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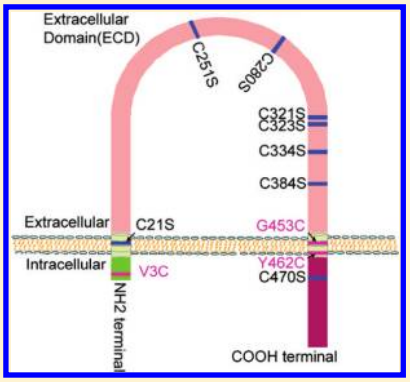
Differential Roles of Cysteine Residues in the Cellular Trafficking, Dimerization, and Function of the High-Density Lipoprotein Receptor, SR-BI

Jie Hu, Zhonghua Zhang, Wen-Jun Shen, Ann Nomoto, and Salman Azhar*

Geriatric Research, Education and Clinical Center (GRECC), VA Palo Alto Health Care System, Palo Alto, California 94304, United States, and Stanford University, Stanford, California 94305, United States

ABSTRACT: The scavenger receptor, class B, type I (SR-BI) binds high-density lipoprotein (HDL) and mediates selective delivery of cholesteryl esters (CEs) to the liver and steroidogenic cells of the adrenal glands and gonads. Although it is clear that the large extracellular domain (ECD) of SR-BI binds HDL, the role of ECD in the selective HDL-CE transport remains poorly understood. In this study, we used a combination of mutational and chemical approaches to systematically evaluate the contribution of cysteine residues, especially six cysteine residues of ECD, in SR-BI-mediated selective HDL-CE uptake, intracellular trafficking, and SR-BI dimerization. Pretreatment of SR-BI-overexpressing COS-7 cells with a disulfide (S−S) bond reducing agent, β-mercaptoethanol (100 mM) or dithiothreitol (DTT) (10 mM), modestly but significantly impaired SR-BI-mediated selective HDL-CE uptake. Treatment of SR-BI-overexpressing COS-7 cells with the optimal doses of membrane permeant alkyl methanethiosulfonate (MTS) reagents, positively charged MTSEA or neutral MMTS, that specifically react with the free sulfhydryl group of cysteine reduced the rate of SR-BI-mediated selective HDL-CE uptake, indicating that certain intracellular free cysteine residues may also be critically involved in the selective cholesterol transport process. In contrast, use of membrane impermeant MTS reagent, positively charged MTSET and negatively charged MTSES, showed no such effect. Next, the importance of eight cysteine residues in SR-BI expression, cell surface expression, dimer formation, and selective HDL-derived CE transport was evaluated. These cysteine residues were replaced either singly or in pairs with serine, and the mutant SR-BIs were expressed in either COS-7 or CHO cells. Four mutations, C280S, C321S, C323S, and C334S, of the ECD, either singly or in various pair combinations, resulted in significant decreases in SR-BI (HDL) binding activity, selective CE uptake, and trafficking to the cell surface. Surprisingly, we found that mutation of the two remaining cysteine residues, C251 and C384 of the ECD, had no effect on either SR-BI expression or function. Other cysteine mutations and substitutions were also without effect. Western blot data indicated that single and double mutations at C280, C321, C323, and C334 residues strongly favor dimer formation. However, they are rendered nonfunctional presumably because of mutation-induced formation of aberrant disulfide linkages resulting in inhibition of optimal HDL binding and, thus, selective HDL-CE uptake. These results provide novel insights into the functional role of four cysteine residues, C280, C321, C323, and C334, of the SR-BI ECD in SR-BI expression and trafficking to the cell surface, its dimerization, and associated selective CE transport function.



Scavenger receptor, class B, type I (SR-BI) is a cell surface glycoprotein with a molecular mass of ~82 kDa that mediates selective uptake of HDL-derived cholesteryl esters (CEs),[1−5] a process in which HDL-CE is taken into cells without parallel uptake and degradation of the HDL particle itself.[6−8] It is expressed most abundantly in the liver and steroidogenic cells of the adrenal gland and ovary,[9−12] where the level of selective uptake of HDL-CE is greatest. [Selective uptake is a major route for delivery of HDL-CE to steroidogenic tissues (for steroid hormone biosynthesis) and the liver (for bile acid synthesis) in rodents[1,2,7,8,13−17] and appears to be a major route for delivery in human steroidogenic cells, as well.[1,8,18,19]] SR-BI expression in steroidogenic cells of the adrenal gland and gonads is regulated by trophic hormones (gonadotropins and adrenocorticotropic hormones) and their second messenger, cAMP, coordinately with the selective uptake and steroidogenesis.[9−12,20,21] The functional expression of hepatic SR-BI, however, is primarily regulated post-transcriptionally via protein−protein interaction with a PDZ (PSD-95, Discs large, ZO-1) domain-containing protein, PDZK1.[22−24] It is of interest that in steroidogenic tissues, SR-BI is preferentially localized on microvilli[11,12,21] that form microvillar channels and constitute a microvillar compartment.[11,12,21,25−27] It is in these microvillar channels that HDL particles are trapped in an effort to boost the efficiency of the selective HDL-CE transport process.[25−27]

