The Efficient Cellular Uptake of High Density Lipoprotein Lipids via Scavenger Receptor Class B Type I Requires Not Only Receptor-mediated Surface Binding but Also Receptor-specific Lipid Transfer Mediated by Its Extracellular Domain*

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The class B type I scavenger receptor, (SR-BI), is a member of the CD36 superfamily of proteins and is a physiologically relevant, high affinity cell surface high density lipoprotein (HDL) receptor that mediates selective lipid uptake. The mechanism of selective lipid uptake is fundamentally different from that of classic receptor-mediated uptake via coated pits and vesicles (e.g. the low density lipoprotein receptor pathway) in that it involves efficient transfer of the lipids, but not the outer shell proteins, from HDL to cells. The abilities of SR-BI and CD36, both of which are class B scavenger receptors, to bind HDL and mediate cellular uptake of HDL-associated lipid when transiently expressed in COS cells were examined. For these experiments, the binding of HDL to cells was assessed using either 125I- or Alexa (a fluorescent dye)-HDL in which the apolipoproteins on the surface of the HDL particles were covalently modified. Lipid transfer was measured using HDL noncovalently labeled by the fluorescent lipid 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate. Although both mSR-BI and human CD36 (hCD36) could mediate the binding of HDL in a punctate pattern across the surfaces of cells, only mSR-BI efficiently mediated the transfer of lipid to the cells. Analysis of point mutants established that the major sites of fatty acylation of mSR-BI are Cys462 and Cys770 and that fatty acylation is not required for receptor clustering, HDL binding, or efficient lipid transfer. Generation of mSR-BI/hCD36 domain swap chimeras showed that the differences in lipid uptake activities between mSR-BI and hCD36 were not due to differences between their two sets of transmembrane and cytoplasmic domains but rather result from differences in their large extracellular loop domains. These results show that high affinity binding to a cell surface receptor is not sufficient to ensure efficient cellular lipid uptake from HDL. Thus, SR-BI-mediated binding combined with SR-BI-dependent facilitated transfer of lipid from the HDL particle to the cell appears to be the most likely mechanism for the bulk of the selective uptake of cholesteryl esters from HDL to the liver and steroidogenic tissues.

The protective role of HDL against cardiovascular disease is commonly attributed to its ability to remove excess cholesterol from cells in the arterial wall and transport it to the liver for disposal, a process called reverse cholesterol transport (1, 2). In addition to delivering cholesterol to the liver, HDL has been shown to be a significant source of cholesterol both for steroidogenesis and for cholesterol storage depots in steroidogenic tissues (adrenal gland, ovary, testis) and cells (3–11). The novel mechanism by which the cholesteryl esters in HDL are transferred to liver and steroidogenic tissues is called selective uptake (Refs. 12–18; reviewed in Refs. 9 and 19) and is distinct from the classic receptor-mediated pathway of lipoprotein particle endocytosis via coated pits and vesicles to lysosomes (20). The first step of selective uptake may involve receptor binding, followed by the reversible incorporation of HDL cholesteryl esters into a plasma membrane pool and then transfer of the lipid to an inaccessible pool, presumably by mechanisms not involving coated pit-mediated endocytosis (Refs. 5, 11, and 18–24; reviewed in Refs. 9, 19, 25, 26).

The multiligand class B type I scavenger receptor, SR-BI (9, 26–30) was recently shown to be the first molecularly well defined cell surface HDL receptor (21). Subsequent studies (reviewed in Refs. 9 and 26) have demonstrated that SR-BI is a physiologically relevant HDL receptor that mediates selective cholesterol uptake (9, 21, 26). In cultured mammalian cells, SR-BI binding to HDL (apparently via its apolipoproteins (Ref. 31; also see Ref. 28) results in efficient selective uptake (21). Although SR-BI has been shown to be a cluster in caveolae-like domains (32), the detailed mechanism of SR-BI-mediated selective uptake has not been defined. For example, it has been uncertain if SR-BI mediates selective uptake merely by bringing HDL in close proximity to the plasma membrane or if SR-BI or other cellular components directly facilitate the lipid transfer after HDL is bound to the cell. In vivo SR-BI is expressed in adult mice (21), rats (33), cows (34), and humans (29, 35) at high levels in precisely those tissues (liver, steroidogenic) that are most active in selective uptake of HDL cholesterol (12–14). The temporal and spatial expression of SR-BI during murine embryogenesis suggests that SR-BI plays a role in delivering cholesterol to the developing fetus (36, 37). Furthermore, in vivo studies with mice and rats, as well as experiments with murine and human cultured cell lines, have shown that SR-BI expression in steroidogenic tissues is coordinately regulated with steroidogenesis (33, 34, 38–40). Flutter et al. have also reported additional strong correlative evidence for the impor-

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1 The abbreviations used are: HDL, high density lipoprotein; SR-BI, scavenger receptor class B type I; BSA, bovine serum albumin; mSR-BI, murine SR-BI; hCD36, human CD36; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate.
tance of SR-BI in selective cholesterol uptake by hepatocytes in vitro and in vivo (41, 42).

Direct evidence for a physiologically relevant role of SR-BI in HDL metabolism and selective uptake has recently appeared (reviewed in Ref. 26). Temel et al. (10) used a SR-BI-specific blocking antibody to provide the first evidence that SR-BI is directly involved in mediating selective uptake in a physiologic system (hormone-stimulated cultured adrenocortical cells). Kozarsky et al. (43) used a recombinant adenovirus to dramatically induce hepatic overexpression of SR-BI in mice. The consequent virtual disappearance of plasma HDL and doubling of biliary cholesterol indicated that SR-BI may play roles in hepatic HDL metabolism, in determining plasma HDL concentrations, and possibly in mediating cholesterol efflux from cells (a suggestion recently confirmed by in vitro studies of efflux (44, 45)). The first targeted disruption (null mutation) of the SR-BI gene in mice by Rigotti et al. (46) and their analysis of the heterozygous and homozygous mutant animals definitively established that SR-BI can play a key role in determining plasma HDL cholesterol levels, almost certainly because reduced expression of SR-BI resulted in decreased selective cholesterol uptake in the liver. These findings were recently confirmed by subsequent analysis of mice that exhibit partially reduced levels of SR-BI expression (47) and that resemble the heterozygous null mutants of Rigotti et al. (46). Based on the in vitro activity and tissue distribution of the human SR-BI (29, 35), it is reasonable to suggest that the function of SR-BI in humans (originally called CLA-1 (48)) may be similar to that in mice. Thus, SR-BI, the first well-characterized receptor for selective uptake, may influence the development and progression of atherosclerosis, and it is an attractive candidate for therapeutic intervention in this disease (9, 21, 26, 43, 46).

