{50}[b]
	μg apolipoprotein/ml	*μM*
Lipoproteins		
HDL_3	25	
Lipid-free apolipoprotein[c]		
apoA-I	140	3.9
apoA-II	130	2.2
apoE-2	130	4.4
apoE-3	160	5.4
apoE-4	320	11
apoE-2, 22 kDa	790	17
apoE-3, 22 kDa	630	14
apoE-4, 22 kDa	1,260	28
Apolipoprotein complexed with PL[c,d]		
apoA-I/DMPC	45	
apoA-II/DMPC	35	
apoE-2/DMPC	50	
apoE-3/DMPC	30	
apoE-4/DMPC	35	
apoE-2, 22 kDa/DMPC	50	
apoE-3, 22 kDa/DMPC	70	
apoE-4, 22 kDa/DMPC	65	

DMPC, 1,2-dimyristoyl phosphatidylcholine; SR-BI, scavenger receptor class B, type I.

[a] IC_{50} values were derived from competitive binding experiments as shown in Figs. 2–5 by transformation of log of competitor concentration versus logit transformation of ^{125}I-HDL_3 binding as described in Experimental Procedures. At least three independent experiments with triplicate determinations in each were combined to obtain IC_{50} values, which are accurate to within 10% (see Experimental Procedures).

[b] Determined by dividing the IC_{50} values in column 2 with the molecular mass of appropriate apolipoproteins; 28, 17, 34, and 22 kDa for apoA-I, apoA-II, apoEs, and apoE-22 kDa fragments, respectively.

[c] The IC_{50} values for lipid-free apolipoproteins are statistically different from apolipoproteins complexed with phospholipids.

[d] Phospholipid-to-protein mass ratio was 2:1.

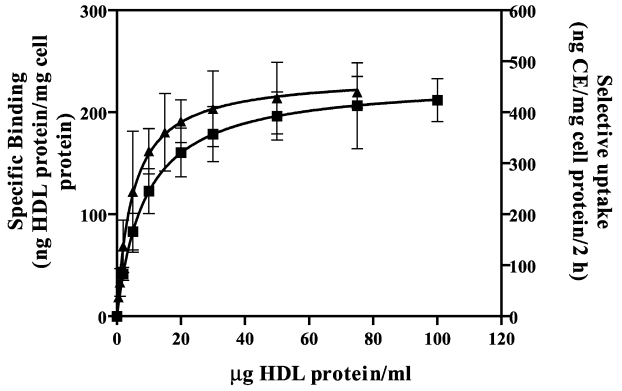

Fig. 1. Concentration-dependence of HDL_3 binding and selective cholesteryl ester (CE) uptake. Hormone-stimulated Y1-BS1 cells were incubated with the indicated concentrations of dual-labeled HDL_3 ([^{3}H]CE and ^{125}I-apo) for 2 h at 37°C. The high-affinity binding (square) and selective CE uptake (triangle) were determined as described before (19). The data are the mean ± SD from two experiments, with triplicate wells in each experiment (n = 6).

