Murine SR-BI, a High Density Lipoprotein Receptor That Mediates Selective Lipid Uptake, Is N-Glycosylated and Fatty Acylated and Colocalizes with Plasma Membrane Caveolae*

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The class B, type I scavenger receptor, SR-BI, was the first molecularly well defined cell surface high density lipoprotein (HDL) receptor to be described. It mediates transfer of lipid from HDL to cells via selective lipid uptake, a mechanism distinct from receptor-mediated endocytosis via clathrin-coated pits and vesicles. SR-BI is expressed most abundantly in steroidogenic tissues (adrenal gland, ovary), where trophic hormones coordinately regulate its expression with steroidogenesis, and in the liver, where it may participate in reverse cholesterol transport. Here we have used immunochemical methods to study the structure and subcellular localization of murine SR-BI (mSR-BI) expressed either in transfected Chinese hamster ovary cells or in murine adrenocortical Y1-B81 cells. mSR-BI, an ~82-kDa glycoprotein, was initially synthesized with multiple high mannose N-linked oligosaccharide chains, and some, but not all, of these were processed to complex forms during maturation of the protein in the Golgi apparatus. Metabolic labeling with [3H]palmitate and [3H]myristate demonstrated that mSR-BI was fatty acylated, a property shared with CD36, another class B scavenger receptor, and other proteins that concentrate in specialized, cholesterol- and glycolipid-rich plasma membrane microdomains called caveolae. OptiPrep density gradient fractionation of plasma membranes established that mSR-BI copurified with caveolin-1, a constituent of caveolae; and immunofluorescence microscopy demonstrated that mSR-BI colocalized with caveolin-1 in punctate microdomains across the surface of cells and on the edges of cells. Thus, mSR-BI colocalizes with caveolae, and this raises the possibility that the unique properties of these specialized cell surface domains may play a critical role in SR-BI-mediated transfer of lipids between lipoproteins and cells.

High density lipoprotein (HDL) and low density lipoprotein (LDL) play important roles in the movement of cholesterol and cholesteryl esters through the plasma and in receptor-mediated sterol uptake by tissues. The mechanisms by which HDL delivers cholesterol and cholesteryl ester to steroidogenic (3) and hepatic (4) cells, although not well understood, clearly differ from the classic LDL receptor pathway, which involves clathrin-coated pits, coated vesicles, endosomes, and lysosomes (1, 2). HDL particles bind to cell surface receptors, but the intact particles are neither transported to lysosomes nor degraded (hydrolyzed). Instead, the core cholesteryl esters are selectively transferred into cells, and the lipid-depleted apolipoprotein-containing shell is released into the extracellular fluid, a process known as selective cholesterol uptake (5–11). The detailed molecular mechanisms underlying selective cholesterol uptake from HDL have not yet been determined.

Recent in vivo and in vitro studies have suggested that the class B, type I scavenger receptor, SR-BI, is a cell surface HDL receptor that mediates physiologically relevant selective cholesterol uptake (12–14). SR-BI is expressed both in steroidogenic tissues, where it is coordinately regulated with steroid hormone production, and in the liver, where its expression in rats is suppressed by estrogen (12–14). cDNAs encoding the hamster (15), human (Ref. 16; “CLA-1”), and murine (12) SR-BI homologues have been cloned, and their sequences establish that SR-BI is a member of the CD36 family of membrane proteins (Refs. 15 and 17–19; see Ref. 17 for a description of their common sequence elements). CD36, along with SR-BI, is a class B scavenger receptor that can bind a variety of ligands (Refs. 15 and 20–22; for review, see Ref. 23). Because of their sequence similarities, SR-BI and CD36 are expected to have similar structures. Topologic studies of CD36 in conjunction with sequence analysis indicate that human CD36 has two transmembrane domains, which sit adjacent to relatively short cytoplasmic N-terminal (~6–8 residues) and cytoplasmic C-terminal (6 residues) domains (23, 24) (however, see Ref. 25 for an alternative view of the topology). Most of CD36’s polypeptide chain, which lies between the two transmembrane domains, represents a large extracellular loop and consists of a multiply N-glycosylated stretch of ~400 residues (23).

