The murine class B, type I scavenger receptor mSR-BI, a high density lipoprotein (HDL) receptor that mediates selective uptake of HDL lipids, contains 11 potential N-linked glycosylation sites and unknown numbers of both endoglycosidase H-sensitive and -resistant oligosaccharides. We have examined the consequences of mutating each of these sites (Asn → Gln or Thr → Ala) on post-translational processing of mSR-BI, cell surface expression, and HDL binding and lipid transport activities. All 11 sites were glycosylated; however, disruption of only two (Asn-108 and Asn-173) substantially altered expression and function. There was very little detectable post-translational processing of these two mutants to endoglycosidase H resistance and very low cell surface expression, suggesting that oligosaccharide modification at these sites apparently plays an important role in endoplasmic reticulum folding and/or intracellular transport. Strikingly, although the low levels of the 108 and 173 mutants that were expressed on the cell surface exhibited a marked reduction in their ability to transfer lipids from HDL to cells, they nevertheless bound nearly normal amounts of HDL. Indeed, the affinity of 125I-HDL binding to the 173 mutant was similar to that of the wild-type receptor. Thus, N-linked glycosylation can influence both the intracellular transport and lipid-transporter activity of SR-BI. The ability to uncouple the HDL binding and lipid transport activities of mSR-BI by in vitro mutagenesis should provide a powerful tool for further analysis of the mechanism of SR-BI-mediated selective lipid uptake.

Scavenger receptor class B, type I, SR-BI, is a 509-residue, ~82-kDa integral membrane cell surface glycoprotein of the CD36 superfamily that was the first high density lipoprotein (HDL) receptor to be characterized in detail (1, 2). SR-BI helps control the structure and metabolism of HDL by mediating the transport of lipids from HDL to cells. The mechanism by which SR-BI mediates this lipid transport differs from the classic LDL receptor pathway of endocytosis, in which the entire lipoprotein is internalized via coated pits and subsequently hydrolyzed by lysosomal enzymes (3). Instead, HDL binds to SR-BI, lipids of HDL (primarily neutral lipids such as cholesterol esters in the core of the particle) are translocated into the cells, and the lipid-depleted particle is released into the extracellular space (1, 2, 4, 5). This mechanism is called selective lipid uptake (2, 4, 5). Numerous studies indicate that SR-BI-mediated selective lipid uptake is a two-step process involving productive binding of HDL (1, 6, 7) followed by binding-dependent lipid transfer. In addition to mediating selective lipid uptake, SR-BI can mediate cholesterol efflux from cells to HDL (8) via a binding-dependent process (6, 7, 9) (however see Ref. 10 for alternative view). The physiologic significance of SR-BI-mediated cholesterol efflux is not clear. In addition to HDL, SR-BI can bind tightly to numerous other ligands, including native and modified LDL (reviewed in Ref. 2).

In vitro analyses of the function of SR-BI, primarily using SR-BI homozygous null mice (11, 12) and murine hepatic overexpression of SR-BI transgenes (13–18), have shown that SR-BI can profoundly influence several physiologic systems (also see Ref. 46). For example, adenovirus-mediated hepatic overexpression of SR-BI increases biliary cholesterol concentration, whereas complete loss of expression lowers biliary cholesterol (12, 13, 19). Female SR-BI knock-out mice are infertile due to lipoprotein-dependent defects in oocyte development (12, 20). Loss of SR-BI expression can disrupt red blood cell development (21) and influence the development of atherosclerosis (SR-BI expression is protective (12, 14, 16, 18)), coronary heart disease, and premature death (23).

The molecular mechanisms underlying SR-BI activity and the relationship of the structure of SR-BI to its functions are not well-understood. To better understand the structure of SR-BI and its mechanism of action, we have conducted several studies involving the generation and characterization of SR-BI mutants (6, 9, 25). Some of these studies have shown that the large, extracellular loop of SR-BI, which is glycosylated, plays a critical role in mediating not only ligand binding but also the selective lipid uptake step (6, also see Ref. 26). To date, the functional role of the extensive N-linked glycosylation of SR-BI has not been explored. Oligosaccharides in glycoproteins can serve a variety of functions, including facilitating protein folding, protecting against proteolysis, directly participating in intermolecular interactions, directing intracellular trafficking and secretion, and influencing cell surface expression and activity (27–29). In some cases it has not been possible to attribute a specific function to a given N-linked glycan.

Here we report the effects of mutating (Asn → Gln or Thr →...
 Ala (each) of the 11 potential N-linked glycosylation sites (Asn-X-Ser/Thr) in the extracellular loop of murine SR-BI on the extent of glycosylation (changes in apparent mass), cell surface expression, 125I-HDL binding, and cellular uptake of lipid from HDL. The results show that all 11 sites are glycosylated and that two of these sites at positions 108 and 173 are essential for normal surface expression and efficient lipid uptake but not HDL binding. To confirm the position 173 mutational sites most influence the apparent binding affinity of HDL or maximal amount of binding. They also show that the HDL binding and lipid transport activities of mSR-BI can be uncoupled.