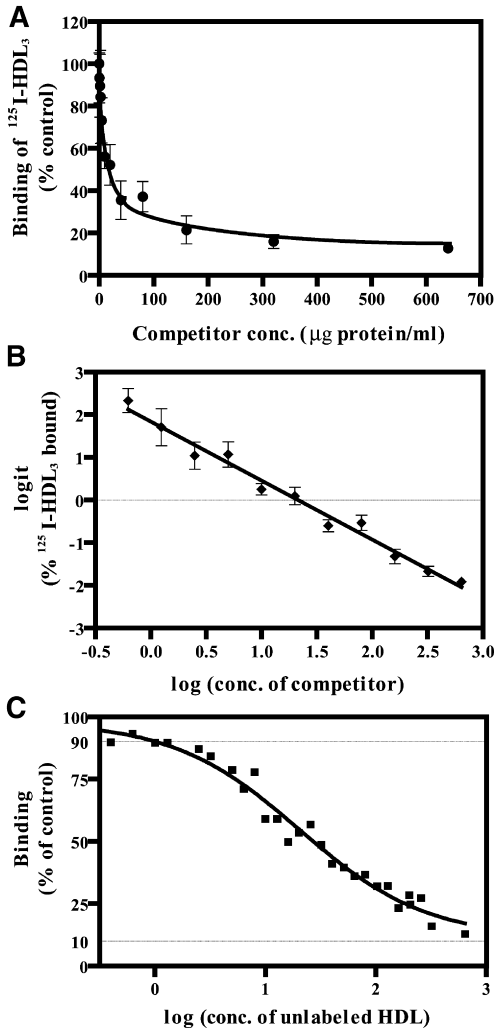

Fig. 2. Competitive binding of ^{125}I-HDL$_3$ and unlabeled HDL$_3$ to scavenger receptor class B, type I (SR-BI). A: Ten micrograms of protein/ml of ^{125}I-HDL$_3$ plus increasing amounts of unlabeled HDL$_3$ were incubated for 2 h at 4°C with SR-BI-expressing Y1-BS1 cells. The binding of ^{125}I-HDL$_3$ is expressed as a percentage of control, where 100% represents wells that did not contain any competitor. Data points are mean of triplicate wells ± SD from a representative experiment of 11 independent experiments. B: Logit transformation of binding versus the logarithmic transformation of the competitor concentration of the data presented in A. The concentration of competitor when the logit value is zero represents the IC_{50}. C: The Hill plot of HDL$_3$ versus ^{125}I-HDL$_3$ from 11 experiments with triplicate wells in each.

concentration and plotting it against the logit transformation (41) of the binding of ^{125}I-HDL$_3$. Such a transformation allows the most accurate determination of the IC_{50} value, the concentration of the competitor required to reduce the binding of ^{125}I-HDL$_3$ to 50%. Unlabeled HDL$_3$ has an IC_{50} value of 25 μg protein/ml (equivalent to about 0.87 μM apoA-I and 0.13 μM HDL$_3$ particle). This value is about three times higher than the K_d for HDL$_3$ binding (~8 μg protein/ml) that we obtained in the same cell by direct binding measurements (19). This is because the K_d and IC_{50} values are similar only under conditions where the radiolabeled ligand, ^{125}I-HDL$_3$, is at trace concentrations [i.e., ^{125}I-HDL$_3$ << K_d of HDL$_3$ (43)]. In our competitive binding experiments, we used 10 μg protein/ml of ^{125}I-HDL$_3$, which is close to the K_d of HDL. In general, when the K_d is close to the concentration of the labeled ligand, the IC_{50} is approximately two times the K_d value (43).

Another way to analyze the competitive binding experiment is shown in Fig. 2C. Several competitive binding experiments were combined, and the concentration of the competitor was logarithmically transformed and plotted against the binding of the ^{125}I-HDL$_3$. From the sigmoidal graph that results from such a transformation, the steepness of the slope of the curve called the Hill slope (44) can be obtained graphically. For HDL, the analysis shows the Hill slope to be −0.76 (95% confidence interval between −1 and −0.49), which is not significantly different from the Hill slope of −1 expected for a single class of binding site on a receptor. Therefore, similar to the results obtained from direct binding of HDL (Fig. 1), the competitive binding experiments indicate the presence of one class of binding site on SR-BI for HDL.

Competitive binding of apoA-I

Using a similar type of experiment and analysis, the major apolipoprotein on HDL, apoA-I, as a complex with DMPC, is slightly less effective than HDL$_3$ in competing with ^{125}I-HDL$_3$ binding (IC_{50} = 45 μg protein/ml) (Table 1). Interestingly, apoA-I complexed with POPC is a better competitor, with an IC_{50} value of 10 μg protein/ml. A similar phospholipid-specific effect in the binding of apoA-I to SR-BI has been reported before (20), suggesting that apoA-I conformation is significant for binding to SR-BI. Consistent with this idea, lipid-free apoA-I is not an effective competitor of ^{125}I-HDL$_3$ binding (**Fig. 3**). The IC_{50} value is 140 μg protein/ml, which is over 3-fold higher

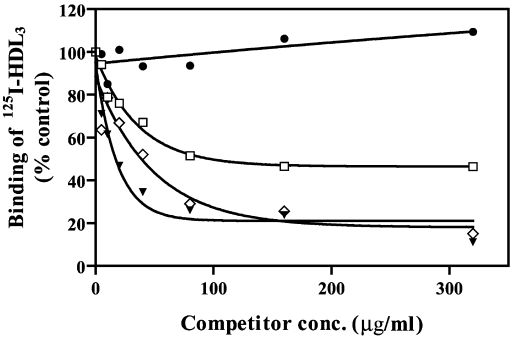

Fig. 3. The effect of lipidation on the affinity of apoA-I for SR-BI. The graph shows competitive binding between 10 μg protein/ml of ^{125}I-HDL$_3$ plus increasing amounts of lipid-free apoA-I and apoA-I complexed with different PC. The ratios of PC to protein were 100:1 mol/mol, or about 2.5:1 w/w in each case. Data points correspond to lipid-free apoA-I (open square), apoA-I/1,2-dimyristoyl phosphatidylcholine (DMPC) complex (open diamond), apoAI/1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) complex (inverted triangle), or POPC/small unilamellar vesicle (SUV) (closed circle). Data are presented as percentage of control, where 100% represents wells that do not contain any competitor. Data points are presented as mean of triplicate wells ± SD from a representative experiment of three independent experiments. The competitor concentrations are micrograms of protein/ml, except for the SUV, where micrograms of PC/ml is plotted.

