Highly Purified Scavenger Receptor Class B, Type I
Reconstituted into Phosphatidylcholine/Cholesterol Liposomes
Mediates High Affinity High Density Lipoprotein Binding and
Selective Lipid Uptake*

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The murine class B, type I scavenger receptor mSR-BI is a high and low density lipoprotein (HDL and LDL) receptor that mediates selective uptake of cholesteryl esters. Here we describe a reconstituted phospholipid/cholesterol liposome assay of the binding and selective uptake activities of SR-BI derived from detergent-solubilized cells. The assay, employing lysates from epitope-tagged receptor (mSR-BI-t1)-expressing mammalian and insect cells, recapitulated many features of SR-BI activity in intact cells, including high affinity and saturable $^{125}$I-HDL binding, selective lipid uptake from $[^3]$H]cholesteryl ether-labeled HDL, and poor inhibition of HDL receptor activity by LDL. The novel properties of a mutated receptor (Q402R/Q418R, normal LDL binding but loss of most HDL binding) were reproduced in the assay, as was the ability of the SR-BI homologue CD36 to bind HDL but not mediate efficient lipid uptake. In this assay, essentially homogeneously pure mSR-BI-t1, prepared by single-step immunoaffinity chromatography, mediated high affinity HDL binding and efficient selective lipid uptake from HDL. Thus, SR-BI-mediated HDL binding and selective lipid uptake are intrinsic properties of the receptor that do not require the intervention of other proteins or specific cellular structures or compartments.

The LDL$^1$ receptor pathway for the delivery of lipoprotein cholesterol to cells involves clathrin-coated pit-mediated endocytosis and subsequent lysosomal degradation of the entire LDL particle (1). Almost 20 years ago a strikingly different mechanism for the cellular uptake of lipoprotein cholesterol, called selective cholesterol uptake, was identified during the analysis of HDL metabolism in vivo (2, 3). Selective cholesterol uptake from HDL and other lipoproteins does not involve endocytosis and subsequent degradation of the entire lipoprotein particle (2, 3; reviewed in Refs. 4 and 5). In the case of HDL, the lipoprotein binds to the cell membrane and transfers its cholesteryl esters to the cell, and then the lipid-depleted HDL particle dissociates from the cell and can re-enter the circulation.

The HDL receptor SR-BI (scavenger receptor, class B, type I) was the first cell surface receptor to be shown to mediate physiologically relevant selective lipid uptake (6–8; reviewed in Ref. 5). In vivo studies have established that SR-BI critically influences HDL structure and metabolism and apparently plays an important role in the transport of cholesterol from peripheral tissues to the liver for recycling or biliary excretion (5, 7–12). This probably accounts for the ability of SR-BI to protect against atherosclerosis in murine models (9, 13–15). Recent studies have established that expression of SR-BI in mice is normally required for red blood cell development (16) and female fertility (9, 17) and can prevent the development of myocardial infarctions, cardiac dysfunction, and premature death in apoE-deficient mice (18).

In vitro studies have shown that SR-B can bind and mediate lipid uptake from LDL as well as HDL (6, 19–22). Strikingly, HDL competes efficiently for LDL binding, whereas LDL is a poor competitor of HDL binding (6, 23). SR-BI also can facilitate the efflux of unesterified cholesterol from cultured cells (24, 25), although the physiologic significance of this is not certain. Several studies support the proposal (26) that SR-BI-mediated transport of lipids between cells and lipoproteins involves two sequential steps: 1) productive lipid binding and 2) binding-dependent, yet distinct, SR-BI-mediated lipid transfer (25–29). It has not yet been determined if SR-BI-mediated selective lipid uptake occurs only at the cell surface, or in some intracellular compartment followed by retroendocytosis (secretion) of the lipid-depleted lipoprotein, or both (30–32; reviewed in Refs. 4 and 5).

A particularly important question regarding the mechanism of SR-BI activity has been: does SR-BI require the participation of one or more other proteins to mediate either ligand binding, lipid transport, or both, or are these activities autonomous properties of SR-BI (independent of other proteins)? This question has arisen, in part, because of the multiple and complex activities of SR-BI (5). A direct approach for studying the autonomous properties of SR-BI and its mechanism of action is to examine in an in vitro system the activity of the receptor purified away from other proteins. Here we describe an in vitro reconstituted liposome assay for SR-BI-mediated ligand binding and selective lipid uptake that reflects many of the characteristics of SR-BI activity in intact cells. This assay, effective with total cell lysates as well as highly purified protein, was used to show that, in the absence of other proteins, SR-BI can
bind HDL and LDL and mediate efficient selective cholesteryl ether uptake from HDL.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human high density lipoprotein (HDL), human low density lipoprotein (LDL), [125I]-labeled HDL, [125I]-labeled LDL, 1,1-diiodoacyl-3,3,3'-tetrathethylinidocarboxyamine perchlorate (DiI)-labeled HDL (DiI-HDL), and human lipoxygen-deficient serum were prepared as previously described (6, 19). The phCD36 expression vector (32) was a generous gift from B. Seed (Massachusetts General Hospital). The KKB-1 antibody was a generous gift from Karen Kozarsky (25). The 1D4 antibody was obtained from the ATCC. The mixture of complete protease inhibitors was purchased from Roche Molecular Biochemicals. All other reagents were purchased from standard suppliers or obtained as indicated below. Cell culture supplies were purchased from Invitrogen, Irvine Scientific, or JRH Biosciences. The peptide TETTSQVAPA was prepared in the biopolymer laboratory at Massachusetts Institute of Technology and was a gift from the G. Khorana laboratory.

[2H]Cholesteryl ether ([2H]CE)-labeled HDL ([2H]CE-HDL) was prepared according to the procedure of Rodrigueza (34) with minor modifications. The labeled [2H]CE-HDL was isolated by ultracentrifugation (225,000 x g) and filtered through a 0.22-μm membrane, and the protein concentration was determined using the method of Lowry et al. (35).