SR-BI is a member of the CD36 superfamily of proteins, which includes CD36 (reviewed in Ref. 49), LIMPII (a lysosomal protein) (50), Smp-P1 (a silk moth olfactory neuron membrane protein) (51), EMP (a Drosophila epithelial membrane protein) (52), and Croquemort (a Drosophila hemocyte/macrophage receptor) (53). SR-BI (509 amino acids) and CD36 (472 amino acids) are class B scavenger receptors (27, 54, 55). Membrane topologies of proteins (Molecular Probes Alexa 488 Protein Labeling Kit Manual; catalog no. A-10235), primarily covalently labeled the two major apolipoproteins on the surface of HDL, Apo-A-I and Apo-A-II (25, 65) (data not shown). In contrast, Dil associated with the HDL particles but did not covalently label the apolipoproteins (not shown). The hCD36 expression vector was the generous gift of B. Seed (Massachusetts General Hospital, Boston).

Cell Culture and Transfection—COS M6 cells were grown in Dulbecco modified Eagle's medium with 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine (medium A) supplemented with 10% fetal bovine serum (medium B) at 37 °C in a humidified 5% CO2, 95% air incubator and were transiently transfected with expression vectors for hCD36 (56) and wild-type, mutant, or chimeric (see below) mSR-BI (21) or with a control plasmid that did not encode a protein product (pcDNA-1) as described previously (27). Briefly, 1.5 × 106 COS M6 cells were plated in 100-mm dishes in medium B on day 0. On day 1, cells were transfected with the plasmid using the DEAE-dextran method as described previously (27). Cells were harvested with trypsin and plated in medium B containing 1 mM sodium butyrate (medium C) on day 2. For 125I-HDL binding and fluorimetric assays, cells were seeded on day 2 in 24-well plates at 150,000 cells/well in 1 ml of medium C. For flow cytometry or metabolic labeling, cells were seeded on day 2 in six-well dishes at a concentration of 500,000 cells/well in 3 ml of medium C. For fluorescence microscopy, cells were transfected on day 0 onto polylsine-coated coverslips (12 × 12 mm) in six-well dishes (3 coverslips/well) at a concentration of 50,000 cells/well in 3 ml of medium C. Cells were analyzed as described below on day 3.

125I-HDL Binding and Association Assays—Cells were washed once with medium A and then refed with medium A containing 0.5% (w/v) fatty acid-free BSA (medium D) and the indicated concentrations of 125I-HDL without (duplicate determinations) or with (single determinations) a 40-fold excess of unlabeled HDL. After 1.5 h of incubation at 37 °C, cells were washed once with Tris wash buffer (50 mM Tris-HCl, 0.15 mM NaCl, pH 7.4) containing 2 mg/ml BSA, followed by two quick washes with Tris wash buffer without BSA. Cells were then solubilized in 1 ml of 0.1 N NaOH for 30 min at room temperature on a shaker, and we determined the amounts of cell-associated radioactivity ([50 μl sampled using an LKB γ-counter] and the protein content (100 μl assayed using the method of Lowry et al. (66)). For 4 °C binding studies, the protocol was identical to that at 37 °C, except that the cells were prechilled on ice for 15 min and incubated with 125I-HDL at 4 °C for 2 h. Specific cell association or binding was determined by subtracting the values obtained in the presence of the excess unlabeled HDL (nonspecific) from those obtained in the absence of the unlabeled HDL (total).

Fluorimetric Assay of Dil Uptake—Cells were washed once with medium A and then refed with medium D containing the indicated concentrations of Dil-HDL without (duplicate determinations) or with (single determination) a 40-fold excess of unlabeled HDL. After incubation at 37 °C for 2 h, cells were washed twice with PBS containing Ca2+ and Mg2+ (5 min wash). Cell-associated Dil was then solubilized in 0.5 ml of MeSO at room temperature for 2 h, and the fluorescence was measured by fluorimetry using a Fluoromax 2 spectrofluorimeter. The amount of Dil in each sample, expressed as equivalent amounts of Dil-HDL (μg of protein), was calculated by comparing the fluorescence intensity of the sample to that from a standard curve (linear in the range 0–1 μg of Dil-HDL protein/ml) generated by dissolving Dil-HDL in MeSO. Specific Dil uptake was determined by subtracting the
values obtained in the presence of the excess unlabeled HDL (nonspecific) from those obtained in the absence of the unlabeled HDL (total). We define the relative uptake efficiency for CD36, or wild-type or chimeric mSR-BI, as the ratio of specific D1 uptake to specific $^{125}$I-HDL association at 37 °C, normalized by dividing this value by the same ratio measured for the scrambled control (31). The ratios, the specific values for each construct were corrected by subtracting the specific background values from cells transfected with the control plasmid pcDNA1 (COS[Control]).