than that for apoA-I associated with DMPC. As evident from Fig. 3, lipid-free apoA-I only reduces the binding of ^{125}I-HDL$_3$ by ~45%, indicating that even at high concentrations, it cannot fully compete with the binding of ^{125}I-HDL$_3$. Since the apolipoproteins were freshly dialyzed from GdnHCl, they should have remained largely monomeric during the 2 h incubation experiments. This means that the lack of competition is probably not due to loss of effective protein concentration resulting from aggregation or self-association of the ligands. Since lipid-free apoA-I does not achieve full competition, we examined the competitive binding result as follows. If the data are analyzed as the concentration of competitor required to achieve 50% of the maximum inhibition (45% for lipid-free apoA-I and 80% for apoA-I/DMPC), then the resulting transformed IC$_{50}$ values are similar (~25 μg protein/ml) for lipid-free apoA-I and apoA-I associated with phospholipid. Interestingly, unlabeled HDL is able to compete effectively with lipid-free ^{125}I-apoA-I and reduce the binding to ~10% of the control value (data not shown). In control competitive binding experiments, BSA and phospholipid vesicles (Fig. 3) did not inhibit the binding of ^{125}I-HDL$_3$, as others have also shown (23). These results support the view that SR-BI exhibits a high-affinity protein-protein interaction with apolipoproteins (26).

Competitive binding of apoA-II and apoE

As seen with apoA-I, other apolipoproteins (i.e., apoA-II and apoE-3) in lipid-free form at high concentrations also reduce the binding of ^{125}I-HDL$_3$ by only 45–50% (**Fig. 4**). Consistently, lipid-free apolipoproteins have a reduced capacity to compete with HDL for binding to SR-BI. The competitive binding data in Fig. 4 show that similar to apoA-I, apoA-II (Fig. 4A) and apoE-3 (Fig. 4B) are both better able to compete with ^{125}I-HDL$_3$ binding when added as lipid complexes. Upon complexing with phospholipids, their capacity to reduce ^{125}I-HDL$_3$ binding approaches 80% to 85%, equivalent to the reduction caused by unlabeled HDL. Table 1 summarizes the effect of lipid association on the IC$_{50}$ values for the major HDL apolipoproteins examined. The data show that the IC$_{50}$ values of full-length apolipoproteins decreased from ~130–320 μg protein/ml to ~35–50 μg protein/ml upon being associated with phospholipids.

The role of apoE in lipoprotein metabolism is well documented (45), and we wanted to explore further if apoE isoform-specific interaction with SR-BI exists. As seen in **Fig. 5**, all three naturally occurring isoforms of apoE can compete effectively with ^{125}I-HDL$_3$. During the preparation of this manuscript, Li et al. (28) reported that the apoE isoforms complexed with phospholipids can bind equally to SR-BI in agreement with our findings. However, our results contradict the recent report by Bultel-Brienne et al. (29) suggesting that lipidated apoE does not bind to SR-BI; the reason for this disagreement is not obvious. The data in Table 1 show that, in the lipid-free state, all three isoforms are not able to compete with ^{125}I-HDL$_3$ as effectively as when lipidated. Furthermore, similar to the full-length proteins, each of the 22 kDa apoE molecules (corresponding to N-terminal residues 1–191) compete