**EXPERIMENTAL PROCEDURES**

**Materials**—(1a,2α,3α)-[1H]Cholesteryl oleoyl ether (1H[CEt], 1 mCi/μl, specific activity of 58 Ci/mmol) was from Amersham BioSciences. Human HDL, COOH-terminal-HDL, Di-labeled HDL, Alexa-labeled HDL, and 1H[CEt]-HDL were prepared as described previously (1, 6, 9, 25). The GenePORTER transfection reagent was from GTS Inc. Rabbit anti-serum against the extracellular domain of mSR-BI was previously described (anti-mSR-BI KKK-1 antiseraum, 1:1000 dilution), a generous gift from K. Kozarsky (9), FITC-conjugated goat anti-rabbit IgG (1:1000 dilution, Cappel, West Chester, PA). All other reagents were obtained from standard commercial sources or as described previously (25, 28, 32, 33). The expression vectors for mSR-BI and mutants were constructed in pCDNA1 (Invitrogen) using standard recombinant DNA techniques. Wild-type mSR-BI expression vectors used for these studies included pmSR-BI 77 (1) and a minor variant ex68, which contained small deletions in the extracellular loop of SR-BI. Site-directed Mutagenesis of mSR-BI—Site-directed mutagenesis to create the mutants was performed on SR-BI cDNA in the pCDNA1 vector using a commercial kit (QuikChangeTM site-directed mutagenesis kit, Stratagene Inc., La Jolla, CA) according to the manufacturer’s instruction. Briefly, a pair of complementary primers with 25–35 bases was designed for each mutation and designed to change asparagine to glutamine or threonine to alanine was placed in the middle of the primers. The wild-type mSR-BI cDNA within pcDNA was amplified for 16 cycles in a DNA thermal cycler using Pfu DNA polymerase with these primers. After digestion, the template DNA with Dpn I, the amplified mutant DNA was transferred into E. coli (MC1061/P3 strain (Invitrogen)). The mutations were confirmed by automated DNA sequencing. The sequences of the mutagenic primers for the Asn→Gln mutations were: 102, 5′-TTG AAG AAA GGC GTC CAT ACC TTC TAC AAT GAC-3′; 108, 5′-ATT ACC TAT ACC TAT GAC CAG GAC ACC GTG TTC TTC-3′; 116, 5′-CTG GTG TTC TTC GTG GAG CAG CAC CAC CAC CTC-3′; 173, 5′-GCT GCT TTT ATG CAG GGC ACA GTT GGT GAG-3′; 212, 5′-CTT GTT GTT ATG GAG GAC AAC TAC AAT TCT GGG-3′; 227, 5′-TTC ACC GGC GTG CAC CAA TTC AGC AGG ATC CAT C-3′; 255, 5′-CTGCCCGGAGGTACCTTGGGTTTTGATATCCCGCGGTGCCTTGCTTCTCC-3′; 258, 5′-GAC TGC CAC ATC ACC GAC GCC GGC GCC GGC TTC ACC TCT GGG GGC-3′; 383, 5′-TCC GTT AGC CCC ACC ATG GCC TGT AAT TCT GGG GAG-3′. The sequences of the mutagenic primers for the Thr→Ala mutants were: 108 (T→A), 5′-TCT AAC AAT AAC AAC GCC GCC GAC GGC GTG TTC TTC G3′; 173 (T→A), 5′-GCT TTT ATG AAC CGT ACA GTA GCC GTC AGC-3′.

**Site-directed Mutagenesis of mSR-BI—** Site-directed mutagenesis was performed on wild-type mSR-BI cDNA in the pCDNA1 vector using a commercial kit (QuikChange™ site-directed mutagenesis kit, Stratagene Inc., La Jolla, CA) according to the manufacturer’s instruction. Briefly, a pair of complementary primers with 25–35 bases was designed for each mutation and designed to change asparagine to glutamine or threonine to alanine was placed in the middle of the primers. The wild-type mSR-BI cDNA within pcDNA was amplified for 16 cycles in a DNA thermal cycler using Pfu DNA polymerase with these primers. After digestion, the template DNA with Dpn I, the amplified mutant DNA was transferred into E. coli (MC1061/P3 strain (Invitrogen)). The mutations were confirmed by automated DNA sequencing. The sequences of the mutagenic primers for the Asn→Gln mutations were: 102, 5′-TTG AAG AAA GGC GTC CAT ACC TTC TAC AAT GAC-3′; 108, 5′-ATT ACC TAT ACC TAT GAC CAG GAC ACC GTG TTC TTC-3′; 116, 5′-CTG GTG TTC TTC GTG GAG CAG CAC CAC CAC CTC-3′; 173, 5′-GCT GCT TTT ATG CAG GGC ACA GTT GGT GAG-3′; 212, 5′-CTT GTT GTT ATG GAG GAC AAC TAC AAT TCT GGG-3′; 227, 5′-TTC ACC GGC GTG CAC CAA TTC AGC AGG ATC CAT C-3′; 255, 5′-CTGCCCGGAGGTACCTTGGGTTTTGATATCCCGCGGTGCCTTGCTTCTCC-3′; 258, 5′-GAC TGC CAC ATC ACC GAC GCC GGC GCC GGC TTC ACC TCT GGG GGC-3′; 383, 5′-TCC GTT AGC CCC ACC ATG GCC TGT AAT TCT GGG GAG-3′. The sequences of the mutagenic primers for the Thr→Ala mutants were: 108 (T→A), 5′-TCT AAC AAT AAC AAC GCC GCC GAC GGC GTG TTC TTC G3′; 173 (T→A), 5′-GCT TTT ATG AAC CGT ACA GTA GCC GTC AGC-3′.
at 25°C) and 2–10 units of endoglycosidase H (endo H) were added, and the mixture was incubated at 37°C overnight. Undigested controls were incubated without the added enzyme.