CD36 is expressed on the cell surface and is one of a number of palmitoylated proteins (24, 26–32) that appear to be concentrated in specialized low density membrane microdomains

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The abbreviations used are: HDL, high density lipoprotein; LDL, low density lipoprotein; mSR-BI, murine SR-BI; CHO, Chinese hamster ovary; FBS, fetal bovine serum; Endo H, endoglycosidase H; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ACTH, adrenocorticotropic hormone.
called caveolae (reviewed in Ref. 33). Caveolae were initially identified as 50–100-nm flask-shaped invaginations in the plasma membrane (34–36). These microdomains are enriched in cholesterol and glycolipids relative to the bulk of the plasma membrane and are resistant to detergent extraction (36–44). In many cells, a major component of caveolae is the ~21–24-kDa protein caveolin-1 (36) or its homologues caveolin-2 (45) and caveolin-3 (46, 47). Caveolae have been implicated in a number of transport and signaling phenomena (48–52), and recent studies suggest that caveolae also participate in the intracellular transport and efflux of unesterified cholesterol (41, 53–55). This raises the possibility that if SR-BI, like CD36, is concentrated in caveolae, these specialized membrane domains might play a role in SR-BI-mediated lipid transport.

In the current study, we have examined the structure of murine SR-BI (mSR-BI) and the possibility that this receptor is concentrated in caveolae. Immunocytochemical techniques were used to examine the synthesis and posttranslational processing of mSR-BI, and cell fractionation and immunofluorescence methods were used to assess its subcellular localization. Most experiments were performed using transfected Chinese hamster ovary (CHO) cells expressing murine SR-BI (ldlA[mSR-BI] cells), and key findings were confirmed by analysis of the endogenous mSR-BI expressed in a murine adrenocortical cell line, Y1-BS1. As has been observed for CD36, mSR-BI was extensively N-glycosylated, with the mature protein containing multiple complex as well as hybrid and/or high mannose chains, and it was fatty acylated; mSR-BI was labeled with both [3H]palmitate and [3H]myristate. In addition, mSR-BI copurified with caveolin-1 in a low density plasma membrane fraction and colocalized with caveolin-1 in punctate microdomains across the surface of cells and on the edges of cells. 

EXPERIMENTAL PROCEDURES

Materials—Reagents (sources in parentheses) were as follows: mouse monoclonal anti-caveolin-1 antibody (clone 2234, gift from John Glenney, Transduction Laboratories); rabbit polyclonal anti-caveolin-1 antibody (C13630, Transduction Laboratories, Lexington, KY); mouse monoclonal anti-chicken antibody (N-18, Santa Cruz Biotechnology, Santa Cruz, CA); anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA); Vectastaining media (Vector Laboratories, Inc., Burlingame, CA); ECL Western blotting kit and Amersham (Amersham Corp.); protein A-Sepharose (Pharmacia Biotech Inc.); fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS); heat-inactivated FBS and horse serum, Ham’s F-10 medium (Sigma); N-glycanase, endoglycosidase H (Endo H) (Enzo Life Sciences, Farmingdale, NY); Sendai virus (recombinant, stored at −80 °C) (ViroScience, Philadelphia, PA); bovine serum albumin; and horse serum, Ham’s F-10 medium (Sigma); 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 μM glutamine (medium D); 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 μM glutamine; medium E (Ham’s F-10 medium supplemented with 2 mM glutamine); and medium G (medium E supplemented with 0.1% (v/v) Triton X-100). 

Cell Culture—Y1-BS1 murine adrenal cells were grown in medium C (Ham’s F-12 medium supplemented with 20 mM HEPES, 2 mM glutamine, and 5% (v/v) FBS). ldlA[mSR-BI] cells (12) are stably transfected with the cDNA encoding human SR-BI (labyrinthin; Ref. 62). They were maintained in medium A (primary medium) supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine, and samples were incubated overnight at 37 °C in 1% (v/v) Triton X-100, 0.1 mM leupeptin, and 1 mM PMSF in 0.1 M Tris-HCl, pH 8. IgG from goat anti-mouse IgG at 37 °C for 1 h. Samples were then incubated overnight at 4 °C on a rotary shaker with 40 μl of a 1:1 suspension of protein A-Sepharose beads in PBS. The beads were washed four times with 1 ml of immunoprecipitation wash solution diluted 1:1 with water. When indicated, the immunoprecipitates were then subjected to various chemical or enzymatic treatments. Subsequently, the precipitates were boiled for 5 min in electrophoresis sample buffer (1.2% (w/v) SDS, 0.05% (w/v) 2-mercaptoethanol, and 0.005% (v/v) bromphenol blue, 10% (v/v) glycerol, 62.5 mM Tris-HCl, pH 6.8) containing 5% (v/v) 2-mercaptoethanol. In experiments with 3H-fatty acid-labeled proteins, the 2-mercaptoethanol was omitted. After electrophoresis (method of Laemmli; Ref. 65), the gels were fixed, treated with Amplify, dried, and exposed to Kodak XAR-5 x-ray film. The estimated apparent molecular weights are based on 14C-methylated protein standards (Amersham) (62). Figures containing autoradiograms and chemiluminescence exposures were prepared after digitizing the images with the aid of the program NIH Image.