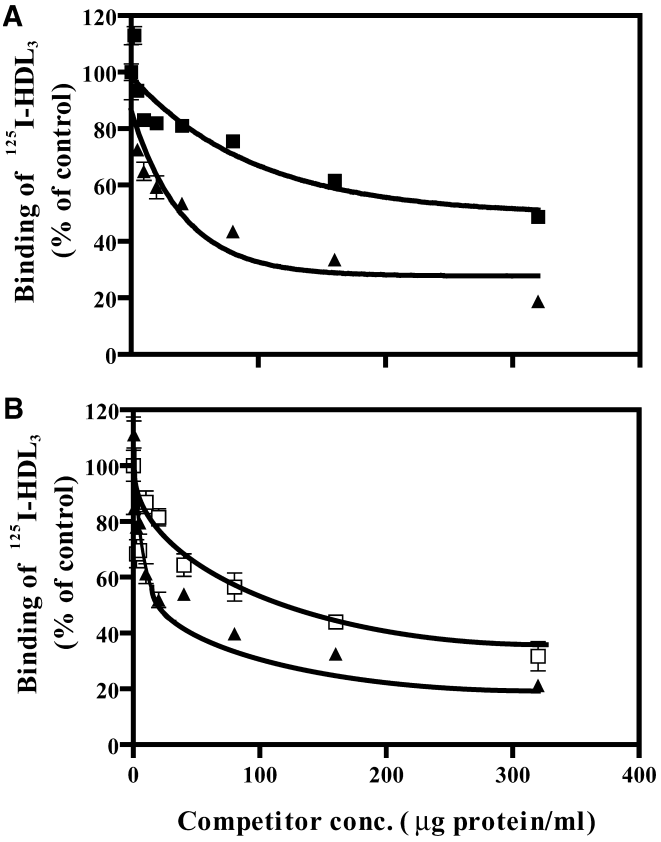

Fig. 4. The effect of lipidation on the affinity of apoA-II and apoE-3 for SR-BI. The graph shows competitive binding between 10 μg protein/ml of ^{125}I-HDL$_3$ plus increasing amounts of competitor for 2 h at 4°C. A: Lipid-free apoA-II (closed square) and apoA-II/DMPC (triangle). B: Lipid-free apoE-3 (open square) and apoE-3/DMPC (triangle). Data are presented as a percentage of control where 100% represents wells that do not contain any competitor. Data points are presented as mean of triplicate wells ± SD. A representative curve from three independent experiments for apoA-II and five for apoE-3 are shown in each case.

effectively with ^{125}I-HDL$_3$ binding when presented as phospholipid complexes. This indicates that there is a recognition domain present in the N-terminal region of the apoE molecule (28). Interestingly, as reflected in their IC$_{50}$ values (Table 1), when compared with the full-length molecules, the 22 kDa fragments in the lipid-free state compete poorly with ^{125}I-HDL$_3$. It appears that the C-terminal domain (residues 191–299) is important in enhancing the interaction of lipid-free apoE with SR-BI.

Competitive binding of apoA-I peptides and model peptides

Amino and carboxy terminal deletion mutants of apoA-I can bind to SR-BI (25), as can the isolated amino and carboxy terminal domains (26), indicating that more than one domain in the apoA-I molecule can mediate binding to SR-BI. Consistent with these results, **Fig. 6A** shows that the terminal CNBr fragments of apoA-I complexed with phospholipids are equivalent in their ability to compete with ^{125}I-HDL$_3$ for binding to SR-BI. To refine these observations, we performed similar experiments using peptides

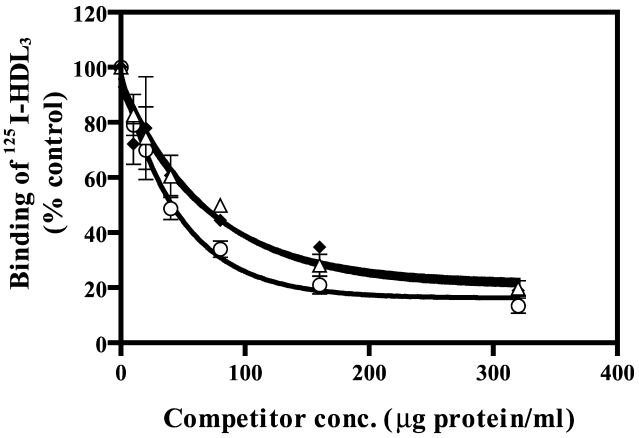

Fig. 5. Competitive binding to SR-BI of ^{125}I-HDL$_3$ and apoE isoforms. Y1-BS1 cells were incubated for 2 h at 4°C with 10 μg/ml of ^{125}I-HDL$_3$ plus either apoE-2, apoE-3, or apoE-4 complexed with DMPC. Data points correspond to apoE-2/DMPC complex (triangle), apoE-3/DMPC complex (diamond), and apoE-4/DMPC complex (circle). Data represent mean of triplicate wells ± SD from a representative experiment of five independent experiments.