Flow Cytometry—Cells were labeled with either DiI-Dil alone, Alexa-HDL alone, or both. For DiI-Dil analysis, cells were incubated at 37 °C for 1 h with 10 μg/ml DiI-Dil and then incubated twice with Ca$^{2+}$ and Mg$^{2+}$-free PBS. Cells were then incubated in Ca$^{2+}$- and Mg$^{2+}$-free PBS containing 0.5% BSA and 2 mM EDTA for 1 h at 4 °C, detached from the plate by gentle pipetting and immediately subjected to flow cytometry. For Alexa-HDL analysis, cells were incubated at 4 °C for 1 h in Ca$^{2+}$- and Mg$^{2+}$-free PBS containing 0.5% BSA, 2 mM EDTA, and 10 μg/ml Alexa-HDL, detached as described above, and then on ice, and then immediately before flow cytometry analysis the cells were pelleted at 500 x g for 2 min and resuspended in Ca$^{2+}$- and Mg$^{2+}$-free PBS containing 0.5% BSA and 2 mM EDTA. For two-color flow cytometry, cells were first labeled with DiI-Dil at 37 °C as described above and then washed once with ice-cold Ca$^{2+}$- and Mg$^{2+}$-free PBS. They were then incubated with Alexa-HDL and analyzed by flow cytometry as described above using two-color flow cytometry. Alexa-HDL cells labeled separately with either DiI-Dil or Alexa-HDL were used to set the compensation to correct the fluorescence signal spill-over to the other channel.

Fluorescence Microscopy—Cells were first washed once with medium A and incubated with 10 μg/ml DiI-Dil or 10 μg/ml Alexa-HDL in medium D at 37 °C for the indicated times. The coverslips were then quickly rinsed by dipping five times in PBS containing Ca$^{2+}$- and Mg$^{2+}$-free PBS at room temperature and immediately observed and photographed without fixation using a fluorescence microscope equipped with standard rodamine (for DiI) and fluorescein isothiocyanate (for Alexa 488) filter sets. To minimize the dissociation of Alexa-HDL from the surfaces of the unfixed cells, we took only two photographs from each coverslip.

For 4 °C binding, cells were first washed once with ice-cold medium A and then refed with 1 ml of ice-cold medium D containing 10 μg/ml Alexa-HDL and incubated at 4 °C for 1 h. The coverslips were then rinsed, and the cells were observed as described above. Fig. 3 was prepared using Adobe Photoshop.

Construction of mSR-BI/hCD36 Chimeras—Five mSR-BI/hCD36 chimeric constructs were generated. The plasmid pmSR-BI-77, which encodes mSR-BI in a pcDNA1 backbone, and a plasmid encoding hCD36 (COS[mSR-BI]) cells labeled separately with either DiI-Dil or DiI-HDL or Alexa-HDL, were used to set the compensation to correct the fluorescence signal spill-over to the other channel.

**RESULTS**

In preliminary studies of a Chinese hamster ovary (CHO) cell-derived line that expresses high levels of the class B scavenger receptor CD36, we found that HDL could compete with acetyl-low density lipoprotein’s binding. This raised the possibility that, as is the case with the other well characterized class B scavenger receptor SR-BI, HDL might bind directly to CD36. To directly compare the binding properties of human CD36 (hCD36) and the murine HDL receptor SR-BI (mSR-BI), we examined the interaction at 37 °C of HDL with COS cells that were transiently transfected with expression vectors encoding mSR-BI (COS[mSR-BI]) or hCD36 (COS[hCD36]), as well as the "empty" vector control (COS[Control]). Fig. 1A shows that 125I-HDL, associated with both COS[mSR-BI] and COS[hCD36] cells with high affinity, (Kd < 10–20 μg of protein/ml), whereas there was only a low level association with the

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incubated with cells for 2 h at 37 °C at the indicated concentrations, and the DiI content of cell extracts was determined by spectrofluorimetry. We observed substantial high affinity uptake of DiI by COS[mSR-BI] cells. In contrast, although the HDL binding activities of the COS[mSR-BI] and COS[hCD36] cells differed by only ~50% in this experiment (see legend), there were essentially only control (background) levels of DiI uptake mediated by hCD36 in the COS[hCD36] cells. The efficiency of lipid uptake (the ratio of DiI uptake/125I-HDL binding) mediated by hCD36 was only about 5–15% of that of mSR-BI. Observations consistent with these were obtained when DiI uptake from DiI-HDL and 125I-HDL binding were compared in CHO-derived cells expressing either mSR-BI or hamster CD36. High levels of DiI were taken up by the mSR-BI-expressing CHO-derived cells, while virtually no DiI uptake was observed in the CD36-expressing cells, although the CD36-expressing cells exhibited 2-fold higher levels of 125I-HDL binding (data not shown).

This distinctive difference in the abilities of mSR-BI and hCD36 to mediate lipid delivery from bound HDL was confirmed by two-color fluorescence flow cytometry (Fig. 2). Cells were incubated with HDL covalently labeled on its apolipoproteins with Alexa 488 for 1 h at 4 °C (Alexa-HDL, green fluorescence, horizontal axes, panels A–C), with DiI-HDL for 2 h at 37 °C (red fluorescence, vertical axes, panels D–F), or a sequential combination of the two (panels G–I, see “Experimental Procedures”). Control experiments established that, compared with 125I labeling, covalent modification of HDL with Alexa 488 did not substantially alter its ability to bind to SR-BI or CD36, as assessed, for example, by measuring the dissociation constant (not shown). The top row in Fig. 2 shows that there were substantially higher levels of labeling by Alexa-HDL of both the COS[hCD36] (B) and COS[mSR-BI] (A) cells compared with that of COS[control] cells (C, compare signals in lower right quadrants of each cytograph). In contrast, little difference in the accumulation of DiI (vertical axes) was observed between the control and hCD36-expressing cells (F and E), while the accumulation of DiI in mSR-BI-expressing cells was much greater (D, compare upper left quadrants). When the cells were labeled with both Alexa-HDL and DiI-HDL (bottom row), two-color fluorescence flow cytometry clearly distinguished between receptor-deficient COS[control] cells (I, signal primarily in lower left quadrant), HDL binding-positive but lipid uptake-negative COS[hCD36] cells (H, strong signal in lower right quadrant), and HDL binding-positive and lipid uptake-positive COS[mSR-BI] cells (G, strong signal in upper right quadrant). The broad, yet nearly linear distribution with a positive slope of the COS[mSR-BI] cells in G indicates that the amount of lipid uptake was directly proportional to the amount of HDL bound to mSR-BI. No such well defined hCD36-dependent correlation was observed (H).