**Construction of Expression Vectors**

**Mammalian Expression Vectors**—mSR-BI cDNA was amplified from pmSR-BI (6) by PCR using the primers BL5, 5'-GAGACTGTTACGA- TATCAGCGGGCACTGAGGCTCCAG-3', and BL3, 5'-CTGTCGACTCCAG-3', and 5'-GCAGCTCGAGGTCACAAGTACATC-3' (the expected sequence was designated "mSR-BI-t1"). The PCR product was treated with restriction endonucleases KpnI and XhoI, and the KpnI/XhoI fragment was cloned into the mammalian expression vector pcDNA3 (+) (Invitrogen) that includes an internal neomycin resistance marker for selection in mammalian cells. The ligation product was used to transform Escherichia coli cells, and the plasmid DNAs of selected clones were isolated and sequenced. One of these with the expected sequence was designated pmSR-BI-t1. The mSR-BI cDNA was then reconstructed into an expression plasmid (36) using an Sall and EcoRV restriction fragment from pmSR-BI-t1. This plasmid was designated pAcmSR-BI-t1 and was used for the generation of the stable cell line HEK[mSR-BI-t1] (see below).

**SE1 Expression Vectors**—Three receptors were expressed in SE1 cells at high levels using the Bac-To-Bac baculovirus expression system (Invitrogen). These were mSR-BI-t1, a double-substitution mutant of mSR-BI-t1 (arginines in place of the glutamines at positions 402 and 418, designated 402R/418R), which retains most of the LDL but little of the HDL selectivity of the lipid uptake process. The 125I-HDL binding assay was performed as previously described (6). The [2H]CE-HDL association assay was similar to the [125I]-labeled HDL binding assay except that the NaOH cell lysis step was preceded by the addition of 1 ml of isopropanol at room temperature for 30 min to extract the incorporated [2H]cholesteryl ether from the cells. Radioactivity in the isopropanol extract was measured using a liquid scintillation analyzer (Packard Instrument Co., Meriden, CT). The amount of [2H]cholesteryl ether associated with the cells (or liposomes in the assay described below) was determined by flow cytometry as previously described (23).

**Preparation of Total Cell Lysates**

HEK[mSR-BI-t1] and untransfected HEK293S cells were plated at 1–2 x 10⁶ cells/plate in 10-cm plates and grown in 15 ml of medium A culture, HEK293S cells were grown with constant gentle stirring at 20–40 rpm in spinner bottles in medium B (HBGr medium (Irvine Scientific) supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine (Invitrogen), and 10% fetal bovine serum) at 37 °C in a humidified incubator in 5% CO2/95% air. For suspension growth, cells were incubated at 1.2–1.5 x 10⁶ cells/ml in 500 ml of medium B and incubated for 5–7 days without changing the medium. Cells were harvested either after reaching confluence on dishes by treatment with trypsin/EDTA (JRHI Biosciences) or after 6 days of growth in suspension.

For transient transfections, 1.5 x 10⁶ COS M9 cells were seeded in 12-well dishes in medium A without antibiotics and incubated at 37 °C overnight. The cells were treated with 10 μg per plate of DNA (pmSR-BI-t1 or the "empty" vector pcDNA3 without an expression cassette insert as a control) using the LipofectAMINE (Invitrogen) method according to the manufacturer’s recommendations. The cells were grown in medium A for an additional 2 days, and then receptor activity assays were performed. Briefly, 24 h post-transfection, the cells were washed with PBS, harvested with trypsin/EDTA, and plated in 1 ml/well of medium A at 150,000 cells/well in 24-well plates. The receptor activity of the cells was analyzed 48 h post-transfection.

A stable cell line expressing high levels of mSR-BI-t1, HEK[mSR-BI-t1] (clone 7), was established as follows: HEK293S cells were transfected with the pmCAmSR-BI-t1 vector using the LipofectAMINE method according to the manufacturer’s recommendations. The infected cells were grown in medium A for 5 days at 27 °C in Grace’s medium, the media were collected as the recombinant baculovirus particle stocks and used for infection of Sf21 cells (procedures carried out according to the manufacturer’s recommendations). The infected cells were grown in Grace’s medium for 72–128 h and harvested by centrifugation. High level expression of the proteins was verified by SDS-PAGE, and immunoblotting analysis of cell extracts, prepared as described below, using polyclonal anti-SR-BI antibody (6) or anti-mChicken antibody (4) supplemented with 0.05% G418. Individual colonies were isolated by screening for their abilities to take up the fluorescent dye DiI from DiI-HDL (10 μg of protein/ml, 2 h at 37 °C) with an inverted fluorescence microscope and by flow cytometry as previously described (26).
mSR-BI-t1 was purified by immunoadfinity chromatography using a modification of the procedure of Reeves et al. (38). Briefly, 1.5 g (wet weight) of HEK[mSR-BI-t1] cells were incubated with 13 ml of lysis buffer A (1.5% (v/v) octyl glucoside and protease inhibitors (1x) in PBS) per 0.1 g (wet weight) of pellet and incubating at 4 °C for 30 min with gentle mixing. The lysates were clarified by centrifugation at 11,951 × g using an SS34 rotor for 20 min at 4 °C, and the supernatants were collected and used as total cell lysates. Protein concentration was determined by the DC protein assay (Bio-Rad) and was typically 1–2 mg/ml. The same procedure was used to prepare lysates from SF21 cells.

**Immunoadfinity Purification of mSR-BI-t1**

For 3–4 days. When the cells were confluent, the plates were washed twice with PBS, and the cells were harvested by scraping with a rubber policeman and concentrated into a pellet by centrifugation at 4 °C at 1,460 × g for 20 min in an SS34 rotor in a Sorvall RC-5B centrifuge (DuPont Instruments). The cell pellets were dissolved by adding 1 ml of lysis buffer A (1.5% (w/v) octyl glucoside and protease inhibitors (1x) in PBS) per 0.1 g (wet weight) of pellet and incubating at 4 °C for 30 min with gentle mixing. The lysates were clarified by centrifugation at 11,951 × g using an SS34 rotor for 20 min at 4 °C, and the supernatants were collected and used as total cell lysates. Protein concentration was determined by the DC protein assay (Bio-Rad) and was typically 1–2 mg/ml. The same procedure was used to prepare lysates from SF21 cells.