While significant progress has been made in understanding the regulation of SR-BI expression and function,[1−5,23,24,28] relatively

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Table 1. List of Primers

primer	sequence
C21S-Forward	5′-CTAGGGCTGCTGAGTGCTGCGCTCG-3′
C21S-Reverse	5′-CGAGCGCAGCACTCAGCAGCCCTAG-3′
C251S-Forward	5′-CTATTGGCATTCGGAACAGAGCAACATGATCAATGGTAC-3′
C251S-Reverse	5′-GTACCATTGATCATGTTGCTCTGTTCCGAATGCCAATAG-3′
C280S-Forward	5′-CTTCAGCCCAGAAGCCAGCAGATCTATGAAGCT-3′
C280S-Reverse	5′-AGCTTCATAGATCTGCTGGCTTCTGGGCTGAAG-3′
C321S-Forward	5′-CTAATGAAGGCTTCAGCCCGTGCCGCGAG-3′
C321S-Reverse	5′-CTCGCGGCACGGGCTGAAGCCTTCATTAG-3′
C323S-Forward	5′-GCTTCTGCCCGAGCCGCGAGTCC-3′
C323S-Reverse	5′-GGACTCGCGGCTCGGGCAGAAGC-3′
C334S-Forward	5′-CAGAATGTCAGCACCAGCAGGTTTGGTGCGC-3′
C334S-Reverse	5′-GCGCACCAAACCTGCTGGTGCTGACATTCTG-3′
C384S-Forward	5′-GGGATCCCCATGAACAGTTCCGTGAAGATGC-3′
C384S-Reverse	5′-GCATCTTCACGGAACTGTTCATGGGGATCCC-3′
C470S-Forward	5′-TACCAACTGCGCAGCCAGGAGAAAAGCTTTTTATTTTG-3′
C470S-Reverse	5′-CAAAATAAAAAGCTTTTCTCCTGGCTGCGCAGTTGGTA-3′
V3C-Forward	5′-ACGCGAACATGGGCTGCAGCTCCAGGGCAC-3′
V3C-Reverse	5′-GTGCCCTGGAGCTGCAGCCCATGTTCGCGT-3′
G453C-Forward	5′-YGCTGCTGGGGCTTGGATGCCTCCTGC-3′
G453C-Reverse	5′-GCAGGAGGCATCCAAGCCCCAGCAGCA-3′
Y462C-Forward	5′-GCTTCTGGTGCCCATCATTTGCCAACTGCGCA-3′
Y462C-Reverse	5′-TGCGCAGTTGGCAAATGATGGGCACCAGAAGC-3′

less is known about the structural requirement and contribution of various components of the SR-BI molecule in selective CE transport function. Previous studies have shown that the extracellular domain of SR-BI is essential for efficient HDL-CE uptake, but the C-terminal domain is also critical for the optimal selective uptake process.[29−31] Our recently published data provide evidence that the physical state of the SR-BI protein (i.e., monomeric, vs dimeric and higher-order oligomeric forms of SR-BI) and architectural changes in the cell surface induced by the expression of SR-BI also play major roles in the functional efficiency of the selective pathway.[12,32,33] In this study, we further examine the structure−function relationships and dynamics of SR-BI activity and focus our efforts on determining the structural and functional contributions of cysteine residues within SR-BI. We elected to examine the contribution of cysteine residues for the following reasons. (a) Cysteine residues are integral for inducing and maintaining the three-dimensional conformation in proteins by forming critical inter- and intramolecular disulfide bond linkages.[34,35] (b) Sulfhydryl (SH) side chains of cysteines are polar, similar to that of the hydroxyl group (OH) of serines, and can participate in hydrogen bonding interactions and facilitate protein−protein interactions.[36] (c) Cysteine side chains are preferred sites for various biological coupling and conjugation reactions such as palmitoylation, isoprenylation, disulfide cross-linking, and thiol−disulfide exchange that are known to play critical roles in intracellular protein trafficking, stability, and/or activity.[36−38] (d) The SR-BI contains several cysteine residues that are highly conserved across the species and uniquely distributed within the different domains of the SR-BI molecule and as such are highly likely to contribute to SR-BI structure and function (Table 1 and Figure 1).