In addition to flow cytometry, we used fluorescence microscopy to assess the amounts and the cellular distributions of bound HDL (Alexa-HDL) and transferred lipid (DiI) after incubation for 10 and/or 60 min at 37 and 4 °C (Fig. 3). There was almost no fluorescence signal for either dye in the COS[control] cells (right column), COS[mSR-BI] (left column) and COS[hCD36] (center column) cells exhibited a punctate pattern of dots or very short strings of fluorescence after incubation with Alexa-HDL (10 μg of protein/ml) at either 4 °C (upper panels) or 37 °C (middle panels). The Alexa-HDL distributions on the COS[mSR-BI] and COS[hCD36] cells were consistent with the previously reported clustering of SR-BI (32) and CD36 (59, 60) in caveolae-like domains. Although there was cell-to-cell variation in the intensity of the Alexa-HDL signals (some of the brighter cells are shown in Fig. 3), the overall visual im-

![Fig. 1. Concentration dependence of HDL binding to (A) and HDL lipid uptake by (B) COS[mSR-BI], COS[hCD36], and COS[control] cells. COS M6 cells were transfected with expression vectors for mSR-BI, hCD36, or the control (“empty”) vector pcDNA1 as described under “Experimental Procedures” and plated in 24-well dishes at a density of 150,000 cells/well in medium C on day 2. On day 3, the indicated amounts of either 125I-HDL (A) or DiI-HDL (B) were added in 0.5 ml of medium D in the absence (duplicate incubations) or presence (single incubations) of a 40-fold excess of unlabeled HDL, and the cells were incubated at 37 °C for either 1.5 h (A) or 2 h (B). The amounts of specific 125I-HDL binding or DiI uptake (spectrofluorimetry) were determined by calculating the differences between measurements made in the absence or presence of unlabeled HDL as described under “Experimental Procedures.” Similar results have been observed in multiple independent experiments, and the data shown are representative. The data for the COS[control] cells in A are from a separate experiment than those for the COS[mSR-BI] and COS[hCD36] cells, and B represents an independent experiment. In the experiment shown in B, 125I-HDL binding was also measured. The specific binding values (ng of protein/1.5 h/mg of cell protein) for COS[mSR-BI], COS[hCD36], and COS[control] cells in this experiment, determined at either 10 or 20 μg/ml HDL, were as follows: for 10 μg/ml HDL, 482, 342, and 19; for 20 μg/ml HDL, 742, 366, and 25. Absolute values for 125I-HDL binding can sometimes vary between different preparations of 125I-HDL. The error bars for all data were smaller than the symbols presented, except for the three data for COS[mSR-BI] cells at the highest DiI-HDL concentrations in B.

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![Fig. 2. Flow cytometry of Alexa-HDL-mediated lipid uptake in COS[mSR-BI], COS[hCD36], and COS[control] cells. The left panel shows the Alexa-HDL (green fluorescence, horizontal axes) and DiI-HDL (red fluorescence, vertical axes) fluorescence signals for two-color flow cytometry of Alexa- and DiI-HDL, respectively. To test the ability of the mSR-BI and hCD36 to mediate lipid uptake, COS[mSR-BI] and COS[hCD36] cells were covalently labeled with Alexa-HDL (left panels), DiI-HDL (middle panels), or both (right panels). Data are presented for Alexa-HDL labeling without DiI-HDL (A–C), Alexa-HDL with 4 h of DiI-HDL (D–F), and Alexa-HDL with 2 h of DiI-HDL (G–I). The top row in Fig. 2 shows that there were substantially higher levels of labeling by Alexa-HDL of both the COS[hCD36] (B) and COS[mSR-BI] (A) cells compared with that of COS[control] cells (C, compare signals in lower right quadrants of each cytograph). In contrast, little difference in the accumulation of DiI (vertical axes) was observed between the control and hCD36-expressing cells (F and E), while the accumulation of DiI in mSR-BI-expressing cells was much greater (D, compare upper left quadrants). When the cells were labeled with both Alexa-HDL and DiI-HDL (bottom row), two-color fluorescence flow cytometry clearly distinguished between receptor-deficient COS[control] cells (I, signal primarily in lower left quadrant), HDL binding-positive but lipid uptake-negative COS[hCD36] cells (H, strong signal in lower right quadrant), and HDL binding-positive and lipid uptake-positive COS[mSR-BI] cells (G, strong signal in upper right quadrant). The broad, yet nearly linear distribution with a positive slope of the COS[mSR-BI] cells in G indicates that the amount of lipid uptake was directly proportional to the amount of HDL bound to mSR-BI. No such well defined hCD36-dependent correlation was observed (H).

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![Fig. 3. Fluorescence micrographs of cells expressing SR-BI and CD36. COS M6 cells were transfected with expression vectors for mSR-BI, hCD36, or the control (“empty”) vector pcDNA1 as described under “Experimental Procedures” and plated in 24-well dishes at a density of 150,000 cells/well in medium C on day 2. On day 3, the indicated amounts of either 125I-HDL (A) or DiI-HDL (B) were added in 0.5 ml of medium D in the absence (duplicate incubations) or presence (single incubations) of a 40-fold excess of unlabeled HDL, and the cells were incubated at 37 °C for either 1.5 h (A) or 2 h (B). The amounts of specific 125I-HDL binding or DiI uptake (spectrofluorimetry) were determined by calculating the differences between measurements made in the absence or presence of unlabeled HDL as described under “Experimental Procedures.” Similar results have been observed in multiple independent experiments, and the data shown are representative. The data for the COS[control] cells in A are from a separate experiment than those for the COS[mSR-BI] and COS[hCD36] cells, and B represents an independent experiment. In the experiment shown in B, 125I-HDL binding was also measured. The specific binding values (ng of protein/1.5 h/mg of cell protein) for COS[mSR-BI], COS[hCD36], and COS[control] cells in this experiment, determined at either 10 or 20 μg/ml HDL, were as follows: for 10 μg/ml HDL, 482, 342, and 19; for 20 μg/ml HDL, 742, 366, and 25. Absolute values for 125I-HDL binding can sometimes vary between different preparations of 125I-HDL. The error bars for all data were smaller than the symbols presented, except for the three data for COS[mSR-BI] cells at the highest DiI-HDL concentrations in B.
pression was that there was very little change in the distribution of Alexa-HDL fluorescence between 10 and 60 min of incubation at 37 °C or between 4 and 37 °C, although there was an apparently small increase in signal intensity at 37 °C relative to that at 4 °C. This is consistent with the previous report that 125I-HDL binding to SR-BI-expressing cells rapidly reaches steadystate (21) and there is almost no 125I-HDL internalization and lysosomal degradation during incubations of less than 5 h.