**Liposome Preparation**

The preparation of multimamellar vesicles was carried out according to Schneider et al. (39) with minor modifications. Briefly, egg yolk L-α-phosphatidylcholine (Avanti) and cholesterol (Sigma Chemical Co.) (molar ratio of 5:1) in ether were dried and resuspended at 2 mg/ml in 50 mM Tris buffer (pH 6.0). Then 0.5 ml of the suspension and 2.4 ml of buffer B (50 mM Tris, pH 6.0, 150 mM NaCl, 2 mM CaCl₂) containing either 100 μg of detergent-solubilized cell lysate or 2 μg of detergent-solubilized purified mSR-BI-t1 were mixed, precipitated by 0.6 volume of ice-cold acetone, and the precipitate was recovered by centrifugation (4 °C at 30,500 × g, 20 min). The liposomes were resuspended in 300 μl of buffer C (20 mM Tris, pH 8.0, 1 mM CaCl₂, 20 mM NaCl, and protease inhibitors).

**Filter Binding Assay**

Liposomes (8 μl) were diluted with 12 μl of assay buffer (20 mM Tris, pH 8.0, 2 mM CaCl₂, 80 mg/ml fat-free bovine serum albumin (Sigma), and protease inhibitors (1x)) and the indicated amounts of 125I-HDL or [3H]CE-HDL (usually 10 μg of protein/ml) in the presence (single incubations) or absence (duplicate incubations) of 40-fold excess of unlabeled HDL. The mixture was incubated at 37 °C for 2 h unless otherwise noted, and the liposomes with bound 125I-HDL were isolated by filtration (38) using 0.45 μm nitrocellulose membranes and a multfilter filtration manifold (Millipore, Milford, MA). Briefly, the filters in the manifold were moistened with wash buffer (20 mM Tris, pH 8.0, 50 mM NaCl, 20 μg/ml fat-free bovine serum albumin), the sample was filtered at room temperature and then the filters were washed three times with wash buffer. The amounts of 125I-bound were measured with a gamma counter (LKB-Wallac, Finland). To measure 1H, the filters were added to 4 ml of Hydrofluor scintillation fluid (National Diagnostics) and radioactivity was measured using a liquid scintillation analyzer (Packard Instrument Co., Meriden, CT).

**RESULTS**

The two goals of this study were 1) to develop an in vitro reconstituted liposome assay of SR-BI-mediated ligand binding and lipid uptake and 2) to use the assay to determine if highly purified SR-BI required the cooperation of other proteins to mediate HDL binding and selective lipid uptake or if it could do so independently of other proteins. To address the later goal, we developed a modification of the method of Reeves et al. (38), developed for the analysis of rhodopsin, to isolate essentially homogeneous pure and functional mSR-BI containing an exogenous epitope tag.

**Comparison of Binding and Uptake Activities of Cells Transfected with Plasmid DNA of Wild-type and Tagged mSR-BI**—To facilitate isolation of pure mSR-BI protein for reconstitution into liposomes, we slightly modified the approach described by Reeves, Thurmond, and Khorana (38) for the generation and purification of recombinant bovine rhodopsin from transfected mammalian cells. Reeves et al. (38) expressed bovine rhodopsin in HEK293S cells and purified the detergent-solubilized protein using monoclonal antibody affinity purification and epitope peptide elution. The anti-rhodopsin antibody 1D4 recognizes the C-terminal 9-amino acid peptide from rhodopsin (40). For the synthesis and purification of mSR-BI, we constructed a mammalian cell expression vector, pmSR-BI-t1, that encodes a chimeric protein (mSR-BI-t1) containing the full-length mSR-BI and, at its C terminus, the C-terminal epitope-tag TETTSQVAPA from bovine rhodopsin. To determine if epitope tagging interfered with the activity of the receptor, we transiently transfected COS M6 cells with expression vectors encoding either wild-type mSR-BI (COS[mSR-BI]), epitope-tagged mSR-BI-t1 (COS[mSR-BI-t1]), or an empty vector (pcDNA) control (COS[control]) and measured the two best defined activities of SR-BI (5): binding of 125I-HDL and uptake of cholesteryl ether from [3H]CE-HDL. The values for receptor-specific binding or lipid uptake are defined as the differences between total binding or uptake and the values determined in the presence of a 40-fold excess of unlabeled ligand (nonspecific values). The lipid uptake data are presented as [3H]CE-HDL protein equivalents (nanograms of [3H]CE-HDL protein that contain the amount of [3H]cholesteryl ether associated with the cells). Fig. 1 shows that the specific 125I-HDL binding (panel A) or [3H]cholesteryl ether uptake (panel C) values for mSR-BI and mSR-BI-t1 were similar. The relative levels of surface expression of the wild-type and tagged receptors were determined using an anti-SR-BI antibody (KKB-1) and quantitative flow cytometry (25). There was a somewhat higher level of expression of the tagged receptor (1.2-fold). When the 125I-HDL binding and [3H]cholesteryl ether uptake values for the wild-type receptor were normalized to account for this difference, the binding and lipid uptake curves for the two receptors were virtually identical (Fig. 1, B and D). The ratios of the maximal [3H]CE-HDL uptake/125I-HDL binding, expressed as equivalent ng of HDL protein/mg of cell protein, were 47 and 44, respectively, clearly showing that both receptors mediated selective lipid uptake. Therefore, the epitope tagging at the C terminus of mSR-BI did not appear to alter the key activities of mSR-BI in transfected COS cells, and this tagged mSR-BI should be useful for the isolation of purified SR-BI (see below) for studying its activity in a reconstituted system.