**Immunoblotting**—Cell lysates were analyzed by immunoblotting with the rabbit anti-sR-BI anti-peptide polyclonal antibody 495 (1, 11), and the blots were visualized with the ECL chemiluminescent detection system (Amersham Biosciences) and x-ray film as previously described (33).

**Immunofluorescence**—On day 2 after plating, cells were fixed with 4% formaldehyde in PBS for 30 min at room temperature, washed twice with PBS, incubated for 15 min with 50 μM NH₄Cl, blocked for 1 h with 10% fetal bovine serum in PBS, incubated overnight at 4°C with KRB-1 antibody (1:1000 dilution), and then incubated with Texas Red-conjugated anti-rabbit secondary antibody (1:500 dilution) at room temperature for 1 h in the dark. Controls included rabbit IgG (10 μg/ml) as primary antibody followed by secondary antibody or omitting the primary antibody and incubating only with secondary antibody. Samples were then washed with PBS three times for 5 min and mounted in Vectashield (Vector Laboratories, Burlingame, CA). The cells were viewed with a Zeiss Axioplan microscope (×63 and 1.3 numerical aperture objective) and a confocal laser scanning system (1024 Bio-Rad MRC equipped with an argon krypton laser). Images were saved as tiff format files and edited with Photoshop 6.0 software.

**RESULTS**

**Identification of N-Linked Glycosylation Sites on mSR-BI by Mutagenesis**—Murine sR-BI contains 11 potential N-linked glycosylation sites (Asn-X-Ser/Thr) in its extracellular domain (1); these include the asparagines at positions: 102, 108, 116, 173, 212, 227, 255, 288, 310, 330, and 383. These sites are conserved in the mouse and rat and, with the exception of positions 116 and 288, in the human and hamster. Previous studies have shown that, when expressed in CHO cells, at least five of these sites in mSR-BI are modified by glycosylation and that N-glycosylation appears to account for most of the difference between the predicted (~57 kDa, based on amino acid sequence) and observed (~82 kDa determined by SDS-PAGE) masses of SR-BI (33). The observed apparent mass of SR-BI is ~82 kDa in a variety of both cultured cells and tissues, suggesting that the extent of N-glycosylation is similar in these cells and tissues. To determine which of the potential N-linked sites were glycosylated, we (a) generated a collection of 11 mutant mSR-BI cDNA expression vectors in which one of each of the 11 sites was mutated (Asn → Gln), (b) expressed the wild-type and mutant proteins in COS M6 cells by transient transfection, and (c) compared their electrophoretic mobilities by SDS-PAGE and immunoblotting with an anti-C terminus antipeptide antibody (1). This antibody can recognize mSR-BI independently of its glycosylation state (Ref. 33 and data not shown). The expectation was that there would be a small increase in electrophoretic mobility (reduced apparent mass) in a mutant mSR-BI relative to the wild-type if the mutated site were glycosylated in the wild-type protein. Fig. 1A shows that each of the 11 mutants (lanes 1–4, 6–9, 11, 13, and 14) exhibited a slightly greater mobility than the wild-type receptor (lanes 5, 10, and 12). Thus, we conclude that all 11 potential N-glycosylation sites in COS cells are normally glycosylated and probably in many other mammalian cells in culture and in vivo.

**Effects of N-Linked Glycosylation Site Mutations on Receptor Processing by the Golgi Apparatus**—Prior to processing in the Golgi apparatus, newly synthesized mSR-BI in the ER contains the high mannose forms of N-linked oligosaccharides that are all sensitive to cleavage by the enzyme endoglycosidase H (endo H (33)). After modification in the Golgi apparatus, the processed form of mSR-BI, when expressed in either stably transfected CHO cells in culture or adrenal glands in vivo, contains two classes of N-linked oligosaccharides: endo H-resistant chains (complex type N-linked sugars) and endo H-sensitive chains (either high mannose or hybrid type N-linked sugars) and the blots were visualized with the ECL chemiluminescent detection system (Amersham Biosciences) and x-ray film as previously described (33). The extent of processing (degree of endo H resistance of the processed receptor) varies depending on the cells/tissue (33).

**Immunoblot** analysis of wild-type mSR-BI (None) and its single N-glycosylation site mutants: electrophoretic mobility shifts endoglycosidase H sensitivity. COS cells were transiently transfected with expression vectors encoding either wild-type mSR-BI (None) or mSR-BI in which a single amino acid mutation in the codon for Asn at each of the 11 potential N-linked glycosylation sites (indicated by the number of its location in the sequence) was mutated to that for Gln. Cell lysates (40 μg of protein) were analyzed by SDS-10% polyacrylamide gel electrophoresis and immunoblotting with an anti-mSR-BI C-terminal anti-peptide antibody (anti-mSR-BI495) without enzymatic treatment (A) or were incubated overnight at 37°C with 0.1 unit/ml of endo H prior to electrophoresis and immunoblotting (B). In the fully lower bands and partially (upper) endo H-sensitive forms of the protein are shown.