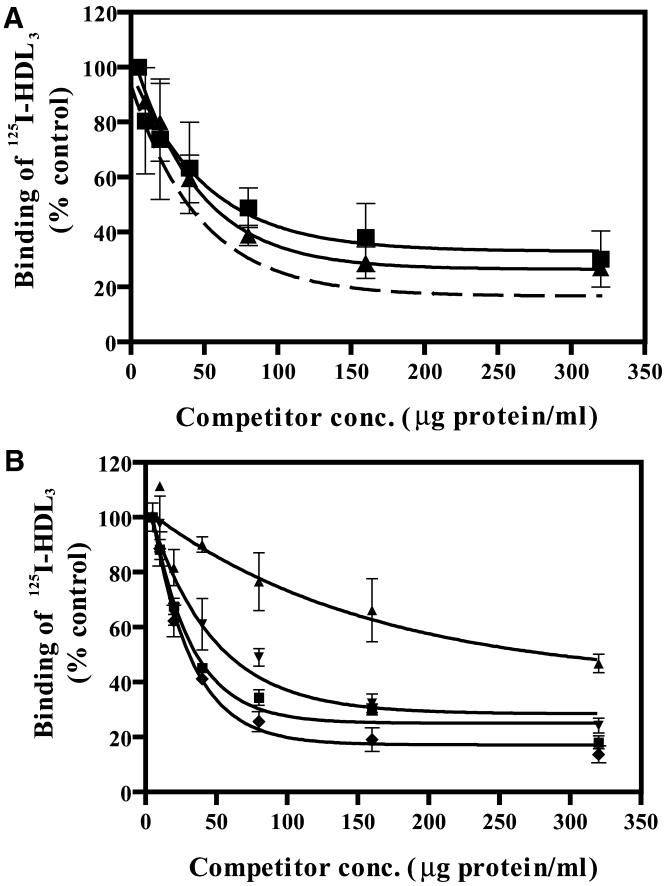

Fig. 6. Competitive binding of various apoA-I peptides complexed with DMPC to SR-BI-expressing cells. Y1-BS1 cells were incubated with 10 μg/ml of ^{125}I-HDL$_3$ plus increasing amounts of competitor. All competitors were complexed with DMPC. A: Shows the competitive binding curves for the cyanogen bromide fragments of apoA-I corresponding to amino terminal residues 1-85 (triangle) and carboxy terminal residues 149–243 (square). The dashed line is the competitive binding curve for the apoA-I/DMPC complex from Fig. 3. B: Shows competitive binding curves for peptides of apoA-I corresponding to residues 1–43 (triangle), 44–65 (diamond), 44–87 (inverted triangle), and 209–241(square). Data are presented as mean of triplicate wells ± SD. A representative experiment of three independent experiments is shown for each.

corresponding to various segments of the apoA-I molecule. The results in Fig. 6B show that the apoA-I peptide corresponding to residues 1–43 is not able to compete effectively with ^{125}I-HDL$_3$, while all other peptides of apoA-I that were examined are effective competitors. The IC$_{50}$ value of 160 μg protein/ml for peptide 1–43 is statistically different from the values for all other peptides examined. The IC$_{50}$ values obtained for the remaining peptides of apoA-I [i.e., 1–85 (N-terminal CNBr fragment), 44–65, 44–87, 149–243 (C-terminal CNBr fragment), and 209–241] were in the range of 30 μg to 65 μg protein/ml; these values are not statistically significantly different (ANOVA P = 0.86). Note that residues 209–241 contain a class Y amphipathic α-helix, while residues 1–43 contain a class G* helix (38). In addition, synthetic amphipathic α-helical peptides (18A and 37pA) with no sequence homology to apoA-I as DMPC complexes are effective competitors of ^{125}I-HDL$_3$ binding to SR-BI (**Fig. 7**); their IC$_{50}$ values are comparable to that of unlabeled HDL$_3$. Note that 37pA, which has two helical segments per molecule, exhibits slightly better competition (IC$_{50}$ of 10 ± 2.8 vs. 4 ± 1.4 μg protein/ml, P = 0.04) than 18A, which contains a single α-helix. The results are in agreement with our prior results (26) using the 37pA peptides in cross-linking experiments to show direct interaction of SR-BI and a class A amphipathic α-helix. Moreover, peptides of identical sequences containing either all D amino acids or a mixture of D and L enantiomers have the same ability to bind to SR-BI (Fig. 7B). These results indicate that the binding site in SR-BI is not stereospecific with respect to the handedness of the α-helical ligand.