The amount and distribution of DiI fluorescence depended on the type of cells and the time of incubation at 37 °C. There was no significant accumulation of DiI fluorescence from DiI-HDL (10 μg of protein/ml) by the COS[mSR-BI] cells after incubation for 10 min, 60 min (center column, bottom), or overnight (not shown). In contrast, there was an initial punctate distribution of DiI fluorescence in COS[mSR-BI] cells after incubation with DiI-HDL for 10 min (left column, bottom). This was probably a consequence of the rapid local selective uptake of DiI (21) at the surface of the cells at the sites of mSR-BI clustering rather than fluorescence due to surface-bound DiI-HDL particles. Under the conditions of this experiment, the fluorescence from the DiI-HDL particles bound to the surface was apparently too weak to detect above background. With increasing time of incubation of DiI-HDL with the COS[mSR-BI] cells, the cell-associated fluorescence intensity increased, and after 60 min there appeared to be a combination of a fairly even distribution across the entire surfaces of the cells with an underlying punctate distribution. In addition, we observed bright, apparently intracellular juxtanuclear sites of fluorescence near the centers of the cells. These images are virtually identical to those previously observed for uptake via mSR-BI in transfected CHO and COS cells (21). This pattern was interpreted to represent selective uptake into the plasma membrane compartment followed by transfer to intracellular sites of unknown identity (21). Thus, it appears that HDL binding to SR-BI is clustered on the surface of the cells, presumably in caveolae-like domains (32), and that after HDL binds, HDL lipids are rapidly transferred initially to membrane sites associated with the clustered receptors, possibly caveolae, and then spread to the bulk of the plasma membrane. Additional studies will be required to define the detailed mechanism of this lipid transfer and spreading.

Taken together, these data clearly demonstrate that, unlike the case with mSR-BI, high affinity binding of HDL to hCD36 did not result in efficient selective uptake of HDL’s lipid. These results therefore strongly suggest that “docking” of HDL to the cell surface is not sufficient for efficient lipid transfer. mSR-BI exhibits a distinctive lipid transfer activity not shared by its structural homologue CD36 (~30% sequence identity, similar proposed membrane topologies; see Introduction).

To begin to define the features of mSR-BI’s structure that contribute to its distinctive lipid transfer activity, we constructed a series of mutant mSR-BI expression vectors and expression vectors for SR-BI/CD36 domain swap chimeras and compared their HDL binding and lipid uptake properties to those of the wild-type mSR-BI in transiently transfected COS cells. Fig. 4A illustrates models of the proposed topologies of mSR-BI (open bar) and hCD36 (solid bar) and also indicates potential sites of fatty acylation in mSR-BI (Gly2, Cys462, and Cys470). Also shown are the structures of the chimeras that were studied (mixed open and solid bars; C1–C5). The potential sites of fatty acylation (underlined) as well as the junction sequences for the chimeras (labeled a, b, and c) are shown in Fig. 4B, and their relative positions are indicated at the top of Fig. 4A. SR-BI (509 amino acids), CD36 (472 amino acids), and most other members of the CD36 superfamily of proteins have been proposed to have similar membrane topologies (see Introduction). These proteins appear to contain two hydrophobic, putative membrane-spanning domains (C-TM and N-TM), one near a short cytoplasmic C terminus (C-cyto), and the other near a short putative cytoplasmic N terminus (N-cyto). The bulk of each protein (Exo) falls between the two hydrophobic domains on the extracellular surface of cells and contains a set of conserved cysteines and putative N-linked glycosylation sites. Both mSR-BI (32) and hCD36 (49, 69) are heavily N-glycosylated, and both have cysteines in similar, but not identical, positions near the interface between the C-TM and C-cyto domains; these cysteines are fatty acylated (Refs. 32 and 57; see below). One of the most notable differences between these proteins is that the C-cyto domain of mSR-BI (45 amino acids) is significantly longer than its hCD36 counterpart (6 residues). In the current work, we asked if fatty acylation was required...
for the punctate localization of mSR-BI and its function in binding HDL and mediating lipid uptake. We also determined if chimeras between hCD36 and mSR-BI, in which the cytoplasmic and transmembrane domains were swapped, could bind HDL and mediate lipid uptake.

To examine the sites of fatty acylation and their roles in mSR-BI function in transfected cells, we generated expression vectors for site-specific mutants in which the potential fatty acylation sites were destroyed. The potential myristoylation site in N-cyto (32) was inactivated by converting Gly2 to Ala2 (G2A). This mutation had no significant effect on the HDL binding or lipid uptake activities of mSR-BI, as measured in COS[mSR-BI:G2A] cells by two-color flow cytometry (Fig. 5) or by [3H]HDL binding (not shown). The G2A mutation also had no effect on the punctate pattern of Alexa-HDL binding at 4 °C (top two rows), two representative images for each cell type taken after 60-min incubations are shown. In all other cases, single images for each cell type under each incubation condition (Alexa-HDL, 4 °C, or 4 °C) are shown.