Enzymatic Digestions of Immunoprecipitates—N-Glycanase and Endo H digestions were performed on washed immunoprecipitates as described previously (62). For sialidase digestions, washed immunoprecipitates were solubilized with boiling for 3 min in 11.1 μl of 0.5% (w/v) SDS and 0.1% (w/v) 2-mercaptoethanol in water. Subsequently, 15.3 μl of 60 mM sodium cacodylate, pH 6.5, containing 2 mM PMSF, 5.6 μl of 7.5% (v/v) Nonidet P-40, and 50 μl of 0.5 units/ml of sialidase were added, and samples were incubated overnight at 37 °C for Endo H/sialidase double digests, the samples were first treated with Endo H (62), exchanged the pH was adjusted to 6.0 rather than 7.5, and then incubated with 5.0 units (0.17 units/ml) of sialidase overnight at 37 °C. For O-glycosidase digestions, samples were treated with sialidase for 1 h at 37 °C prior to the addition of 3 milliliters of 0.09 units/ml of O-glycosidase and incubation overnight at 37 °C. Control reactions without enzymes were performed in parallel.

Immunoblotting of Lysates from Murine Adrenal Glands and ldlA[mSR-BI] Cells—Whole adrenal gland homogenates were prepared as described previously (13, 66). ldlA[mSR-BI] cells were plated on day 0 (3 × 106 cells/150-mm dish in 10 ml of medium B), and on day 2 the cells were washed twice in ice-cold PBS and harvested by scraping into cold PBS. Cells were lysed, and postnuclear supernatants from both ldlA[mSR-BI] cells and adrenal gland homogenates were prepared as described previously (13). Protein concentrations were determined by the method of Lowry et al. (67). Cell extracts or adrenal gland homogenates containing 20 μg of protein were then boiled for 5 min in a volume of 200 μl containing 0.5% SDS (w/v), 2% (v/v) 2-mercaptoethanol, 1 mM PMSF, and 50 mM sodium phosphate, pH 5.5. Subsequently, 1 ml of 2% (w/v) sodium azide and 2.5 milliliters (0.1 unit/ml) of Endo H were added, and the samples were incubated overnight at 37 °C. Control reactions without Endo H were performed in parallel. Samples were boiled for 5 min in reducing electrophoresis sample buffer, subjected to 8% SDS-polyacrylamide gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, 0.22-μm pore size) (68). Immunoblot analysis was performed using anti-mSR-BI (65) and chemiluminescence detection (ECL) as described previously (13).
Analysis of Fatty Acids Incorporated into mSR-BI—IdlA[mSR-BI] cells were labeled with [3H]palmitate or [3H]myristate, and cell extracts were immunoprecipitated using anti-mSR-BI 495 antibody and protein A-Sepharose beads as described above. To determine the hydroxylamine sensitivity of the incorporated radiolabel, the beads were resuspended in 100 μl of either 1.0 M hydroxylamine-HCl, pH 7, or 1.0 M Tris-HCl, pH 7, and allowed to react at room temperature for 1 h (69). Beads were pelleted and washed twice with 1.0 ml of IP wash solution diluted 1:1 with water prior to analysis by 8% SDS-polyacrylamide gel electrophoresis as described above. To analyze the nature of the incorporated label, immunoprecipitates of cell extracts labeled with [3H]palmitate were subjected to alkaline hydrolysis in 100 μl of 1.5 N NaOH at 37 °C for 36 h (69). The hydrolysate was acidified with 1.1 equivalents (27.5 μl) of 6 N HCl and extracted twice with 480 μl of chloroform:methanol (1:2) according to the method of Bligh and Dyer (70). The pooled chloroform phases were dried under vacuum in a SpeedVac Concentrator. The residue was dissolved in 75 μl of chloroform and analyzed by thin layer chromatography on Whatman KC 18 paper (69). TLC plates were sprayed with EN' HANCE fluorography spray and exposed to Kodak XAR-5 x-ray film overnight.