DISCUSSION

A two-step model of SR-BI-mediated selective uptake from a particle such as HDL not only requires binding, but also appropriate organization of the bound HDL and the extracellular domain of the receptor. We demonstrated this previously by showing that apoA-I-mediated selective CE uptake is more efficient than apoE-mediated selective CE uptake in COS-7 cells transfected with SR-BI (18). The idea of a "productive complex" was also shown in a mutation of apoA-I that exhibited normal binding but a decreased efflux of cholesterol via SR-BI (28). Additionally, HDL from apoA-I-deficient mice binds to SR-BI with the same or greater affinity compared with HDL from wild-type mice, but apoA-I-deficient HDL shows a 2- to 3-fold reduced V_{max} for selective CE uptake (46). It follows that understanding SR-BI/apolipoprotein interaction is critical for describing the lipid transport process (Step 2, see Appendix) at the molecular level.

Both the phospholipid and protein components of HDL have the potential to bind to the receptor. Thus,

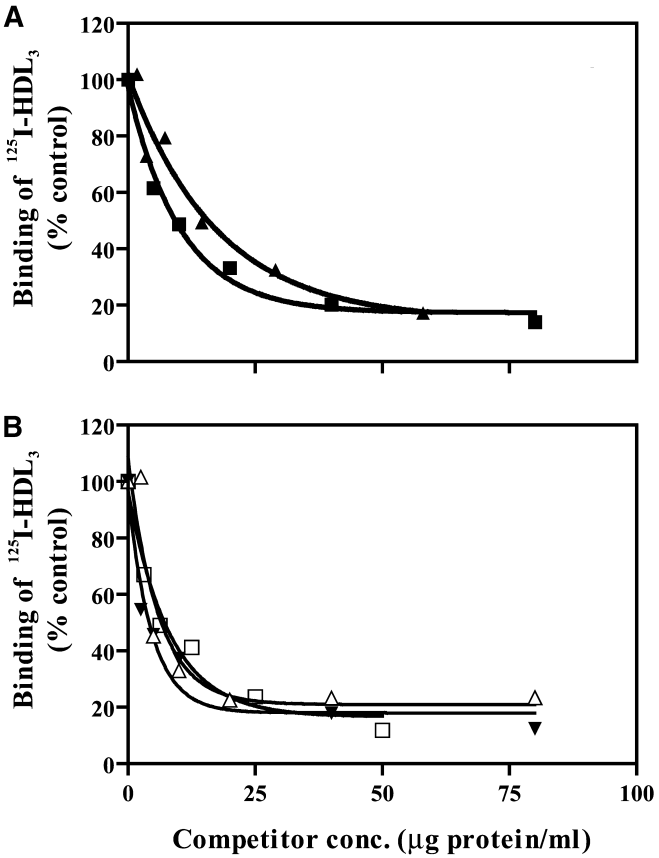

Fig. 7. Competitive binding of synthetic amphipathic helical peptides complexed with DMPC to SR-BI-expressing cells. Y1-BS1 cells were incubated with 10 μg/ml of ^{125}I-HDL$_3$ plus increasing amounts of competitor. All competitors are complexed with DMPC. A: Shows the competitive binding curves for D-18A (closed triangle) and L-18A (closed square). B: Shows competitive binding curves for DL-37pA (open square), L-37pA (open triangle), and D-37pA (inverted closed triangle). Data are mean of duplicate well determinations.

while POPC-SUVs do not compete the binding of ^{125}I-HDL (Fig. 2) (23), measurements of direct binding showed that the vesicles do bind to SR-BI and that this binding can be inhibited by rHDL (18). In the case of particles such as HDL, which contain apolipoproteins, the binding to SR-BI is predominately via the apolipoprotein. The experiments with apoA-I peptides (Fig. 6B), CNBr fragments of apoA-I (Fig. 6A), apoE isoforms (Fig. 5) (28), and synthetic amphipathic peptides 18A and 37pA (Fig. 7) indicate that the specific amino acid sequence of an apolipoprotein is not critical for binding to SR-BI, but rather some structural motif common to the exchangeable apolipoproteins is necessary. Our data (Fig. 6A) support and extend the finding that multiple domains of apoA-I can mediate binding to SR-BI (26).