We chose the rat SR-BI as a prototype for our studies because it contains more conserved cysteine sequences than SR-BI from any other species (Table 2). Its sequence contains a total of eight cysteine (C) residues (Figure 1 and Table 2). With the exception of C21, the seven remaining residues are highly conserved in other species, including mouse, hamster, rabbit, pig, cow, and human. Five residues (C280, C321, C323, C334, and C384) are clustered in the C-terminal half of the putative extracellular domain (ECD). The three remaining

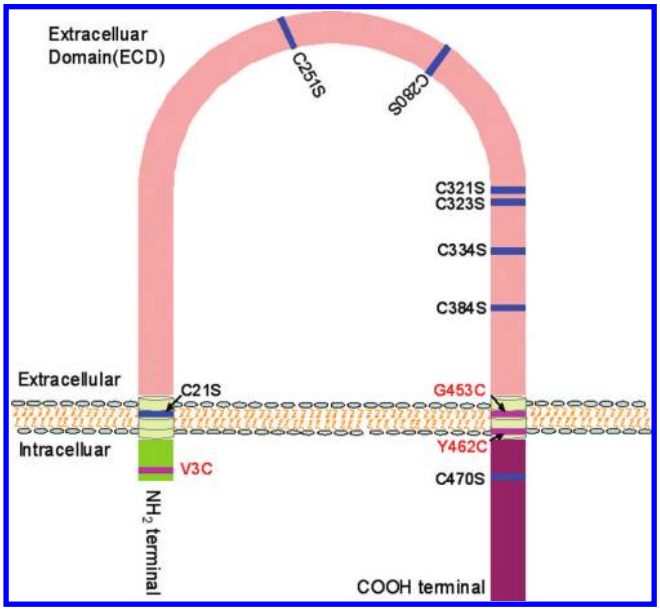

Figure 1. Schematic diagram showing the position of rat SR-BI cysteine residues that were subjected to site-directed mutagenesis and location of artificially created cysteines. Its sequence contains a total of eight cysteine (C) residues (C21, C251, C280, C321, C323, C334, C384, and C470). Five residues (C280, C321, C323, C334, and C384) are clustered in the C-terminal half of the putative extracellular domain (ECD). The three remaining cysteine residues are equally distributed in the N-terminal transmembrane domain (C21), the N-terminal half of the ECD (C251), and the C-terminal domain (C470). With the exception of C21, the seven remaining residues are highly conserved in other species, including mouse, hamster, rabbit, pig, cow, and human. Among the three artificially created cysteine residues, two (G453C and Y462C) were located in the C-terminal transmembrane domain, while the third (V3C) was in the N-terminal domain.

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Table 2. Positions of Cysteine Residues in Mouse, Rat, Hamster, Rabbit, Pig, Cow, Dog, Northern Tree Shrew, and Human SR-BI

	ND	NTD	ECD	ECD	ECD	ECD	ECD	ECD	CTD	CTD	CD
mouse (9)	–	C21	C251	C280	C321	C323	C334	C384	–	C462	C470
rat (8)	–	C21	C251	C280	C321	C323	C334	C384	–	–	C470
hamster (8)	–	C21	C251	C280	C321	C323	C334	C384	C453	–	C470
rabbit (8)	–	C21	C251	C280	C321	C323	C334	C384	–	–	C470
pig (8)	–	–	C251	C280	C321	C323	C334	C384	C453	–	C470
cow (8)	–	–	C251	C280	C321	C323	C334	C384	C453	–	C470
dog (12)[a]	–	–	C251	C280	C321	C323	C334	C384	C453	–	C470
tree shrew (9)	–	C21	C251	C280	C321	C323	C334	C384	C453	–	C470
human (11)	C3	C21	C251	C280	C321	C323	C334	C384	C453	C462	C470

[a]The predicted dog SR-BI sequence indicates the presence of four additional cysteine residues at positions 45, 53, 92, and 100 in the ECD. Positions of cysteine residues obtained from protein sequences using GenBank entries NM_016741, NP_113729.1, U11453, NM_001082788, NM_213967, DAA20570, XM_543366, EU379936, and NM_001082959 for mouse, rat, hamster, rabbit, pig, cow, dog, Northern tree shrew, and human, respectively. Abbreviations: ND, N-terminal domain; NTD, N-terminal transmembrane domain; ECD, extracellular domain; CTD, C-terminal transmembrane domain; CD, C-terminal domain.

cysteine residues are equally distributed in the N-terminal transmembrane domain (C21), the N-terminal half of the ECD (C251), and the C-terminal domain (C470). Given that the extracellular domain contains six conserved cysteine residues, these could theoretically form up to three disulfide bonds and, in turn, could help to stabilize the conformation of SR-BI, participate in its dimerization, or contribute to SR-BI-mediated selective HDL-CE uptake. Using a combination of mutational and chemical approaches, we provide evidence that four cysteine residues, C280, C321, C323, and C334, of the extracellular domain (ECD) are crucial for preserving normal SR-BI (HDL) binding activity, selective CE uptake, and trafficking to the cell surface. The mutation of the two remaining cysteine residues, C251 and C384 of the ECD, has no effect on either SR-BI expression or function. Other cysteine mutations and substitutions also had no effect.