**Effects of N-Linked Glycosylation Site Mutations on Receptor Processing by the Golgi Apparatus**—Prior to processing in the Golgi apparatus, newly synthesized mSR-BI in the ER contains the high mannose forms of N-linked oligosaccharides that are all sensitive to cleavage by the enzyme endoglycosidase H (endo H (33)). After modification in the Golgi apparatus, the processed form of mSR-BI, when expressed in either stably transfected CHO cells in culture or adrenal glands in vivo, contains two classes of N-linked oligosaccharides: endo H-resistant chains (complex type N-linked sugars) and endo H-sensitive chains (either high mannose or hybrid type N-linked sugars) and the blots were visualized with the ECL chemiluminescent detection system (Amersham Biosciences) and x-ray film as previously described (33). The extent of processing (degree of endo H resistance of the processed receptor) varies depending on the cells/tissue (33). Fig. 1B shows the effects of endo H treatment of cell lysates on the electrophoretic mobilities of wild-type mSR-BI (lane 1) or the single Asn → Gln glycosylation mutants (lanes 2–12) expressed transiently in COS cells. As was previously observed in CHO cells, two immunoreactive bands were observed for the endo H-digested wild-type receptor in COS cells (lane 1). The lower bands is the fully endo H-sensitive form of the receptor and presumably represents the precursor form of the protein prior to Golgi processing (33–35). The upper band represents a processed, partially endo H-resistant form. Most of the N-linked oligosaccharides in COS cells were endo H-sensitive after Golgi processing, the mobility of the processed, partially endo H-resistant form was only slightly less than that of the fully endo H-sensitive form and significantly greater than that of the receptor not subjected to endo H digestion (Fig. 1A, lanes 5, 10, and 12).

The doublet patterns of precursor and processed forms after endo H treatment were similar to that of the wild-type receptor for seven of the mutants: 383, 330, 310, 288, 227, and 102 (Fig. 1B, lanes 2–7 and 12). The relative ratios of the intensities of the upper and lower bands varied, raising the possibility that processing rates or stabilities of the mutants might have differed from those of the wild-type in some cases. Although these results are consistent with all of these seven sites in the wild-type receptor normally carrying endo H-sensitive chains, alternative explanations are possible (e.g. loss of one glycosylation site might alter the processing at another site). Indeed, the reproducibly lower mobility of the processed bands in the 212 and 116 mutants (lanes 8 and 10) is most likely due to a change in the structure of one or more oligosaccharides at one or more other sites (e.g. additional endo H-resistant chains or altered structure of the endo H-resistant chains). The most striking abnormalities in the doublet patterns were seen for the 173 and 108 mutants, where very little (108, lane 1) or no (173, lane 9) processed forms were detected. Although it is possible that all of the N-linked sugars on these two mutants remain endo H-resistant, the lower bands were not endo H-sensitive (Fig. 1B, lane 9), raising the possibility that the endo H-resistant sugars on these two mutants might have dif-

![Fig. 1. Immunoblot analysis of wild-type mSR-BI (None) and its single N-glycosylation site mutants: electrophoretic mobility shifts and endoglycosidase H sensitivity. COS cells were transiently transfected with expression vectors encoding either wild-type mSR-BI (None) or mSR-BI in which a single amino acid mutation in the codon for Asn at each of the 11 potential N-linked glycosylation sites (indicated by the number of its location in the sequence) was mutated to that for Gln. Cell lysates (40 μg of protein) were analyzed by SDS-10% polyacrylamide gel electrophoresis and immunoblotting with an anti-mSR-BI C-terminal anti-peptide antibody (anti-mSR-BI495) without enzymatic treatment (A) or were incubated overnight at 37°C with 0.1 unit/ml of endo H prior to electrophoresis and immunoblotting (B). In B the fully lower bands and partially (upper) endo H-sensitive forms of the protein are shown.](image-url)
Typically, the fluorescence intensities for control cells transfected with anti-SR-BI polyclonal antibody (KKB-1) and an FITC-labeled secondary antibody were determined by flow cytometry using an analyzed as described under Experimental Procedures. A, cell surface receptor-specific expression was determined by flow cytometry using an anti-SR-BI polyclonal antibody (KKB-1) and an FITC-labeled secondary antibody. B and C, receptor-specific HDL binding activity was determined after a 1.5-h incubation with 125I-HDL (10 μg of protein/ml). D and E, receptor-specific lipid uptake activity was determined by flow cytometry after a 2-h incubation with DiI-HDL (10 μg of protein/ml). In all cases, the receptor-specific values were calculated as the differences between the determinations from cells transfected by the wild-type or mutant SR-BI cDNAs and those from the cells transfected with the empty vector control. In some experiments, incubations with DiI-HDL were immediately followed by incubations with the antibodies to permit simultaneous determination of lipid uptake and surface expression by two-color flow cytometry. In A, B, and D, the 100% of control values are those of the wild-type mSR-BI measured in the same assay. The measured values for A and D were fluorescence intensities in arbitrary units. Typically, the fluorescence intensities for control cells transfected with an empty vector were 2–3% (surface expression) and 5–10% (DiI uptake) of those of cells expressing wild-type mSR-BI. In B, the 100% of control binding values were typically ~100 ng of 125I-HDL protein/mg of cell protein with empty vector control binding values of ~1–10%. The surface expression-corrected values for binding and lipid uptake in C and E, respectively, were calculated by dividing the percentage of control value for each of these parameters by the corresponding value for surface expression measured in the same experiment. All of the values shown represent the means from at least four and as many as nine independent transfections and are from multiple independent experiments. Each error bar represents the standard error of the mean.