**Reconstituted Liposome Assay for the Cell-free Analysis of SR-BI Function**—We have developed a reconstituted liposome filter binding assay for measuring the binding of 125I-HDL and the uptake of [3H]cholesteryl ether from [3H]CE-HDL mediated by SR-BI derived from detergent-solubilized cultured cells based on the LDL receptor binding assay of Schneider et al. (39) (see “Experimental Procedures”). In brief, phosphatidilycholine/cholesterol vesicles were prepared using detergent-solubilized whole cell lysates or detergent-solubilized purified receptor protein. After incubation with radiolabeled lipoprotein (standard conditions: 5–10 μg of protein/ml of lipoprotein for 2 h at 37 °C, variations indicated below), the liposome-associated radioactivity was determined by ultrafiltration and counting. Standard controls included: preparation of liposomes either with lysates from cells that do not express recombinant receptor or without added cell lysate or purified receptor; and incubation with a 40-fold excess of unlabeled lipoprotein to compete for the specific binding of the ligands to the receptor.
shown in the presence of cell surface expression relative to that directed to account for the 1.2-fold difference in surface expression of mSR-BI-expressing cells.

The specificity of the receptor was determined using the polyclonal anti-mSR-BI antibody KKB-I by flow cytometry and the control "empty" vector pCDNA. Two days later, the cells were incubated for 2 h at 37 °C with the indicated concentrations of either 125I-HDL or [3H]CE-HDL in the absence (single determinations) or absence (duplicate determinations) of a 40-fold excess of unlabeled HDL. Specific 125I-HDL binding (Δ) and [3H]CE uptake (C) were determined as described under "Experimental Procedures." The relative levels of cell surface expression of mSR-BI and mSR-BI-t1 were determined using an ELISA assay validated using cell lysates from insect cells (Sf21) expressing high levels of tagged SR-BI (22). The reconstituted liposome assay was initially validated using cell lysates from insect cells expressing high levels of SR-BI-t1 (pACmSR-BI-t1) and isolated a clone expressing high levels of the receptor, HEK[mSR-BI-t1] (clone 7). Detergent-solubilized lysates of these cells were reconstituted into liposomes, and the binding of 125I-HDL and the uptake of [3H]cholesterol ether from [3H]CE-HDL were measured. Fig. 5A shows results similar to those seen using extracts from the insect cells, i.e. high affinity binding and uptake (apparent $K_d$ 8.5 μg of protein/ml, $P_{max}$ 12.8 ng of HDL protein/assay) and selective lipid uptake (apparent $K_d$ 29.4 μg of protein/ml, $P_{max}$ 0.3 ng of protein/ml) by mSR-BI-t1-containing insect cell lysate-reconstituted liposomes. The specificity of the receptor's activity in mSR-BI-t1-containing mammalian cell lysate-reconstituted liposomes was examined by comparing the abilities of unlabeled HDL and LDL to inhibit [3H]cholesterol ether uptake from [3H]CE-HDL (10 μg of protein/ml). Previous studies of SR-BI expressed in intact cells established that, although LDL can bind to SR-BI with nonspecific background binding of 125I-HDL varied from 10 to 50% of the total binding and appeared to depend critically on the quality of the preparation, e.g. extent of radiolabeled decompostion of the HDL, which depends on the age and specific activity of the preparations and can occur rapidly (41). The absolute values for binding and lipid uptake constants (apparent $K_d$ and $P_{max}$ (binding maximum) and $U_{max}$ (uptake maximum)) also varied somewhat between different receptor preparations and dependent on the quality of the radilabeled lipoprotein (41).

Validation of the Reconstituted Liposome Assay—The reconstituted liposome assay was initially validated using cell lysates from insect cells (SF21) expressing high levels of tagged receptor (not shown) due to infection with a baculovirus encoding mSR-BI-t1. Previous studies have shown that insect cells can express on their surfaces functional mammalian SR-BI (42, 43). Fig. 2A shows the ligand concentration dependence of the binding of 125I-HDL (squares) and the uptake of [3H]cholesterol ether from [3H]CE-HDL (circles) by mSR-BI-t1-containing insect cell lysate-reconstituted liposomes (open symbols) and control liposomes prepared with lysates from cells infected with baculovirus without the infection (control, filled triangles). Specific 125I-HDL binding to the mSR-BI-t1-containing liposomes (open squares) was high affinity (apparent $K_d$ 0.14 μg/ml, similar to that seen in mSR-BI-expressing mammalian cells (6, 41)) and saturable ($B_{max}$ 15 ng of protein/assay), whereas there was very little specific binding to the mSR-BI-t1-negative control liposomes (filled triangles). Similar results were obtained for the uptake of [3H]cholesterol ether from [3H]CE-HDL (open circles, apparent $K_d$ 11 μg of protein/ml, $U_{max}$ 106 ng of protein/assay); the maximal value for lipid uptake was 7-fold greater than that for binding, indicating selective uptake.