Isolation of Caveolae and Immunoblotting—Cell fractionation (Opti-Prep method) and immunoblotting with polyclonal anti-caveolin antibody or whole anti-mSR-BI 495 antiserum were performed as described previously (71) with the following modification. The second OptiPrep density gradient was composed of 9 ml of 23% OptiPrep plus sample overlayed with 1 ml of 15% OptiPrep and 0.5 ml of 5% OptiPrep. The caveola-enriched membranes were collected from the 5–15% interface.

Immunofluorescence Microscopy—Cells were stained with either one or a combination of the following: anti-mSR-BI 495 IgG, polyclonal anti-caveolin-1 antibody diluted 1:500, mouse monoclonal anti-caveolin-1 antibody (2334), or mouse monoclonal anti-clathrin antibody (X22) diluted 1:1000.2

RESULTS

We have used immunochemical methods and immunofluorescence microscopy to study the structure, biosynthesis, and subcellular distribution of mSR-BI. The experiments were performed using anti-mSR-BI 495, a rabbit polyclonal antibody raised against a peptide corresponding to the carboxyl terminus of mSR-BI, which previously has been shown to recognize an ~82-kDa protein in immunoblots of mSR-BI-expressing cultured cells and tissues (12–14). To determine if anti-mSR-BI 495 could immunoprecipitate mSR-BI, we metabolically labeled cultured cells with a mixture of [35S]methionine and [35S]cysteine ([35S]Met/Cys) for 5 h at 37 °C, lysed the cells, and subjected the lysates to immunoprecipitation followed by SDS-polyacrylamide gel electrophoresis and autoradiography. Cultured cell lines used in these experiments included untransfected IdlA cells (LDL receptor-negative CHO cell mutants that exhibit very little HDL receptor activity) and IdlA[mSR-BI] cells (IdlA cells that were stably transfected with a mSR-BI expression vector). IdlA[mSR-BI] cells express high levels of mSR-BI and exhibit its associated HDL binding and selective cholesterol ester uptake activity (12). Fig. 1 shows that anti-mSR-BI 495 immunoprecipitated a major ~82-kDa band from IdlA[mSR-BI] cells (lane 1). The ~82-kDa band was not detected in IdlA[mSR-BI] cells using preimmune serum (lane 2) or in untransfected IdlA cells using anti-mSR-BI 495 (lane 3). When analyzed under nonreducing conditions, mSR-BI's electrophoretic mobility was somewhat higher than that seen under reducing conditions (not shown). Thus, anti-mSR-BI 495 can specifically immunoprecipitate mSR-BI from metabolically labeled IdlA[mSR-BI] cells.

While the observed apparent mass of mSR-BI based on immunoblot and immunoprecipitation experiments is ~82 kDa, the mass predicted from the receptor's deduced primary amino acid sequence is ~57 kDa (12). A likely source of this discrepancy is cotranslational and posttranslational glycosylation. To determine if mSR-BI is N-glycosylated and, if so, how many of its 10 potential N-glycosylation sites are modified with oligosaccharides, we examined the effects of N-glycanase digestion on the apparent mass of [35S]Met/Cys-labeled mSR-BI immunoprecipitates using SDS-polyacrylamide gel electrophoresis and autoradiography. Fig. 2 shows that digestion with a low concentration of N-glycanase (0.06 units/ml) led to a time-dependent increase in mobility (reduction in apparent mass) from ~82 to ~54 kDa during a 23-h incubation. At least four distinct intermediate forms could be observed, suggesting that at least five of the N-glycosylation sites were occupied by oligosaccharides. Increasing the amount of enzyme to 0.5 units/ml did not dramatically alter the mobilities of the products after 23 h of digestion (Fig. 2, right lane). These results and the results of digestions with Endo H shown below suggest that N-glycosylation can account for most of the difference between the predicted and observed masses of mSR-BI.

The biosynthesis and stability of mSR-BI in IdlA[mSR-BI] cells were examined in the pulse-chase experiment shown in Fig. 3. Cells were pulse-labeled with [35S]methionine for 30 min
and chased in medium containing excess unlabeled methionine for the indicated times, and immunoprecipitates were analyzed by gel electrophoresis and autoradiography. The only major band detected immediately following the pulse labeling (0 chase) was a heterogeneous mixture of labeled precursor and mature forms, all of which was slowly degraded at 37 °C with the indicated amounts of N-glycanase for the indicated times. Samples were analyzed by 10% SDS-polyacrylamide gel electrophoresis and autoradiography. The mobility of undigested mSR-BI is indicated.