Effects of N-Linked Glycosylation Site Mutations on Receptor Activity—Two characteristic activities of SR-BI are its abilities to bind HDL and to transfer lipids such as cholesteryl esters or the hydrophobic fluorescent dye DiI from HDL to cells. These activities in transiently transfected COS cells were measured using 125I-HDL (10 μg of protein/ml, lipoprotein binding activity) and DiI-labeled HDL (DiI-HDL, 10 μg of protein/ml, lipid transfer activity) as described under “Experimental Procedures.” Fig. 2B shows that the absolute levels of 125I-HDL binding to COS cells expressing all but two of the mutants were similar to that of the cells expressing the wild-type receptor. The exceptions were the 173 and 108 mutants that exhibited substantially lower binding. The result is consistent with the possibility that these mutations might have interfered with exit of the receptors from the ER, substantially reducing Golgi-glycosylation independent (see “Experimental Procedures”)

Effects of N-Linked Glycosylation Site Mutations on Cell Surface Expression—To address this later possibility, we used a rabbit polyclonal antibody (KKB-1) that recognizes apparently glycosylation independent (see “Experimental Procedures”) epitopes in the extracellular domain of mSR-BI to measure cell surface expression of the wild-type mSR-BI and the mutants by transiently transfected COS cells. Transfected cells were incubated with KKB-1, washed, and incubated with an FITC-labeled anti-rabbit IgG secondary antibody, and then cellular fluorescence was quantitated by flow cytometry as previously described (6, 7, 9, 25). Fig. 2A shows that mutations at positions 108 and 173 substantially reduced cell surface immunodetectable mSR-BI, whereas the other single glycosylation mutations had little effect on surface expression relative to that of the wild-type mSR-BI (SR-BI). These experiments suggest that the N-linked oligosaccharide chains at positions 108 and 173, but not the others, are critical determinants of cell surface expression. It was possible that the conversion of the Asn side chains themselves at positions 108 or 173, rather than the loss of N-glycosylation, was responsible for the reduced processing and surface expression of these mutants. Therefore, a different class of mutation, Thr → Ala, in the consensus glycosylation sequence (Asn-X-Thr) that would prevent N-glycosylation was introduced at these sites. Fig. 3A shows that surface expression was even lower in the Thr → Ala mutants than the Asn → Gln mutants (reduction relative to wild-type control: 173, 92% versus 58%; 108, 84% versus 67%). Thus, glycosylation at positions 173 and 108 appear to be required for normal mSR-BI expression on the surface of COS cells.
finding that there was substantially lower surface expression of these mutant receptors (Fig. 2A). When the binding data were corrected to normalize for the extent of surface expression (Fig. 2C), there were no statistically significant differences among the mutant and wild-type receptors, including the 173 and 108 mutants. This suggests that the mutations at positions 173 and 108 did not interfere with the capacity to bind HDL of those relatively few mutant receptor proteins that were able to be transported to the cell surface. Virtually identical results were obtained when quantitative binding of fluorescently labeled Alexa-HDL (6) was measured using flow cytometry (data not shown).

There was greater mutant-to-mutant variation in Dil uptake than in \( ^{125}\text{I}-\text{HDL} \) binding (Fig. 2D). Relative to the wild-type receptor, small, but reproducible, reductions in receptor-mediated lipid uptake were exhibited by the 310, 255, and 212 mutants, even after correction for surface expression (Fig. 2, D and E). As expected, there was dramatically less Dil uptake by the cells expressing 173 and 108 mutants than those expressing the wild-type receptor (Fig. 2D). These substantially reduced levels of lipid uptake were striking even after correction for surface expression (Fig. 2E). This suggested that, unlike the apparently normal binding activities, the lipid transport activities of these two mutants were significantly reduced relative to wild-type controls. Almost identical results for cell surface expression corrected Dil uptake were observed using the Thr \( \rightarrow \) Ala mutations at positions 173 and 108 in place of the Asn \( \rightarrow \) Gln mutations (Fig. 3, B and C).

The low level of expression of the 173 and 108 mutants made it difficult to characterize further the properties of these mutants in transiently transfected COS cells. For example, we wanted to determine if these mutations affected the uptake of cholesteryl ether from HDL in a fashion similar to that for Dil uptake. We therefore attempted to generate stable cell lines expressing higher cell surface levels of these mutants by transfection of ldlA-7 cells and selection of cells with high surface expression using the KKB-1 antibody and flow cytometry. The ldlA-7 cells are LDL-receptor-deficient mutants that were isolated from mutagen-treated Chinese hamster ovary cells (32). We were unsuccessful in isolating transfected expressing high levels of the 108 mutant but did succeed with the 173 mutant.