Fig. 3 shows the time dependence of 125I-HDL binding (open squares) and [3H]cholesterol ether uptake from [3H]CE-HDL (open circles) by mSR-BI-t1-containing insect cell lysate-reconstituted liposomes at 37 °C. The 125I-HDL binding reached a steady state after about 1 h, whereas the [3H]cholesterol ether uptake increased until approximately 3 h of incubation. Similar differences in the kinetics of SR-BI-mediated HDL binding and lipid uptake have been observed in intact cultured cells (6). There was little binding or lipid uptake by the receptor-negative control liposomes (solid triangles). Fig. 4 shows the temperature dependence of 125I-HDL binding and [3H]cholesterol ether uptake from [3H]CE-HDL at 10 μg of protein/ml by mSR-BI-t1-containing insect cell lysate-reconstituted liposomes. The value for 125I-HDL binding at 0 °C was somewhat lower (79%) than at 37 °C (100%), whereas there was a more substantial reduction in [3H]cholesterol ether uptake at 0 °C (25%) compared with that at 37 °C (100%). It has previously been noted that SR-BI-mediated binding of HDL (23, 34) and selective uptake (34) are lower at 4 °C than at 37 °C and that the temperature sensitivity of selective uptake is greater than that of HDL binding (34). To determine if the liposome assay could be used for recombinant SR-BI produced by mammalian cells, we transfected HEK293S cells with a mammalian expression vector for mSR-BI-t1 (pACmSR-BI-t1) and isolated a clone expressing high levels of the receptor, HEK[mSR-BI-t1] (clone 7). Detergent-solubilized lysates of these cells were reconstituted into liposomes, and the binding of 125I-HDL and the uptake of [3H]cholesterol ether from [3H]CE-HDL were measured. Fig. 5A shows results similar to those seen using extracts from the insect cells, i.e. high affinity HDL binding (apparent $K_d$ 8.5 μg of protein/ml, $P_{max}$ 12.8 ng of HDL protein/assay) and selective lipid uptake (apparent $K_d$ 29.4 μg of protein/ml, $U_{max}$ 220.9 ng of HDL protein/assay). The specificity of the receptor's activity in mSR-BI-t1-containing mammalian cell lysate-reconstituted liposomes was examined by comparing the abilities of unlabeled HDL and LDL to inhibit [3H]cholesterol ether uptake from [3H]CE-HDL (10 μg of protein/ml). Previous studies of SR-BI expressed in intact cells established that, although LDL can bind to SR-BI with
liposomes as described under Experimental Procedures. The liposomes were incubated with 125I-HDL or [3H]CE-HDL at the indicated concentrations at 37 °C for 3 h in the presence (single determinations) or absence (duplicate determinations) of a 40-fold excess of unlabeled HDL, isolated, and washed by filtration, and the amounts of specific 125I-HDL binding and [3H]cholesterol ether uptake were determined as described under “Experimental Procedures.” Error bars represent the range of variation in duplicate determinations. The nonspecific background values for 125I-HDL binding ranged between 19 and 28% (mSR-BI-t1) and 29 and 45% (CD36) of the total binding. The values for the no receptor lysates were very low and overlap for specific 125I-HDL binding and [3H]cholesterol ether uptake (broken lines). Panel C shows with an expanded scale the binding of 125I-HDL to the liposomes containing mSR-B1-t1 (open squares) and CD36 (filled squares).

The [3H]cholesteryl ether uptake from [3H]CE-HDL (10 μg of protein/ml) by mSR-BI-t1-expressing mammalian cell lysate-reconstituted liposomes exhibited essentially no [3H]cholesterol ether uptake activity. Although the above data support the validity of the liposome assay, we further tested the assay by determining the receptor activities of liposomes reconstituted with insect cell lysates containing in place of mSR-BI-t1 either a mutant form of mSR-BI-t1 or human CD36, another class B scavenger receptor (5, 19). We have isolated a set of mutant mSR-BIs, which exhibit altered ligand-binding properties when expressed in transfected mammalian cells (23, 25). One of these has a double substitution of arginines for the glutamines at positions 402 and 418 (designated 402R/418R). This 402R/418R mutant is as effective as wild-type mSR-BI in functioning as an LDL receptor in transfected mammalian cells, mediating high affinity LDL binding, uptake of metabolically active cholesterol from LDL, and efflux of cholesterol to LDL; however, it has lost most of the corresponding HDL receptor activity exhibited by the wild-type receptor (23). Lysates from insect cells expressing mSR-BI-t1, the 402R/418R mutant form of mSR-BI-t1, or no recombinant protein (control) were reconstituted into liposomes, and the abilities of the liposomes to bind 125I-HDL or 125I-LDL were determined. Fig. 6B shows that, as expected, the specific binding of 125I-LDL (5 μg of protein/ml) to the 402R/418R mutant (light gray bar) was similar to that of mSR-BI-t1 (open bar) and substantially greater than that of the receptor-free control (dark filled bar). In contrast, Fig. 6A shows that the binding of 125I-HDL (5 μg of protein/ml) to the 402R/418R mutant was much lower than that of mSR-B1-t1 and was not significantly different from that of the receptor-free control. Because the binding specificities of the whole insect lysate-reconstituted liposomes reflected the specificities of the corresponding intact mammalian cells, it seems likely that the mechanism of SR-BI-mediated lipoprotein binding in the lysate-reconstituted liposomes is similar to that in intact mammalian cells.

CD36 is a class B scavenger receptor that is structurally similar to SR-BI and shares a number of ligand-binding activities (19, 26, 42, 44). For example, CD36 binds HDL with an affinity similar to that of mSR-BI (26, 27, 42); however, CD36 cannot mediate efficient selective uptake of cholesterol from high affinity LDL binding. LDL is a poor inhibitor of HDL binding to SR-BI (6, 25). Fig. 5B shows that, as is the case for intact cells, HDL was an effective inhibitor of mSR-BI-t1-mediated lipid uptake activity from [3H]CE-HDL, whereas LDL was less effective. The [3H]cholesterol ether uptake from [3H]CE-HDL (10 μg of protein/ml) by mSR-BI-t1-containing mammalian cell lysate-reconstituted liposomes was inhibited by the polyclonal anti-SR-BI antibody KKB-1 to the same extent as by excess unlabelled HDL (Fig. 5C), whereas uptake was not inhibited by control pre-immune antibody. Control untransfected HEK293S cell lysate-reconstituted liposomes exhibited essentially no [3H]cholesterol ether uptake activity.

Experimental Procedures.
HDL to cells (26, 27). Fig. 2, B and C show that CD36-containing insect cell lysate-reconstituted liposomes bound 125I-HDL (filled squares) at a level comparable to that of the corresponding mSR-BI-t1-containing liposomes (Fig. 2A, open squares), $B_{\text{max}}$ = 15 ng of protein/assay (apparent $K_d$ 30.3 µg of protein/ml). In contrast, the CD36-containing liposomes exhibited almost no [3H]cholesteryl ether uptake from [3H]CE-HDL (Fig. 2B, filled circles, $U_{\text{max}}$ = 3.5 ng of protein/assay) compared with that of mSR-BI-t1-containing liposomes (Fig. 2A, open circles, $U_{\text{max}}$ = 106.3 ng of protein/assay). Thus, the lipid uptake activities of mSR-BI-t1 and CD36 in whole insect lysate-reconstituted liposomes reflected their activities when expressed on the surfaces of intact mammalian cells.