A particularly important structural motif recognized by SR-BI is the amphipathic α-helix in exchangeable apolipoproteins (26). Presumably, binding of the HDL particles involves interaction of the exposed polar faces of amphipathic α-helices in apolipoproteins with SR-BI. The most abundant type of amphipathic α-helix present in apolipoproteins is the so-called class A helix, which is characterized by clusters of basic amino acids at the polar-nonpolar interfaces of the helix and acidic residues located at the center of the polar face (47). Although class A amphipathic α-helices interact well with SR-BI (Fig. 7), binding does not require this particular distribution of charged residues because class Y helices (e.g., apoA-I residues 209–241), defined by having an additional cluster of basic residues in the middle of the polar face, can also mediate binding to SR-BI (Fig. 6). In comparison, a class G* helix (e.g., apoA-I residues 1–43), which has a random distribution of acidic and basic residues in the polar face, does not bind well to the receptor. The explanation for these differences is not clear at present, but it demonstrates that the subtle variations among the amphipathic α-helices of apolipoproteins can modulate interaction with SR-BI. This subtle variation in the helical organization may in part be the molecular explanation for some of the reported differences between apoA-I and apoA-II binding and selective CE uptake (20, 27), despite the fact that both proteins contain putative amphipathic α-helices.

The L-18A and D-18A peptides form right- and left-handed α-helices, respectively, so the distribution of amino acid sidechains along the amphipathic α-helix is opposite in the two peptides. The fact that this structural difference does not alter binding to SR-BI (Fig. 7B) is consistent with the observation that a specific sequence of amino acids along the amphipathic α-helix is not required. Given that the polar face of a lipidated amphipathic α-helix is exposed for potential binding to SR-BI, it seems that the general characteristics of the distribution of charged residues in the polar face of the helix is the feature that is critical for binding. The binding site(s) on the receptor may contain clusters of charged residues that complement the exposed basic and acidic sidechains of amino acids in the amphipathic, α-helical ligand. α-Helices can function independently in the sense that single helical peptide molecules bind to SR-BI (Fig. 7), but in proteins such as apoA-I, cooperative interactions of covalently linked helices may be involved in binding to the receptor. This notion is supported by the observation that synthetic peptides composed of 37 amino acids (two α-helices) are slightly better at binding to the receptor than those containing only 18 amino acids (one α-helix) (Fig. 7A).

Analysis of HDL direct binding to SR-BI shows a hyperbolic isotherm (Fig. 1), indicative of a single class of binding sites on the receptor. At first sight, this seems to be inconsistent with the competitive binding data in Table 1, showing that there are 3- to 10-fold differences in IC_{50} values for lipid-free and lipid-associated apolipoproteins. However, the IC_{50} values cannot be used simply to derive information about relative affinities of the lipid-free and lipid-associated apolipoproteins for SR-BI, because the maximal degrees of competition are different. Nonetheless, the IC_{50} values in Table 1 indicate that the various apolipoproteins compete similarly when they are lipid bound. If the binding site on SR-BI were the same for both lipidated HDL (discoidal rHDL and spherical HDL) and lipid-free apolipoproteins, at some point the lipid-free

apolipoproteins should overcome the binding of ^{125}I-HDL. But this is not the case; lipid-free apolipoproteins bind with reduced capacity, as judged by the limiting ability to complete the binding of ^{125}I-HDL$_3$ (Figs. 3, 4) and lower B_{max} (26), than when they are associated with lipid. This suggests that the binding sites on SR-BI are distinct for lipid-free versus lipidated apolipoprotein. Therefore, our data suggest that the effect of lipidation on apolipoproteins is to increase the amount of binding to the receptor (B_{max}) and not necessarily to alter the affinity (K_d) of apolipoproteins for the receptor. An explanation for this increase in B_{max} is that multiple apolipoprotein molecules are present on an HDL particle so that, although only a single apolipoprotein molecule may be interacting directly with SR-BI, the other apolipoprotein molecules on the HDL are also bound by virtue of the fact that they are present on the same particle. In the case of lipid-free apolipoproteins, a single molecule in the monomeric state is involved in binding to the receptor.

The current competitive binding data are consistent with a model involving two binding sites on SR-BI, sites A and B. HDL and lipidated apolipoproteins can bind to both sites with equal affinity, while lipid-free apolipoproteins can bind only to site B. Because lipid-free apolipoproteins can bind solely to site B, they can compete for the binding of ^{125}I-HDL only at this site. Hence, site A is still available for ^{125}I-HDL to bind. Therefore, the model predicts that the maximum amount by which lipid-free apolipoproteins can compete the binding of ^{125}I-HDL is 50%, which is consistent with our data. In direct binding experiments, it is not possible to distinguish the second class of binding site since the labeled ligand, be it HDL or lipid-free apolipoproteins, will bind to its binding site irrespective of the other binding sites present. Since the ~40 kDa extracellular domain of SR-BI is comparable in size to apoA-I, it is likely that only one apoA-I molecule (either lipid-free or lipidated) can bind to a given receptor molecule at a given time (i.e., sites A and B on the same receptor molecule cannot be occupied simultaneously). This scenario of multiple binding sites is consistent with the observation that lipidated apolipoproteins and HDL can fully compete with lipid free ^{125}I-apoA-I. Furthermore, other investigators have observed a similar lack of cross competition between HDL and LDL (3), and identified an SR-BI mutant that binds LDL but not HDL (10). These observations support the idea that there are multiple binding sites on the receptor that can be distinct or overlapping, depending on the type of lipoprotein particle and/or the lipidation state of the apolipoproteins. As the tertiary and quaternary structure (e.g., receptor oligomerization) of SR-BI and fundamental information such as the stoichiometry of interaction between the receptor and the ligand become known, it should be possible to generate a molecular description of the ligand binding site(s) on SR-BI and of how it functions in CE selective uptake.