Fig. 4A shows the concentration dependence of \( ^{125}\text{I}-\text{HDL} \) binding to untransfected control cells (ldlA-7, circles), and stably transfected cells expressing the wild-type receptor (ldlA[mSR-BI] (1), open circles) or the 173 mutant (ldlA[N173Q], filled circles). Although the absolute amount of binding of \( ^{125}\text{I}-\text{HDL} \) to ldlA[N173Q] cells was lower than that to ldlA[mSR-BI] cells, it was significantly higher than that of the control ldlA-7 cells. When the transfected receptor-specific binding data (calculated as the difference between binding to transfected and untransfected cells) were corrected by normalizing for the level of receptor surface expression, the extents of binding to the wild-type receptor and the 173 mutant were similar and there was apparently no difference in the binding affinities (Fig. 4C). Thus, the results for cell surface expression-corrected \( ^{125}\text{I}-\text{HDL} \) binding in stably transfected ldlA-7 cells were similar to those in the transiently transfected COS cells.

Fig. 4B shows the concentration dependence of \( ^{3}\text{H}-\text{CEt} \) uptake from HDL in a fashion similar to that for Dil uptake. The nonhydrolyzable cholesteryl ether was used rather than cholesteryl ester to simplify the analysis by preventing intracellular hydrolysis. There was robust selective uptake of the \( ^{3}\text{H}-\text{CEt} \) from \( ^{3}\text{H}-\text{CEt-HDL} \) by the ldlA[mSR-BI]
cells, but uptake by ldlA[N173Q] cells was almost the same as that by the untransfected controls. After correction for the differences in surface expression, it is clear that the cholesteryl ether uptake mediated by the 173 mutant was substantially less efficient than that by the wild-type receptor. As additional controls, we isolated stable transfectants of ldlA-7 cells expressing the 102, 116, 212, 227, 255, 288, 310, and 330 mutants and observed (data not shown) that the results for surface expression, \(^{125}\)I-HDL binding, and lipid uptake (\(^{3}\)H)CEt uptake from \(^{3}\)H)CEt-HDL were all similar to those obtained with the COS cells (Fig. 2).

The lower surface expression of the 173 mutant relative to that of wild-type mSR-BI raised the possibility that the reduced lipid uptake mediated by this mutant may have been a consequence of its reduced surface expression rather than of its altered intrinsic activity. This might arise if the expression level (concentration of receptors on the cell surface) can influence lipid transport activity. For example, this might occur if cell surface concentration-dependent oligomerization of receptor proteins were important for lipid transfer activity. This issue was addressed by analysis of the ldlA[mSR-BI] and ldlA[N173Q] cells using two-color flow cytometry that simultaneously measured on individual cells both surface expression of mSR-BI (binding of the KKB-1 antibody) and lipid uptake (DiI accumulation during a 2-h incubation with DiI-HDL at 37 °C). Fig. 5 shows flow cytograms in which the extent of KKB-1 binding, detected with a fluorescent secondary antibody, is shown on the horizontal axis and cellular DiI fluorescence (lipid uptake) is presented on the vertical axis. The arrows in panels A (ldlA[mSR-BI]) and B (ldlA[N173Q]) represent the surface expression to lipid uptake distribution observed in the ldlA[mSR-BI] cells (panel A). At every level of receptor surface expression above the untransfected cell background, lipid uptake was greater for the cells expressing the wild-type receptor than those expressing the 173 mutant (i.e. the distribution in panel B falls below the arrow). The boxes in panels A and B define sets of cells that exhibit almost identical average levels of surface expression (114 and 112 arbitrary units/cell, panel C). Despite similar levels of surface receptors, there was substantially greater uptake of DiI by the ldlA[mSR-BI] cells (panel C). Thus, differing mean levels of surface expression do not account for the differences in the efficiency of DiI uptake from DiI-HDL mediated by wild-type mSR-BI and the 173 mutant. Furthermore, immunofluorescence analysis of cells using the KKB-1 antibody did not show any gross differences in the surface distributions of the receptors on ldlA[mSR-BI] and ldlA[N173Q] cells (Fig. 6). Thus, in addition to reducing the level of cell surface expression of the receptor, the mutation at position 173 substantially reduced the intrinsic ability of the receptor to mediate selective uptake without reducing its intrinsic ability to bind HDL.

**Discussion**

The HDL receptor SR-BI has been shown to mediate physiologically relevant selective lipid uptake and, as a consequence, play an important role in lipoprotein-mediated cholesterol transport (2). Previous studies have established that the mature form of SR-BI has multiple (at least five) \(N\)-linked oligosaccharides, some of which are endo H-resistant (complex structure) and some of which are endo H-sensitive (high mannose or hybrid structures) (33). In the current study we used site-directed mutagenesis of each potential \(N\)-linked glycosylation site (extracellular Asn-X (not Pro)-Ser/Thr) on murine SR-BI and transient expression in COS cells to determine which sites were glycosylated and which, if any, of the glycosylation sites were required for cell surface expression and receptor function.