Taken together the data in Figs. 2–6 show that the whole cell lysate/liposome assay recapitulated many key features of cellular SR-BI-mediated HDL receptor activity: 1) 125I-HDL binding and [3H]cholesteryl ether uptake from [3H]CE-HDL were high affinity and saturable (6); 2) binding reached a steady state more rapidly than lipid uptake (6); 3) LDL was a poor inhibitor of HDL binding and lipid uptake (6, 25); 4) lipid transfer was specifically inhibited by an anti-SR-BI blocking antibody (25); 5) lipid uptake was substantially more temperature-sensitive than binding (34); and 6) lipid transfer occurred via selective uptake (6). Furthermore, in this assay the activities of a mutant form of SR-BI (405R/m418R) and the homologue CD36 also recapitulated those in intact cells. Therefore, the reconstituted liposome system provides a valid assay for the HDL binding and lipid uptake activities of SR-BI in detergent-solubilized, whole cell lysates.

One-step Immunooaffinity Purification of mSR-BI-t1—To examine the function of SR-BI using the reconstituted liposome assay in the absence of other proteins, we isolated highly purified mSR-BI-t1 from HEK[mSR-BI-t1] cells using a modified version of the rhodopsin purification of Reeves et al. (38). The receptor was purified from octyl glucoside-solubilized cells by immunoaffinity chromatography using an antibody to its C-terminal rhodopsin peptide epitope tag. Results of a typical purification are shown in Fig. 7, in which specimens obtained throughout the procedure were fractionated by SDS-PAGE and visualized by silver staining of the gel. Both the starting cell lysate (lane 1) and the initial column flow-through (material not retained by the column, lane 2) were highly complex protein mixtures. After washing the column so that no additional protein was detected by silver staining (lane 3), we eluted bound material with the rhodopsin C-terminal peptide and recovered virtually homogeneously pure mSR-BI-t1 (lanes 4–8). The electrophoretic mobility of the bulk of the purified material corresponded to 82 kDa, as expected from previous studies (6). Immunoblotting of a replicate gel with anti-mSR-BI KKB-1 antibody established that the major protein band detected by silver staining, as well as the very low abundance minor bands (e.g. see lane 5), was either mSR-BI-t1 or minor proteolytic or aggregated forms of mSR-BI-t1 (not shown). Immunoblotting also revealed that a very small amount of mSR-BI-t1 was present in the column flow-through and wash fractions (corresponding to lanes 2 and 3, not shown). We estimate from quantitative immunoblotting that the overall recovery of purified mSR-BI-t1 from the lysate was ~80%. The yield of mSR-BI-t1 was 100–150 µg/liter of suspension cell culture.

Activity of Purified mSR-BI-t1 Reconstituted into Liposomes—With the availability of pure mSR-BI-t1 and a fully validated in vitro reconstituted liposome assay, we were able to address the main question of this study: could mSR-BI, independently of any other protein, mediate HDL binding and selective lipid uptake? Fig. 8 shows the results of an experiment in which we measured as a function of ligand concentration 125I-HDL binding to (open squares) and [3H]cholesteryl ether uptake from [3H]CE-HDL by (open circles) liposomes reconstituted with the immunooaffinity-purified receptor. Fig. 8 (open squares) shows that specific 125I-HDL binding was of high affinity (apparent $K_d$ of 11.9 µg of protein/ml) and saturable ($B_{\text{max}}$ = 16.6 ng of protein/assay). The maximal binding, corrected for the amount of protein incorporated in the liposomes, was 311 ng of protein/µg of liposome protein, a value 65-fold higher than the corresponding value for binding of 125I-HDL to liposomes reconstituted with HEK[mSR-BI-t1] whole cell lysate ($B_{\text{max}}$ of 4.8 ng of protein/µg of liposome protein). Fig. 8 (open circles) shows the specific [3H]cholesteryl
ether uptake from \( ^{3}H \)cholesterol was also high affinity and saturable. For \( ^{3}H \)cholesterol ether uptake, the apparent \( K_d \) was 13.8 \( \mu g \) of protein/ml and the \( U_{max} \) was 134 ng of protein/assay or 2500 ng of protein/\( \mu g \) of liposome protein. The relative amount of lipid uptake was substantially greater than that of binding, with a ratio of the maximal \( ^{3}H \)CE-HDL uptake/\( ^{125}I \)HDL binding of 8, clearly showing that the pure receptor mediated selective lipid uptake from HDL. The LDL binding and lipid uptake activities of the mSR-BI-t1-containing liposomes increased linearly with the amount of mSR-BI-t1 incorporated into the liposomes (data not shown).

We conducted three additional experiments to determine if the selective lipid uptake activity of the purified receptor reconstituted into liposomes exhibited characteristics similar to those of SR-BI in intact cells. First, we compared the abilities of unlabeled HDL and LDL to inhibit \( ^{125}I \)HDL binding and \( ^{3}H \)cholesterol ether uptake from \( ^{125}I \)HDL (10 \( \mu g \) of protein/ml). Fig. 9 shows that excess unlabeled HDL (open squares and circles) effectively inhibited \( ^{125}I \)HDL binding (panel A) and \( ^{3}H \)cholesterol ether uptake (panel B), whereas unlabeled LDL (filled squares and circles) did not. These results were similar to those observed using liposomes reconstituted with mSR-BI-t1-containing mammalian cell lysates (Fig. 5B) and mSR-BI expressed in intact mammalian cells (6, 25). We did note that the extent of LDL competition for \( ^{125}I \)HDL binding and \( ^{3}H \)cholesterol ether uptake from \( ^{3}H \)CE-HDL varied somewhat from experiment to experiment (maximum percent inhibition of 8–20% for binding and 0–25% for uptake), perhaps reflecting the effects that small changes in the state of the HDL (e.g. oxidation) can have on lipoprotein binding affinities (41). Second, we tested the anti-SR-BI antibody KK-1-specific inhibition of \( ^{3}H \)CE-HDL uptake. Fig. 10 shows that the KK-1 antibody inhibited the \( ^{3}H \)cholesterol ether uptake by mSR-BI-t1-reconstituted liposomes, but the control antibody from preimmune serum did not. These results were similar to those obtained with the mSR-BI-t1 mammalian cell lysate-reconstituted liposomes (Fig. 5C) and mSR-BI-transfected intact mammalian cells (25). Third, we examined the temperature dependence of \( ^{125}I \)HDL binding to (Fig. 11A) and \( ^{3}H \)cholesterol ether uptake from \( ^{3}H \)CE-HDL (Fig. 11B) at 37 °C (open bars) or 0 °C (shaded bars). The lower temperature slightly lowered the \( ^{125}I \)HDL binding (4.1 versus 3.3 ng of protein/assay at 37 °C and 0 °C, respectively, 19.5% reduction), whereas lipid
uptake was substantially reduced at the lower temperature (35.7 versus 14.6 ng of protein/assay at 37 °C and 0 °C, respectively, 59.1% reduction). These findings were consistent with those obtained with mSR-BI-t1-containing cell lysate-reconstituted liposomes (Fig. 4) and intact cells (34). Thus, SR-BI needed no additional protein co-factors to allow it to mediate HDL binding and selective cholesteryl ether uptake.