Based on the analysis by Tao et al. (57) of the cysteine palmitoylation of CD36, it seemed likely that there would be two potential sites of cysteine fatty acylation in mSR-BI, Cys462 and Cys470. Cys462 is located adjacent to the HDL receptor in the N-terminal cytoplasmic domain of CD36 and the two cysteines adjacent to the myristate binding site. Based on the analysis of parallel [3H]palmitate labeling of mSR-BI, Cys462 and Cys470 is present in all SR-BI sequences reported to date (21, 27, 34, 48, 70) but not in SR-BII (an alternatively spliced form of SR-BI (71), while Cys462 is only found in murine and human SR-BI. We generated expression vectors for the single mutants in which a cysteine was replaced by serine (mSR-BI:C462S and mSR-BI:C470S) as well as the corresponding double mutant (mSR-BI:C462S and mSR-BI:C470S) as well as the corresponding double mutant (mSR-BI:C462S and mSR-BI:C470S). The transiently transfected COS cells were metabolically labeled with [3H]palmitate, and cell extracts were subjected to immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and autoradiography. The intensity of labeling of each of the single mutants was approximately half of that of wild-type mSR-BI, while there was essentially no detectable labeling of the double mutant (Fig. 6, upper panel, lanes 1–5). The levels of wild-type and mutant protein expression appeared to be comparable, based on the analysis of parallel [35S]methionine-labeled cells (not shown). The levels of wild-type and mutant protein expression appeared to be comparable, based on the analysis of parallel [35S]methionine-labeled cells (not shown) as well as activity measurements (see below). Therefore, it seems likely that both of these cysteines are fatty acylated in the wild-type molecule and that these are the only significant sites of palmitoylation in mSR-BI. This work follows the lead of Lublin and colleagues (57), who showed using point mutagenesis that the two cysteines in the N-terminal cytoplasmic domain of CD36 and the two cysteines adjacent to the interface between the C-TM and C-cyto domains, while Cys462 is in the C-cyto domain (Fig. 4). Cys470 is present in all SR-BI sequences reported to date (21, 27, 34, 48, 70) but not in SR-BII (an alternatively spliced form of SR-BI (71), while Cys462 is only found in murine and human SR-BI. We generated expression vectors for the single mutants in which a cysteine was replaced by serine (mSR-BI:C462S and mSR-BI:C470S) as well as the corresponding double mutant (mSR-BI:C462S and mSR-BI:C470S). The transiently transfected COS cells were metabolically labeled with [3H]palmitate, and cell extracts were subjected to immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and autoradiography. The intensity of labeling of each of the single mutants was approximately half of that of wild-type mSR-BI, while there was essentially no detectable labeling of the double mutant (Fig. 6, upper panel, lanes 1–5). The levels of wild-type and mutant protein expression appeared to be comparable, based on the analysis of parallel [35S]methionine-labeled cells (not shown) as well as activity measurements (see below). Therefore, it seems likely that both of these cysteines are fatty acylated in the wild-type molecule and that these are the only significant sites of palmitoylation in mSR-BI. This work follows the lead of Lublin and colleagues (57), who showed using point mutagenesis that the two cysteines in the N-terminal cytoplasmic domain of CD36 and the two cysteines adjacent to the interface between the C-TM and C-cyto domains of CD36 are all palmitoylated. Interestingly, the single and double Cys-Ser
mutations in mSR-BI affected the incorporation of \(^{3}H\)myristate in the same way as they did the incorporation of \(^{3}H\)palmitate described above (Fig. 6, lower panel, lanes 1–5); in particular, \(^{3}H\)myristate incorporation was almost completely abolished in the double Cys-Ser mutant (Fig. 6, lower panel, lane 5). Taken together with the data for the G2A mutant, this observation suggests that most of the previously described myristoylation of mSR-BI (32) was due to incorporation of this fatty acid (or a metabolic derivative) as a thioester at one or both of these cysteine positions rather than incorporation at the N-terminal potential myristoylation site.

The single and double cysteine-to-serine mutations had no effect on the HDL binding or lipid uptake activities of mSR-BI, as measured in transfected COS cells by two-color flow cytometry (Fig. 5, E–G) or by \(^{125}I\)-HDL binding (not shown). These mutations also had no effect on the punctate pattern of Alexa-HDL binding at 4 °C observed by fluorescence microscopy (not shown). Therefore, analysis of mSR-BI with point mutations indicates that fatty acylation is not critical for the surface clustering of mSR-BI or for its HDL binding and lipid uptake functions in cultured cells. Thus, differences in the sites or extents of fatty acylation between mSR-BI and CD36 are unlikely to account for the ability of mSR-BI to mediate efficient lipid uptake. The significance of the fatty acylation, perhaps more relevant to the location and function of SR-BI in natural tissues than in transfected cultured cells, remains to be elucidated.

To further explore the molecular basis for the marked differences in lipid uptake activity of mSR-BI and CD36, we compared (Table 1) the abilities of the wild-type receptors and mSR-BI/hCD36 chimeras transiently expressed in COS cells to bind \(^{125}I\)-HDL and accumulate Dil from Dil-HDL (spectrofluorimetric assay). The values shown represent high affinity binding or lipid uptake as well as the ratios (uptake efficiency index) of Dil uptake to \(^{125}I\)-HDL binding (see “Experimental Procedures”). Analysis of these indices permits direct comparisons of lipid uptake efficiency corrected for any differences in the amounts of surface expression of the proteins due to differ-

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**Fig. 4. Proposed topologies of mSR-BI, hCD36 and domain-swap chimeras (A) and comparison of the sequences near their N and C termini (B).** A, the relative locations of the N-terminal cytoplasmic domains (N-cyto), N-terminal transmembrane domains (N-TM), extracellular domains (Exo), C-terminal transmembrane domains (C-TM), and C-terminal cytoplasmic domains (C-cyto) for mSR-BI (white) and hCD36 (black) are illustrated along with the junction sites used to construct mSR-BI/hCD36 domain swap chimeras (a, b, c). Also shown are representations of the chimeras (C1–C5; white corresponds to mSR-BI sequence, and black corresponds to hCD36 sequence) and the positions of the putative fatty acylation sites (Gly2 (G); Cys462 and Cys470 (C)). B, the sequences of mSR-BI and hCD36 at their N termini (top) and C termini (bottom) are shown with the proposed intersections of the topologically defined domains indicated. The junction sites for the domain swap chimeras (a, b, c) and the mutations in the putative fatty acylation sites (Gly2 \(\rightarrow\) Ala, Cys462 \(\rightarrow\) Ser, and Cys470 \(\rightarrow\) Ser) are also shown.
ent rates of synthesis or degradation. We also used two-color fluorescence flow cytometry with Alexa-HDL and DiI-HDL to assess lipid uptake efficiency (Fig. 5).