It was suggested recently that the N-terminal domain of apoE contains the SR-BI binding sequence (28). However, the situation may be more complicated because the four-helix bundle structure adopted by the N-terminal domain of the apoE isoforms in the lipid-free state binds less well to the receptor than the full-length apoE molecules, indicating that there is a contribution from the C-terminal region (Table 1). When complexed with phospholipids, the 22 kDa fragments bind to SR-BI similarly to the full-length proteins (Table 1). In this situation, the N-terminal four-helix bundle is open (48), which shows that the conformation of the apoE molecule significantly affects interaction with SR-BI.

APPENDIX

The scavenger receptor class B, type I (SR-BI)-mediated selective uptake of lipids (L) into cells can be described as a two-step process. The reaction sequence is as follows:

$$LD + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} LD\text{-}R \qquad \text{step 1 (fast)} \quad \textit{(Eq. 1A)}$$

$$LD\text{-}R \xrightarrow[k_3]{} D\text{-}LR \qquad \text{step 2 (slow)} \quad \textit{(Eq. 2A)}$$

where LD is the lipid-donating particle and LD-R is LD bound to the receptor R (SR-BI). Step 1 involves a diffusion-dependent binding of LD to R. From the law of mass action and assuming a single class of independent binding sites, the binding (B) of LD to R can be described by the hyperbolic (Scatchard) equation:

$$B = \frac{[LD]\, B_{max}}{[LD] + K_d} \qquad \textit{(Eq. 3A)}$$

where B_{max} is the maximum amount of LD binding (i.e., ng LD bound/mg cell protein) and K_d is the equilibrium dissociation constant equal to the ratio of the rate constants k_2/k_1. At low LD concentrations (i.e., $[LD] << K_d$), Eq. 3A indicates that B is proportional to [LD]. At concentrations where $LD >> K_d$, the binding sites are saturated and the amount of LD-R formation is independent of [LD]. Step 2 involves the dissociation of lipid L from the bound donor particle LD (rate constant = k_3), and its diffusion down a concentration gradient into the cell plasma membrane via SR-BI to form D-LR (lipid-depleted donor particle bound to the receptor) (19). Under initial velocity conditions, this step is essentially irreversible and it is rate limiting (i.e., $k_3 < k_1$). The rate (V) of selective lipid uptake exhibits a hyperbolic dependence on the concentration of LD.

$$V = \frac{[LD]\, V_{max}}{[LD] + K_m} \qquad \textit{(Eq. 4A)}$$

This two-step kinetic model of cellular lipid uptake is analogous to the Michaelis-Menten description of the rate of enzyme-substrate interaction and the formation of product. A K_m for selective lipid uptake equivalent to the term in the Michaelis-Menten equation can be obtained from the hyperbolic dependence of V on the concentration of LD (Eq. 4A). The experimentally derived values of K_d and K_m are similar (Fig. 1), indicating that steps 1 and 2 are coupled.

If the binding of donor particle LD to SR-BI is mediated by an apolipoprotein, step 2 leads to the selective transfer of lipid molecules from the LD-R complex to the cell membrane. If LD contains different classes of lipid, the relative rates of selective uptake of these lipids are determined by the values of the rate constants (k_3) defining their dissociation from the bound LD. The details of step 2 are quite different if the binding of the do-

nor particle LD to SR-BI is not mediated by an apolipoprotein. When the binding of the donor particle LD to SR-BI is mediated by the lipid component L, the selectivity of lipid uptake disappears. The rate constant k_3, defining the dissociation of lipid molecules from LD, is no longer relevant and is replaced by the rate constant describing the cellular uptake of intact LD. The molecular mechanism of the latter process is consistent with an SR-BI-mediated fusion of the donor lipid particle and the cell plasma membrane (18).

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