In an attempt to identify distinct precursor and mature forms of mSR-BI, we metabolically labeled the cells with [35S]Met/Cys for 5 h at 37 °C, immunoprecipitated the mixture of labeled precursor and mature forms, and chased in medium containing excess unlabeled methionine for the indicated times, and immunoprecipitates were analyzed by gel electrophoresis and autoradiography. The only major band detected immediately following the pulse labeling (0 chase) was a heterogeneous mixture of labeled precursor and mature forms, all of which was slowly degraded at 37 °C with the indicated amounts of N-glycanase for the indicated times. Samples were analyzed by 10% SDS-polyacrylamide gel electrophoresis and autoradiography. The mobility of undigested mSR-BI is indicated.

To determine whether or not the partial Endo H resistance of the mature form of mSR-BI observed in ldlA[mSR-BI] cells was an artifact of high level expression in a transfected cell line, we used immunoblotting to compare the Endo H sensitivities of mSR-BI in these cells with endogenous mSR-BI expressed in vivo in murine adrenal glands (Fig. 5). In contrast to the metabolic labeling, pulse-chase experiments described above, where only newly synthesized receptor incorporated label and was therefore visible by autoradiography, immunoblotting allowed the steady state population of receptors to be examined. Lanes 1 and 2 in Fig. 5 show that in ldlA[mSR-BI] cells, the steady state population of mSR-BI predominantly consisted of molecules in the mature form (lane 2, m-Endo H) with only a small fraction of the total in the precursor form (p-Endo H). Virtually identical results were observed for mSR-BI in the murine adrenal gland (lanes 3 and 4), except that the signal was weaker and it was difficult to detect any precursor form. In the adrenal specimens, the lower electrophoretic mobilities of the undigested mSR-BI and the m-Endo H form relative to the samples from the transfected cells suggest that there were probably some differences in the posttranslational modification of mSR-BI in the cultured transfected cells and the adrenal tissue. The similar Endo H digestion patterns of mSR-BI in both ldlA[mSR-BI] cells and murine adrenal gland (lanes 2 and 4) strongly suggest that the presence of a mixture of complex and high mannose or hybrid N-linked oligosaccharides is an intrinsic feature of the mature mSR-BI structure and not a cell culture artifact.

To extend the analysis of the oligosaccharide structure of mSR-BI expressed in ldlA[mSR-BI] cells, we metabolically labeled the cells with [35S]Met/Cys for 5 h at 37 °C, immunoprecipitated the mixture of labeled precursor and mature forms,
and digested the immunoprecipitates with sialidase, with or without prior digestion by Endo H. Fig. 6 shows that the electrophoretic mobility of the molecules treated with sialidase only (lane 2) was somewhat greater than that of the undigested sample (lane 1), although there was clearly some overlap in the relatively broad bands. This indicated that some, but probably not all, of the molecules contained sialic acid on oligosaccharide chains. This result was confirmed by the analysis of the effects of sialidase treatment of Endo H-digested samples (lanes 3 and 4). The electrophoretic mobility of the m-Endo H, but not the p-Endo H, form was shifted by sialidase digestion, indicating that sialic acids had been added to the complex type oligosaccharides of mSR-BI in the trans Golgi and trans Golgi network of the cells (73–75). It seems likely that most, if not all, of the sialic acid on mSR-BI is on N-linked oligosaccharides, because we could not detect the presence of O-linked sugars by electrophoretic analysis after sialidase/O-glycosidase digestion (data not shown).

Because mSR-BI is a member of the CD36 family of proteins (12, 15, 16) and CD36 has recently been shown to be palmitoylated (24, 76), we determined if mSR-BI were similarly modified by fatty acylation. Cells were metabolically labeled with either [3H]palmitate (2 h) or [35S]methionine (5 h), and mSR-BI was analyzed by immunoprecipitation and autoradiography (Fig. 7). In addition to IdIA[mSR-BI] cells, we analyzed [3H]palmitate incorporation into mSR-BI in Y1-BS1 cells, a murine adrenocortical cell line that exhibits ACTH-regulated mSR-BI expression (13) and selective cholesteryl ester uptake (77). Fig. 7 shows that label from [3H]palmitate was efficiently incorporated into mSR-BI in IdIA[mSR-BI] cells (lane 3). Palmitoylation generally involves covalent modification of cysteines via a thioester linkage, which is sensitive to neutral hydroxylamine or base treatments (69). Treatment of the immunoprecipitate with neutral hydroxylamine for 1 h removed all of the [3H]palmitate-derived label (lane 4) without loss of signal from the control [35S]methionine-labeled samples (lanes 1 and 2). Thin layer chromatographic analysis of fatty acids removed by base hydrolysis from labeled immunoprecipitates established that all of the label comigrated with palmitic acid and was therefore not due to metabolic conversion to some other adduct (data not shown). The results for Y1-BS1 cells (Fig. 7, lanes 5–8) were virtually identical to those for IdIA[mSR-BI] cells. Thus, mSR-BI is the second member of the CD36 family shown to be palmitoylated. Furthermore, we found that [3H]myristate was incorporated into mSR-BI in IdIA[mSR-BI] and Y1-BS1 cells (not shown). In contrast to palmitoylation, myristoylation usually involves covalent modification of amino-terminal glycine residues via an amide linkage, which is resistant to neutral hydroxylamine or base treatment (69). Some of the incorporated label was indeed resistant to neutral hydroxylamine treatment, even after incubations for as long as 20 h (not shown), a result virtually identical to that previously reported by Lublin and colleagues (28) for the analysis of myristoylation of several Src family protein-tyrosine kinases. Thus, mSR-BI appears to be myristoylated as well as palmitoylated.