At each of the 11 potential \(N\)-linked sites, mutation of the essential Asn to Gln increased the electrophoretic mobility of the receptor, indicating that all 11 sites in the wild-type mSR-BI are \(N\)-glycosylated. In the two cases examined (posi-
Potential N-linked glycosylation sites in members of the CD36 superfamily of proteins

| Asn position | 102 | 108 | 116 | 173 | 212 | 227 | 255 | 288 | 310 | 330 | 383 |
|--------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| SR-BI        | 6/6 | 6/6 | 2/6 | 6/6 | 6/6 | 5/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 |
| CD36         | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 |
| LIMPII       | 0/3 | 3/3 | 0/3 | 0/3 | 0/3 | 3/3 | 3/3 | 3/3 | 0/3 | 0/3 | 0/3 |
| SNMP-1       | 0/4 | 2/4 | 0/4 | 0/4 | 4/4 | 4/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| Dros. M.     | 1/2 | 2/2 | 0/2 | 0/2 | 2/2 | 2/2 | 0/2 | 1/2 | 1/2 | 0/2 | 1/2 |
| C. elegans   | 0/1 | 1/1 | 0/1 | 1/1 | 1/1 | 0/1 | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 |

* Number of sequences from different species containing a potential N-linked oligosaccharide at this site/number of sequences from different species in the data base.

* Displaced by three amino acids.

N-Linked Glycosylation of SR-BI

SR-BI, murine, rat, hamster, bovine, porcine, human; CD36, murine, rat, hamster, bovine, rabbit, human; LIMPII, murine, rat, human; SNMP-1, bombyx, heliothis, manduca, moth; Dros. M., Drosophila melanogaster, EMP, croquemort; C. elegans, EST, GenBank Z54270. Alignment was performed using ClustalW (available at dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) (44).

As has been shown for other N-glycosylated proteins, it is likely that glycosylation of positions 108 and 173 in mSR-BI was important for folding in the ER and consequent transport to and through the Golgi, where the protein develops partial endo H resistance, and then to the cell surface (45). Glycosylation of these sites may also have affected the stability of the receptor. The relatively few molecules of the 108 and 173 mutants that were expressed on the cell surface could bind 125I
dHL at least as well as wild-type receptors, but were very inefficient at mediating cellular uptake of lipids (DiI, [3H]CEt) from HDL. Thus, the activities of these mutants are similar to those of the SR-BI homolog CD36, except CD36 is expressed at high levels on the surfaces of cells, such as transiently transfected COS cells (6, 22). Construction and characterization of SR-BI/CD36 chimeras established that differences in the extracellular loops of these two receptors, wherein the 108 and 173 sites reside, were responsible for the differences in their abilities to efficiently transport lipids (6, 22, 26). Indeed, although all six known SR-BI sequences have the potential glycosylation sites at positions 108 and 173, one has the site at position 173. Thus, it is possible that the oligosaccharides at these positions may not only be important for folding and export from the ER, they may contribute to the lipid transport process either by indirectly leading to the formation of key conformations (or conformational changes) of the receptor or perhaps by directly participating in the lipid transport process.

We detected using immunofluorescence no gross differences in the surface distributions of the wild-type and N173Q receptors expressed in stably transfected cells. Although this suggests that the relative amounts of receptor clustering in membrane microdomains, including caveolae (33), may not have been influenced by the altered glycosylation at this site, further ultrastructural and/or biochemical analyses will be required to determine if any glycosylation-dependent shifts in the distribution of the receptors might have influenced their lipid transport activity. Recent examination of the activity of essentially homogeneously pure mSR-BI reconstituted into phosphatidylcholine/cholesterol liposomes has shown that 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 (24). However, the effects on the glycosylation-dependent selective lipid uptake activity of differing membrane lipid compositions by mSR-BI, such as those of caveolae and the bulk plasma membrane, remain to be explored.

Additional studies will be required to determine precisely how mutations at sites 108 and 173 reduced the surface expression and lipid transport activity of mSR-BI without substantially altering its ability to bind HDL. The ability to uncouple the HDL binding and lipid transport activities of mSR-BI by in vitro mutagenesis should provide a powerful tool for further analysis of the mechanism underlying SR-BI-mediated selective lipid uptake.
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REFERENCES