Replacement of the long C-cyto domain of mSR-BI with its short counterpart from hCD36 (chimera C1) did not interfere either with the receptor's ability to bind $^{125}$I-HDL or with lipid uptake (Figs. 4 and 5, Table I). This establishes that the long cytoplasmic domain of mSR-BI is not required for efficient SR-BI-mediated lipid uptake. This is also consistent with studies that have identified and characterized an alternative splice form of SR-BI, designated SR-BII, in which the C-terminal cytoplasmic domain of SR-BI is replaced by a segment of approximately the same length but different sequence (71, 72). mSR-BII can also mediate selective lipid uptake from HDL; however, Webb et al. (72) reported that selective uptake mediated by mSR-BII is 4-fold less efficient than that by mSR-BI. Thus, in some circumstances, the precise nature of the cytoplasmic C terminus may be able to influence, directly or indirectly, the efficiency of lipid uptake.

When both the C-cyto and C-TM domains of mSR-BI were replaced with their hCD36 counterparts (chimera C2), both $^{125}$I-HDL binding and DiI uptake were significantly decreased (20–30% of the mSR-BI control) (Figs. 4 and 5; Table I). Possible explanations for the low absolute activity of chimera C2 include reduced steady state level of the protein, reduced cell surface expression, reduced binding affinity or stoichiometry, or some combination of these or other features of this chimera. Preliminary immunoblot analysis indicated that the steady...
state levels of both C1 and C2 in the transfected cells were similar to that of mSR-BI. Additional studies will be required to determine why the relative activity of C2 is low. Nevertheless, for a given amount of HDL bound, chimera C2 was as efficient at mediating lipid uptake as was wild-type mSR-BI (compare uptake efficiency indices (Table I) and slopes of the linear distributions in the two-color fluorescence flow cytograms (Fig. 5, A, H, and I)). The significance of the slightly higher efficiency of C2 relative to control remains to be determined. This indicates that C-TM domain sequence differences between mSR-BI and hCD36 (only 15% sequence identity) are not responsible for the marked differences in lipid uptake efficiencies of these receptors.

To determine if the two N-terminal domains of mSR-BI (N-cyto and N-TM) play critical roles in lipid uptake, these domains in mSR-BI were replaced with their hCD36 counterparts (chimera C3). The absolute amounts of 125I-HDL binding and DiI uptake were significantly decreased relative to the wild-type mSR-BI control; however, there was only a small decrease in the relative efficiency of lipid uptake (Figs. 4 and 5, Table I). Almost identical results were observed when both the N-cyto and N-TM domains along with the C-cyto and C-TM domains of mSR-BI were replaced with those of hCD36 (chimera C4). These results establish that the N-cyto and N-TM domains of mSR-BI are not responsible for its lipid uptake activity and that there is no marked sequence-specific synergism between the C- and N-terminal domains required for efficient lipid uptake. Consistent with this conclusion, we observed that replacement of the N-cyto and N-TM domains of hCD36 with those of mSR-BI (chimera C5) did not significantly alter the ability of CD36 to bind 125I-HDL, nor did it confer lipid uptake activity on this receptor (Figs. 4, 5, and Table I). Taken together, these data suggest that there are no novel sequence-specific interactions that are essential for efficient lipid uptake between the putative membrane anchors or the transmembrane domains of mSR-BI with either the cell membrane or other cellular components. Thus, the extracellular loop of mSR-BI appears to be directly responsible for this receptor’s novel ability to mediate efficient lipid uptake. Whether the extracellular domain of SR-BI has an intrinsic and autonomous lipid uptake activity or if it interacts with other essential cellular lipid-uptake components remains to be determined.

**TABLE I**

| Transfected COS cells | 125I-HDL cell association a | DiI-HDL uptake b | Uptake efficiency index c |
|-----------------------|-----------------------------|-----------------|--------------------------|
| Control               | 33                          | 151             |                          |
| mSR-BI                | 597                         | 3217            | 100                      |
| hCD36                 | 178                         | 279             | 16                       |
| C1                    | 564                         | 3606            | 120                      |
| C2                    | 128                         | 906             | 146                      |
| C3                    | 202                         | 893             | 81                       |
| C4                    | 175                         | 888             | 96                       |
| C5                    | 191                         | 341             | 22                       |

*a Transfected COS M6 cells expressing mSR-BI, hCD36, domain swap chimeric receptors, or controls were generated as described under "Experimental Procedures" and plated in 24-well dishes at a density of 150,000 cells/well in medium C on day 2.

*b On day 3, 10 μg of protein/ml of 125I-HDL or 10 μg of protein/ml of DiI-HDL were added in 0.5 ml of medium D in the absence (duplicate incubations) or presence (single incubations) of a 40-fold excess of unlabeled HDL, and the cells were incubated at 37 °C for either 1.5 (125I-HDL) or 2 h (DiI-HDL). The amounts of specific 125I-HDL binding or DiI uptake (spectrofluorometry) were determined by calculating the differences between measurements made in the absence or presence of the unlabeled HDL as described under "Experimental Procedures." The data are compiled from two independent experiments.