Fatty acylation is a feature of several proteins, including CD36, which are concentrated in glycolipid- and cholesterol-enriched, detergent-insoluble domains of the plasma membrane, such as caveolae (24, 26–33). Caveolae were originally described as nonclathrin-coated invaginations in the plasma membrane (34, 35), and they are often identified by the presence of the protein caveolin (33, 36). Therefore, we used anti-mSR-BI495, anti-caveolin-1, and anti-clathrin antibodies combined with both subcellular fractionation and immunofluorescence microscopy to determine if mSR-BI was localized in such domains.
Fig. 8. mSR-BI fractionates with caveolin-1 in a low density subfraction of the plasma membrane. On day 0, ldlA[mSR-BI] cells were set in 10-cm dishes (100,000 cells/dish) in medium B containing 10% calf serum rather than 5% FBS. On day 3, cells (90–100% confluent) from 10 dishes (10 mg of total protein) were harvested, and plasma membranes were prepared as described previously (71). Purified plasma membranes were sonicated and then fractionated by a two-step OptiPrep density gradient centrifugation into high density and low density fractions as described previously (71). Ten micrograms of protein from each fraction was subjected to 12.5% SDS-polyacrylamide gel electrophoresis, electrophoretic transfer onto nylon membranes, and immunoblotting with anti-mSR-BI495 whole antiserum or anti-caveolin-1 polyclonal antibody. The positions of mSR-BI and caveolin-1 are shown.

Fractions are as follows: postnuclear supernatant (lane 1), cytosol (lane 2), internal membranes (lane 3), total plasma membrane (lane 4), caveolin-poor plasma membrane fractions (high density) (lane 5), and caveolin-rich plasma membrane fractions (low density) (lane 6), as defined previously (71).

Fig. 9 shows the results of a subcellular fractionation experiment in which plasma membranes from ldlA[mSR-BI] cells were isolated by Percoll density gradient centrifugation and then subjected to a two-step OptiPrep density gradient centrifugation analysis (71). Using this approach, we isolated several distinct membrane compartments (see Ref. 71 for details) and used immunoblotting to examine the relative amounts of mSR-BI and caveolin-1 in these compartments. These compartments include the postnuclear supernatant (PNS, lane 1), cytosol (lane 2), internal (plasma membrane-depleted) membranes (lane 3), total plasma membranes (lane 4), caveolin-poor plasma membranes (lane 5), and low density, caveolin-rich plasma membranes (lane 6). Equal amounts of protein from each fraction were loaded on the gel. Caveolin-1 and mSR-BI were highly enriched in the same low density plasma membrane fraction (lane 6). Although caveolin is present in the total plasma membrane fraction (71), its relative abundance is sufficiently low that it was not detectable under the loading and detection conditions used here. At longer exposure times, a significant signal for mSR-BI could be seen in the caveolin-poor plasma membrane fraction (not shown). These results suggest that mSR-BI preferentially, but not exclusively, associated with caveola in these transfected cells.

This preferential association of mSR-BI with caveola is further supported by immunofluorescence localization experiments. Fig. 9 shows two distinct features in the pattern of anti-mSR-BI495 staining of Triton X-100-permeabilized ldlA[mSR-BI] cells (panels A and D) observed using confocal microscopy. In those cells that exhibited intense staining, there was a distinct bright linear staining along portions of the cells’ borders as well as a heterogeneous, punctate staining over the body of the cells. The relative amounts of surface punctate versus edge staining depended on the depth of the confocal section relative to the thickness of the cells. A similar pattern has previously been described for anti-caveolin-1 staining of fibroblasts (31, 41, 78). Virtually no staining was seen in untransfected ldlA cells or in ldlA[mSR-BI] cells that were not detergent-permeabilized (not shown). Thus, the signal was specific for mSR-BI, and its C terminus was apparently exposed at the cytoplasmic face of the plasma membrane, a topology consistent with that reported for CD36 (23, 24).