**DISCUSSION**

We have developed an in vitro reconstituted liposome assay to measure the ligand binding and lipid transport activities of detergent-solubilized forms of the HDL receptor SR-BI. An epitope-tagged form of the recombinant receptor (mSR-BI-t1) in detergent-solubilized whole insect or mammalian cell lysates, or mSR-BI-t1 purified by immunoaffinity chromatography essentially to homogeneity, can be assayed using this liposome system. The liposome assay recapitulated many features of the HDL receptor activity of SR-BI expressed in intact cells (5): 1) 125I-HDL and 125I-LDL binding from 125I-HDL or 125I-LDL in the presence (single determinations) or absence (duplicate determinations) of a 4-fold excess of the corresponding unlabelled lipoprotein at 37 °C for 2 h, isolated, and washed by filtration, and the amounts of specific 125I-HDL (A) and 125I-LDL (B) binding were determined as described under "Experimental Procedures." The 100% of control values for 125I-HDL and 125I-LDL binding were 6.99 and 6.95 ng of protein/assay, respectively. The nonspecific background values for 125I-HDL binding were 23% (mSR-BI-t1) and 25% (Q402R/Q418R mutant) of the total binding.

**FIG. 6.** 125I-HDL and 125I-LDL binding by liposomes reconstituted with insect (Sf21) cell lysates containing mSR-BI-t1 (open bars), the Q402R/Q418R mutant of mSR-BI (light gray bars), or no recombinant receptor (dark filled bars). Sf21 cells were infected with baculoviruses encoding mSR-BI-t1 (open bars), the Q402R/Q418R mutant of mSR-BI (light gray bars), or no receptor (empty virus, dark filled bars) and grown at 27 °C. Cell lysates were prepared in 1.5% octyl glucoside lysis buffer and reconstituted into liposomes as described under "Experimental Procedures." The liposomes were incubated with 5 μg of protein/ml of 125I-HDL or 125I-LDL in the presence (single determinations) or absence (duplicate determinations) of a 4-fold excess of the corresponding unlabeled lipoprotein at 37 °C for 2 h, isolated, and washed by filtration, and the amounts of specific 125I-HDL (A) and 125I-LDL (B) binding were determined as described under "Experimental Procedures." The 100% of control values for 125I-HDL and 125I-LDL binding were 6.99 and 6.95 ng of protein/assay, respectively. The nonspecific background values for 125I-HDL binding were 23% (mSR-BI-t1) and 25% (Q402R/Q418R mutant) of the total binding. Error bars represent the range of variations in duplicate determinations. *, the range of variation was <50%.

**FIG. 7.** Immunoaffinity purification of mSR-BI-t1 from HEK[mSR-BI-t1] cell lysates. HEK[mSR-BI-t1] cells were grown in suspension culture and lysed, and the lysates were subjected to immunoaffinity chromatography using the 1D4 monoclonal anti-C-terminal epitope tag antibody. The bound protein was eluted from the column with the peptide epitope as described under "Experimental Procedures." Samples of the cell lysate, column flow-through (unbound material), column wash, and peptide-eluted fractions (10 μl of each fraction) were fractionated by 10% SDS-PAGE, and the proteins in the gel were visualized using a Bio-Rad silver-staining kit. The mobilities of molecular weight standards are indicated on the left.
FIG. 8. $^{125}$I-HDL binding and $[^3H]$CE-HDL uptake by liposomes reconstituted with immunoaffinity-purified mSR-BI-t1. Immunoaffinity-purified mSR-BI-t1 isolated from HEK[mSR-BI-t1] cells was reconstituted into liposomes as described under "Experimental Procedures". Control liposomes without added receptor were prepared in parallel. The liposomes were incubated with the indicated concentrations of $^{125}$I-HDL (squares or triangles) or $[^3H]$CE-HDL (circles or inverted triangles) at 37 °C for 3 h in the presence (single determinations) or absence (duplicate determinations) of a 40-fold excess of unlabeled HDL, isolated, and washed by filtration, and the amounts of specific $^{125}$I-HDL binding and $[^3H]$cholesteryl ether uptake were determined as described under "Experimental Procedures". The binding and lipid uptake values for the control (no receptor) liposomes (filled squares, partially obscured by the squares) determined at labeled lipoprotein concentrations of 10 µg of protein/ml were 0.8 and 0.9 ng/assay, respectively. Error bars represent the range of variations in duplicate determinations. The nonspecific background values for $^{125}$I-HDL binding at 2.5, 10, 25, 70, and 100 µg of protein/ml were 20, 24, 26, 30, 42, and 50% of total binding, respectively. Inset, expanded scale for $^{125}$I-HDL binding.