1. Acton, S., Rigotti, A., Landschulz, K. T., Xu, S., Hobbs, H. H., and Krieger, M. (1998) Science 271, 518–520
2. Krieger, M. (1999) Ann. Rev. Biochem. 68, 523–588
3. Brown, M. S., and Goldstein, J. L. (1986) Science 232, 34–47
4. Glass, C., Pittman, R. C., Weinstein, D. B., and Steinberg, D. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5435–5439
5. Stein, Y., Dabach, Y., Hollander, G., Halperin, G., and Stein, O. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 7165–7170
6. Gu, X., Trigatti, B., Xu, S., Acton, S., Babitt, J., and Krieger, M. (1998) J. Biol. Chem. 273, 26338–26344
7. Liu, T., Krieger, M., Kan, H. Y., and Zannis, V. I. (2002) J. Biol. Chem. 277, 21576–21584
8. Ji, Y., Jiao, B., Wang, N., Sun, Y., Moya, M. L., Phillips, M. C., Rothblat, G. H., Swaney, J. B., and Tall, A. R. (1997) J. Biol. Chem. 272, 20982–20985
9. Gu, X., Kozarsky, K., and Krieger, M. (2000) J. Biol. Chem. 275, 29993–30001
10. de la Llera-Moya, M., Rothblat, G. H., Connelly, M. A., Kellner-Weibel, G., Sakr, S. W., Phillips, M. C., and Williams, D. L. (1999) J. Lipid Res. 40, 575–580
11. Rigotti, A., Trigatti, B. L., Penman, M., Rayburn, H., Herz, J., and Krieger, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12610–12615
12. Trigatti, B., Rayburn, H., Vinals, M., Braun, A., Miettinen, H., Penman, M., Hertz, M., Schrenzel, M., Amigo, L., Rigotti, A., and Krieger, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9322–9327
13. Kozarsky, K. F., Donahee, M. H., Rigotti, A., Iqbal, S. N., Edelman, E. R., and Krieger, M. (1997) Nature 387, 414–417
14. Ueda, Y., Geng, E., Royer, L., Cooper, P. F., Francone, O. L., and Rubin, E. M. (2000) J. Biol. Chem. 275, 20368–20373
15. Ueda, Y., Royer, L., Geng, E., Zhang, J., Cooper, P., Francone, O., and Rubin, E. (1999) J. Biol. Chem. 274, 7165–7171
16. Arai, T., Wang, N., Beznarowski, M., Welch, C., and Tall, A. R. (1999) J. Biol. Chem. 274, 2366–2371
17. Wang, N., Arai, T., Rinninger, F., and Tall, A. R. (1998) J. Biol. Chem. 273, 32920–32926
18. Kozarsky, K. F., Donahee, M. H., Glick, J. M., Krieger, M., and Rader, D. J. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 721–727
19. Mardones, P., Quinones, V., Amigo, L., Moreno, M., Miquel, J. F., Schwarz, M., Miettinen, H. E., Trigatti, B. L., Krieger, M., VanPatten, S., Cohen, D. E., and Rigotti, A. (2001) J. Lipid Res. 42, 170–180
20. Miettinen, H. E., Rayburn, H., and Krieger, M. (2001) J. Clin. Invest. 108, 1717–1722
21. Holm, T. M., Braun, A., Trigatti, B. L., Brugnara, C., Sakamoto, M., Krieger, M., and Andrews, N. C. (2002) Blood 99, 1817–1824
22. Connelly, M. A., Klein, S. M., Azhar, S., Abumrad, N. A., and Williams, D. L. (1999) J. Biol. Chem. 274, 41–47
23. Braun, A., Trigatti, B. L., Post, M. J., Sato, K., Simons, M., Edelberg, J. M., Rosenberg, R. D., Schrenzel, M., and Krieger, M. (2002) Circ. Res. 90, 270–276
24. Liu, B., and Krieger, M. (2002) J. Biol. Chem. 277, 34125–34135
25. Gu, X., Lawrence, R., and Krieger, M. (2000) J. Biol. Chem. 275, 9120–9130
26. Connelly, M. A., de la Llera-Moya, M., Monaco, P., Yancey, P. G., Drazul, D., Stoudt, G., Fournier, N., Klein, S. M., Rothblat, G. H., and Williams, D. L. (2001) Biochemistry 40, 5249–5259
27. Helenius, A. (1994) Mol. Cell. 3, 253–265
28. Opdenakker, G., Rudd, P. M., Ponting, C. P., and Dwek A. R. (1993) FASEB J. 7, 1330–1337
29. Imperiali, B., and Rickert, K. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 97–101
30. Krieger, M. (1983) Cell 33, 413–422
31. Acton, S. L., Scherer, P. E., Lodish, H. F., and Krieger, M. (1994) J. Biol. Chem. 269, 21065–21069
32. Ning, C., Dimauro, J. L., Lagoueux, M., Hoffmann, J., and Ezekowitz, R. A. (1998) Immunity 4, 431–442
33. Bai, M., Quinn, S., Trivedi, S., Kifor, O., Pearce, S. H., Pollak, M. R., Krappcho, K., Hieber, S. C., and Brown, E. M. (1996) J. Biol. Chem. 271, 19537–19545
34. Calvo, D., Dapalo, Z., and Vega, M. A. (1995) Genomics 25, 109–116
35. Oppedisano, P., Hundi, E., Lawler, J., and Seed, B. (1989) Cell 58, 95–101
36. Vega, M. A., Segui-Real, B., Garcia, J. A., Cales, C., Rodriguez, F., Vanderkruk, S., and Sandoval, I. V. (1991) J. Biol. Chem. 266, 16818–16824
37. Hart, K., and Wilcox, M. (1993) J. Mol. Biol. 234, 249–253
38. Krieger, M. (1997) J. Biol. Chem. 272, 13242–13249
39. Franz, N. C., Dimarcoq, J. L., Lagoueux, M., Hoffmann, J., and Ezekowitz, R. A. (1988) Immunity 4, 431–442
40. Franz, N. C., Heitzler, P., Ezekowitz, R. A., and White, K. (1999) Science 284, 1991–1994
41. Rogers, M. E., Sun, M., Lerner, M. R., and Vogt, R. G. (1997) J. Biol. Chem. 272, 14792–14799
42. Altschul, S., Madden, T., Senter, S., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. (1997) Nucl. Acids Res. 25, 3389–3402
43. Thompson, J. D., Higgins, D., and Gibson, T. (1994) Nucl. Acids Res. 22, 4673–4680
44. Rose, J. K., and Doms, R. W. (1988) Annu. Rev. Biochem. 4, 257–288
45. Varban, M. L., Rinninger, F., Wang, N., Fairchild-Huntress, V., Dunmore, J., Fang, Q., Gozoilo, M. L., Dixon, D. L., Deeds, J. D., Acton, S. L., Tall, A. R., and Huszar, D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4619–4624