When the ldlA[mSR-BI] cells were costained with anti-mSR-BI495 (Fig. 9A, green) and a monoclonal antibody to caveolin-1 (anti-caveolin, B, red) and the images were analyzed by superposition (panel C), considerable colocalization was seen both for the punctate patterns over the cell bodies and for the linear patterns at the edges of cells (panel C; yellow indicates colocalization).
tion), although there were clearly segments of cell borders that were stained with one, but not the other, of these antibodies (panel C, pure red or green). In contrast, when ldlA[mSR-BI] cells were costained with anti-mSR-BI<sup>495</sup> (panel D, green) and a monoclonal anti-clathrin antibody (panel E, red), there was almost no colocalization of the punctate mSR-BI and clathrin staining over the cell bodies and essentially no strong linear edge staining by the anti-clathrin antibody (combined images in panel F). Similar results were obtained when Y1-B51 cells were costained with anti-mSR-BI<sup>495</sup> and anti-caveolin-1 (panels G, H, and I) or with anti-mSR-BI<sup>495</sup> and anti-clathrin (not shown). While all of the transfected ldlA[mSR-BI] cells were strongly stained with the anti-caveolin or anti-clathrin antibodies, only a subset was stained with anti-mSR-BI<sup>495</sup>, indicating that the expression of mSR-BI in these cells was heterogeneous. Ectopic overexpression of mSR-BI in at least some ldlA[mSR-BI] cells may be responsible for the observation that mSR-BI did not exclusively copurify or colocalize with caveolin-1.

Thus, cell fractionation methods have demonstrated that mSR-BI is enriched in low density, caveolin-1-containing domains of the plasma membrane, and immunofluorescence experiments have shown that there was significant colocalization of mSR-BI and caveolin-1 at the resolving power of confocal microscopy. Taken together, these results suggest that a significant portion of mSR-BI is indeed associated with caveolae. Additional studies will be required to determine the compositions and structures of those sites in ldlA[mSR-BI] cells in which caveolin-1 or mSR-BI, but not both, are enriched.

**DISCUSSION**

The murine class B, type I scavenger receptor, mSR-BI, is an HDL receptor that mediates the selective cellular uptake of cholesteryl esters from HDL by a mechanism that differs markedly from receptor-mediated endocytosis (12). As a first step toward determining the mechanisms underlying mSR-BI function, we used a polyclonal anti-peptide antibody, anti-mSR-BI<sup>495</sup>, which recognizes the C terminus of mSR-BI, in conjunction with immunochemical techniques to examine the biosynthesis, structure, and subcellular localization of mSR-BI. Most experiments were performed using transfected CHO cells expressing murine SR-BI (ldlA[mSR-BI]), and key findings were confirmed by analysis of the endogenous mSR-BI expressed either in a murine adrenocortical cell line, Y1-BS1, or in murine adrenal tissue.

mSR-BI is initially synthesized with multiple high mannose N-linked oligosaccharide chains, and some, but not all, of these oligosaccharides are processed to Endo H-resistant, sialic acid-containing complex forms during the maturation of the protein in the Golgi apparatus. Similar heterogeneity in the structures of the N-linked chains on CD36 has been reported (23, 79). Although the electrophoretic mobilities in 8% SDS-polyacrylamide gels of the precursor and mature forms of mSR-BI were virtually indistinguishable, these forms could be readily differentiated by electrophoresis after Endo H digestion. The electrophoretically determined apparent mass of the mature form of mSR-BI in the transfected cells was ~82 kDa and was reduced to ~54 kDa after removal of the N-linked chains. The apparent mass of the deglycosylated form is similar to the mass calculated from the predicted amino acid sequence (~57 kDa). In the Y1-BS1 cells, the apparent mass of the mature form was somewhat higher than that seen in the transfected cells, possibly due to differences in the structures of the oligosaccharide chains. There appears to be little or no O-glycosylation of the protein.