FIG. 9. Unlabeled HDL and LDL inhibition of $^{125}$I-HDL binding and $[^3H]$CE-HDL uptake by liposomes reconstituted with immunoaffinity-purified mSR-BI-t1. Immunoaffinity-purified mSR-BI-t1 isolated from HEK[mSR-BI-t1] cells was reconstituted into liposomes as described under "Experimental Procedures." Control liposomes without added receptor were prepared in parallel. The liposomes were incubated in duplicate with 5 µg of protein/ml of $^{125}$I-HDL (A) or 10 µg of protein/ml of $[^3H]$CE-HDL (B) in the presence of the indicated concentrations of unlabeled HDL (open symbols) or LDL (filled symbols) at 37 °C for 2 h, isolated, and washed by filtration, and the amounts of $^{125}$I-HDL binding and $[^3H]$cholesteryl ether uptake were determined as described under "Experimental Procedures." The values for the control (no receptor) liposomes (triangles) determined in the absence of competitor or in the presence of 400 µg of protein/ml of HDL or LDL were: $^{125}$I-HDL binding, 1.98, 0.94, and 1.25 ng/assay, respectively; and $[^3H]$CE uptake 5.03, 5.34, and 3.93 ng/assay, respectively.

HDL binding (23) were reproduced in the liposome assay, as was the ability of the SR-BI homologue CD36 to bind HDL but not mediate efficient lipid uptake (26, 27, 42). It should be possible to use this assay to explore in detail many features of the mechanism underlying the complex ligand binding and lipid transport activities of SR-BI. It should be noted that, prior to and after the discovery of SR-BI and its role as an HDL receptor for selective lipid uptake, several groups reported that intact, not solubilized, membranes isolated from adipocyte, steroidogenic, or hepatic tissues or cells could mediate selective lipid uptake (32, 45–49).

The first question regarding the mechanism of SR-BI activity that we addressed using this assay was: does SR-BI require the cooperation of other proteins to mediate HDL binding and selective lipid uptake, or are these activities autonomous properties of the receptor (independent of other proteins)? Several observations raised the possibility that the complex activities mediated by SR-BI might require the intervention or collaboration of other proteins. For example, SR-BI can be found in specialized membrane microdomains, including caveolar-like domains under certain conditions in some cultured cells (50) and microvillar channels in mammalian steroidogenic cells in vivo (51, 52). Indeed, SR-BI expression in cultured cells can induce the formation of microvillar channel-like structures (49) and plays a role in the formation and/or stability of microvillar channels in steroidogenic cells in vivo (53, 54). In addition, SR-BI has been shown to bind, via its C-terminal cytoplasmic tail, to a multiple PDZ domain-containing scaffold protein called CLAMP (55). The only unequivocal way to determine if other proteins are essential for key SR-BI activities was to examine the activity of the receptor purified away from other proteins. The liposome assay together with the highly efficient immunoaffinity purification of an epitope tagged form of the receptor permitted an unequivocal answer to this question. Essentially homogeneously pure mSR-BI-t1 incorporated into phosphatidylcholine/cholesterol liposomes did mediate high affinity and saturable binding of $^{125}$I-HDL that was accompanied by efficient selective uptake of $[^3H]$cholesteryl ether from $[^3H]$CE-HDL. These findings do not address the question of whether or not other proteins can or do modulate SR-BI’s intrinsic ability to mediate HDL binding to and selective lipid uptake by intact cells. Neither do they address the role, if any, of lipoprotein internalization by cells in selective uptake. They do, however, establish that SR-BI itself has the...
capacity to function as an HDL receptor for selective lipid uptake in liposomes without the required intervention of other proteins or cellular structures or compartments. Therefore, these results suggest that SR-BI itself is primarily responsible for the lipid transfer step during SR-BI-mediated selective lipid uptake in vivo.

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**Fig. 10.** Blocking antibody (KKB-1) inhibition of \[^{3}H\]CE-HDL uptake by liposomes reconstituted with immunoaffinity-purified mSR-BI-t1. Immunoaffinity-purified mSR-BI-t1 isolated from HEK[mSR-BI-t1] cells was reconstituted into liposomes as described under “Experimental Procedures.” Control liposomes without added receptor were prepared in parallel. The liposomes were incubated in triplicate at 37°C for 2 h with 10 \(\mu\)g of protein/ml of \[^{3}H\]CE-HDL in the absence (−) or presence (+) of unlabeled HDL (400 \(\mu\)g of protein/ml), KKB-1 antibody (50 \(\mu\)g/ml), or preimmune IgG (50 \(\mu\)g/ml), isolated, and washed by filtration, and the amounts of \[^{3}H\]cholesteryl ether uptake were determined as described under “Experimental Procedures.” The 100% of control value for lipid uptake by the receptor-containing liposomes in the absence of inhibitors was 39.9 ng of protein/assay. Error bars represent the range of variations in triplicate determinations.

**Fig. 11.** Temperature dependence of \(^{125}\)I-HDL association with and \[^{3}H\]cholesterol ether uptake by liposomes reconstituted with immunoaffinity-purified mSR-BI-t1. Immunoaffinity-purified mSR-BI-t1 isolated from HEK[mSR-BI-t1] cells was reconstituted into liposomes as described under “Experimental Procedures.” The liposomes were incubated with 10 \(\mu\)g of protein/ml of \(^{125}\)I-HDL (A) or \[^{3}H\]CE-HDL (B) at 37°C (open bars) or 0°C (filled bars) for 2 h in the presence (single determinations) or absence (duplicate determinations) of a 40-fold excess of unlabeled HDL, isolated, and washed by filtration, and the amounts of specific \(^{125}\)I-HDL binding and \[^{3}H\]cholesterol ether uptake were determined as described under “Experimental Procedures.” The 100% of control values for binding and lipid uptake at 37°C were 4.1 and 35.7 ng of protein/assay, respectively. The specific values for the control (no receptor) liposomes determined at 37°C were: \(^{125}\)I-HDL binding, 28%; and \[^{3}H\]CE uptake, −6% (not shown). The nonspecific background values for \(^{125}\)I-HDL binding were 24% (37°C) and 22% (0°C) of the total binding. Error bars represent the range of variations in duplicate determinations.
SR-BI the procedure for the purification of rhodopsin developed by them and their colleagues and to Karen Kozarsky for providing the KKB-1 antibody used in these studies.

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Highly Purified Scavenger Receptor Class B, Type I Reconstituted into Phosphatidylcholine/Cholesterol Liposomes Mediates High Affinity High Density Lipoprotein Binding and Selective Lipid Uptake

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