*c Uptake efficiency index = [(b - 151)/(a - 33)] × (100/5.436); for wild-type mSR-BI, a = 597, b = 3217, and (b - 151)/(a - 33) = 5.436.

**DISCUSSION**

In this study, we have compared the abilities of two class B scavenger receptors, mSR-BI and hCD36, transiently expressed in COS cells to bind HDL and mediate cellular uptake of HDL-associated lipid. For these experiments, the binding of HDL particles to cells was assessed using either 125I- or Alexa (a fluorescent dye)-HDL in which the apolipoproteins on the surface of the HDL particles were covalently modified with the labels. Lipid transfer was measured using HDL with covalently associated DiI, a fluorescent lipid whose selective transfer from HDL to cells via mSR-BI has been shown to be similar to that of cholesteryl esters and ethers in HDL (21, 33, 36, 43, 68). Spectrofluorimetry, flow cytometry, and fluorescence microscopy were used to quantify the association of the fluorescent dyes with the cells and explore the distribution of the dyes in the cells. We have shown that both receptors can bind HDL with high affinity and that the bound HDL is distributed in a punctate fashion over the surface of mSR-BI- and hCD36-expressing cells. The pattern is consistent with the previously reported clustering of these receptors in caveolae or caveolae-like membrane domains (32, 59, 60). During the preparation of this manuscript, Calvo and colleagues independently reported the binding of several DiI-labeled lipoproteins to CD36, including HDL (64). The issue of differential lipid uptake was not addressed by those investigators.

A striking difference between mSR-BI and hCD36 was that, unlike mSR-BI, hCD36 was unable to mediate the efficient transfer of HDL lipid to cells. Thus, although fluorescence microscopy indicated that HDL binding to both mSR-BI and hCD36 was restricted to clusters on the surface of the cells, possibly caveolae or caveolae-like domains (32, 59, 60), only after binding to mSR-BI, but not hCD36, were the HDL lipids transferred efficiently from the lipoprotein particles to the cells. Examination of the cellular distribution of DiI fluorescence as a function of time after DiI-HDL addition suggests that HDL lipids are initially transferred to caveolae-like membranes and subsequently spread to the bulk of the plasma membrane. The ability of hCD36 to bind HDL (Ref. 64 and this work) but not mediate efficient lipid uptake from HDL may, at least in part, help account for the previous reports that the extent of HDL binding to cells need not always be correlated with the rate of selective uptake into the cells (73, 74).

Analyses of the activities of several SR-BI/CD36 domain swap chimeras established that the differences in mSR-BI and hCD36 lipid uptake activities were not due primarily to differences between their two sets of transmembrane and cytoplasmic domains. In addition, destruction of mSR-BI’s major fatty acylation sites at Cys(462) and Cys(470) in its C-terminal cytoplasmic and transmembrane domains by point mutagenesis (Cys to Ser) established that fatty acylation is not required for surface clustering of HDL binding or efficient lipid uptake. Thus, although the differences between the C- and N-terminal domains in mSR-BI and hCD36 (amino acid sequence and length differences, number and positions of sites of fatty acylation) are substantially greater than those of their large, glycosylated extracellular loop domains (see Fig. 4), the distinct ability of mSR-BI to mediate efficient HDL lipid uptake appears to be a consequence of structures and molecular interactions in its extracellular loop.

There are a number of distinct, but not necessarily mutually exclusive, molecular mechanisms that may underlie physiologically relevant selective lipid uptake. 1) Uptake may be due to spontaneous transfer as a consequence of collisions between HDL particles and the plasma membrane and be nonspecific.
with respect to the composition of the membrane domain with which the HDL collides. In this mechanism, transfer is not facilitated by a defined protein mediator. The extremely low solubility of cholesteryl esters in aqueous solution apparently precludes aqueous diffusion-based mechanisms suggested for transfer of unesterified cholesterol between cells and lipoproteins (75). It has been suggested that such collisions need not involve the fusion of the cell’s membrane leaflet with the HDL phospholipid coat (76). 2) Spontaneous collisional transfer may be specific in that it might depend on cellular membrane lipid composition, potentially with a preference for caveolar or caveola-like domains (9, 32, 77) but would otherwise not be receptor- or transporter-facilitated. 3) Receptor-mediated binding, also referred to as docking oranchoring, of HDL to the cell surface may enhance the rate of spontaneous (not facilitated) collision-mediated transfer. 4) Uptake may depend on receptor-mediated binding combined with receptor-facilitated transfer of lipid from HDL particles to the cell membranes. The receptor alone might mediate both the binding (docking) and the lipid transfer, or additional components (proteins, lipids) might work in concert with the receptor to mediate lipid transfer. 5) For each of the mechanisms listed above, remodeling of the surface and core lipids of HDL by lipases (e.g. hepatic lipase (78–83), lipoprotein lipase (84–86)) or transfer proteins (e.g. cholesteryl ester transfer protein (87), phospholipid transfer protein (88)) or enhancement of lipoprotein binding by some of these proteins (82, 83, 86) might potentiate either facilitated (e.g. receptor-mediated) or nonfacilitated transfer of lipid to cells (9, 39, 78, 82). We and others have noted that the tissue distribution of hepatic lipase is similar to that of SR-BI, raising the possibility that hepatic lipase and SR-BI may act in concert in mediating selective cholesterol uptake (9, 39).

The current studies establish that high affinity binding to a cell surface receptor (hCD36) is not sufficient to ensure efficient lipid uptake from HDL. Thus, the fourth mechanism listed above, receptor-mediated binding combined with receptor-facilitated transfer of lipid from the HDL particle to the cell appears to be the most likely mechanism for SR-BI-facilitated selective lipid uptake. Future studies will be required to determine the molecular mechanism responsible for the SR-BI-facilitated lipid transfer reaction and the relevance, if any, of the clustering of SR-BI in caveolar or caveola-like membrane domains. It should also be possible to determine if SR-BI alone mediates the lipid transfer reaction, presumably due to sequences in its extracellular loop, or if the transfer requires specific interactions of SR-BI with additional cellular components.

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