In addition to glycosylation, mSR-BI is palmitoylated via a linkage that has the characteristics of a thioester (sensitivity to hydroxylamine and base), a feature shared with CD36 (24, 76). Palmitoylation is believed to be restricted to cysteine residues located at the cytoplasmic face of the plasma membrane (80, 81). Based on this criterion, and by analogy to the analysis of palmitoylation of CD36 mutants, the most likely sites of palmitoylation of mSR-BI are Cys<sup>462</sup> and Cys<sup>470</sup>. The predicted amino acid sequence of mSR-BI and the topology of CD36 suggest that, in mSR-BI, Cys<sup>462</sup> lies at and Cys<sup>470</sup> is near the junction of a putative transmembrane domain (residues 444–464) and the cytoplasmic C terminus (residues 465–509). This assignment is consistent with our observation that detergent permeabilization was necessary to detect mSR-BI by immunofluorescence with anti-mSR-BI<sup>495</sup>, an antibody that recognizes the C terminus of the protein. Further studies will be required to establish which of the Cys residues in the protein are palmitoylated. Metabolic labeling studies also indicated that mSR-BI is myristoylated as well as palmitoylated. This modification has been shown to require the removal of the N-terminal methionine prior to amide formation with the amino group of the adjacent glycine (82). Indeed, the N-terminal eight residues of mSR-BI (Met-Gly-Gly-Ser-Ser-Lys-Ala-Lys) are compatible with, but not identical to, consensus sequences for myristoylation based on Src family sequences (83), and the activity of the yeast N-myristoyltransferase (82).

The functional significance of the fatty acylation of mSR-BI remains to be established; however, fatty acylation with palmitate and/or myristate has been shown to affect the subcellular distribution of proteins by facilitating their interactions with the cytoplasmic face of the plasma membrane (84, 85). For example, dual myristoylation and palmitoylation has been shown to be both necessary and sufficient to localize tyrosine kinases, such as p59<sup>fyn</sup> (27, 28) and endothelial NO synthase (30–32) to caveolae. Based on the suggestion that CD36, which is palmitoylated (24, 76), is enriched in caveolae (26), we used cell fractionation and immunofluorescence methods to determine the subcellular localization of mSR-BI. Cell fractionation indicated that a substantial fraction, but not all, of the mSR-BI copurified with caveolin-1 in a caveolae-enriched, low density plasma membrane fraction. Similarly, immunofluorescence demonstrated that a significant portion, but not all, of the mSR-BI colocalized with caveolin-1 in a punctate pattern over the surfaces of cells and in a linear pattern along cell edges. Ectopic overexpression of mSR-BI in at least some ldlA[mSR-BI] cells may be responsible for the observation that mSR-BI did not exclusively copurify or colocalize with caveolin-1. Higher resolution studies will be required to determine if mSR-BI is located in morphologically well defined invaginated and vesicular caveolar structures. The current biochemical and immunofluorescence data, however, provide very strong support for the model that mSR-BI is preferentially concentrated in caveolae. Caveolae may represent one subset of several types of plasma membrane microdomains with similar glycolipid-rich, detergent-insoluble properties, such as detergent-insoluble glycolipid (DIG) or glycolipid-enriched membrane (GEM) domains (44, 86), whose distributions may be similar to that of caveolin-1-rich caveolae. Additional studies will be required to determine if mSR-BI is also concentrated in noncaveolar microdomains.

The localization of mSR-BI in low density, caveolin-enriched membrane domains on the cell surface may have implications for the mechanism of SR-BI-mediated selective lipid uptake by cells. The distinctive physical-chemical properties of these glycolipid- and cholesterol-rich membrane domains may be critical for the transfer of lipid from lipoprotein to cell and perhaps for the reverse transport of lipid from cells to lipoprotein (87). Indeed, recent studies have suggested that caveolae may serve as sites of cholesterol efflux from cells (55). In addition, caveolin appears to be a cholesterol binding protein (88, 89) and it
may be involved in intracellular cholesterol transport (41, 53, 54). The concentration of SR-BI in caveolae may bring the receptor into proximity with caveolin or other resident proteins that may be critical for direct lipid transfer into the cell. Furthermore, the ability of caveolae to internalize molecules (po- tocytosis (50) and transcytosis (48, 49)) may be essential for lipid transport. Recent immunohistochemical studies indicate that, in the cortical cells of the murine adrenal gland, mSR-BI is associated with surface structures that have the morphology of microvilli-rich canaliculi (13). It has been suggested that these regions play a role in HDL binding and selective lipid uptake (90, 91). A comparison of the composi- tions and transport properties of these adrenocortical canaliculi and caveolae may provide further insights into the molec- ular mechanisms underlying selective lipid uptake.

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