EXPERIMENTAL PROCEDURES

Materials. ^{125}I radionucleotide (carrier-free, ~629 GBq/mg, ~17 Ci/mg) and [1α,2α(N)-^{3}H]cholesteryl oleolyl ether (1.78 TBq/mmol, 48.0 Ci/mmol) were obtained from GE Health Care/Amersham (Arlington Heights, IL). Molecular biology reagents were purchased from Roche (Basel, Switzerland), New England Biolabs (Ipswich, MA), Strategene (Agilant Technologies, La Jolla, CA), Bio-Rad (Hercules, CA), and Qiagen (Valencia, CA). Cell culture growth medium and antibiotics were obtained from either Sigma-Aldrich Corp. (St. Louis, MO) or Invitrogen (Life Technologies, Carlsbad, CA). Fetal calf serum was supplied by HyClone Laboratories (Logan, UT). Rabbit polyclononal antibody (NB400-134) against a peptide from the extracellular domain (amino acid residues 230−380) of SR-BI/SR-BII was purchased from Novus Biologicals (Littleton, CO). A rabbit polyclonal antibody raised against a peptide to the carboxyl terminus of mouse SR-BI (amino acid residues 489−509) was prepared as described previously;[20] this antibody cross-reacts with mouse, rat, and human SR-BI. The rabbit anti-calnexin polyclonal antibody (ER marker), goat anti-mouse IgG Alexa Fluor488, and goat anti-rabbit IgG Alexa Fluor568 were obtained from Abcam (Cambridge, MA). The sulfhydryl-specific alkylating MTS reagents, MTSEA, MTSES, MTSET, and MMTS were purchased from Toronto Research Chemicals, Inc. (Toronto, ON). Cholesteryl BODIPY FL C12 (BODIPY-CE) was supplied by Molecular probes/Life Technologies Corp. (Carlsbad, CA). All other reagents used were of analytical grade.

Site-Directed Mutagenesis of SR-BI. The role of disulfide bonds and cysteine residues of rat SR-BI in SR-BI expression and function was examined by systematically mutating to serine each of the cysteine residues within the extracellular and transmembrane domains. These mutations generated a complete series of SR-BI mutants having either single C21S, C251S, C280S, C321S, C323S, C334S, C384S, and C470S or paired C21S/C251S, C251S/C384S, C280S/C323S, C280S/C334S, C251S/C321S, C280S/C321S, C334S/C384S, C321S/C323S, and C323S/C334S mutations. Three artificial cysteine residues, V3C, G453C, and Y462C, were also introduced into the native rat SR-BI via mutagenesis. In addition, double and triple mutants, G453C/Y462C, G453C/Y462C/C470S, and G453C/Y462C/C470A, were created. All receptor mutations were integrated into the coding sequence of a rat SR-BI construct that was placed downstream of the constitutive cytomegalovirus enhancer/promoter in eukaryotic expression vector pcDNA6/V5-His as described previously.[32] Single-codon mutations were introduced into this rat SR-BI-V5-His/pcDNA template in whole-plasmid polymerase chain reactions using the QuickChange II site-directed mutagenesis kit supplied by Stratagene Inc. (La Jolla, CA) and appropriate sets of complementary forward and reverse mutagenic primers listed in Table 1. Multiple-codon exchanges were introduced into SR-BI-V5 coding sequences possessing an appropriate single mutation through successive rounds of QuickChange mutagenesis. The production of the correct mutations and the absence of coding errors in the SR-BI mutant constructs were confirmed by automated DNA sequencing. The primers for various cysteine mutations were designed using QuickChange Primer Design Program (Stratagene on-line software) and synthesized by Elim Biopharmaceuticals Inc. (Hayward, CA).

Cell Culture and Transfection. Two different cell lines, COS-7 and CHO, were employed. They were obtained from American Type Culture Collection (Manassas, VA), maintained as monolayers in DMEM containing 10% fetal bovine serum (Thermo Scientific Hyclone, Logan, UT), 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (complete culture medium) in a humidified atmosphere consisting of 5% CO_2 and 95% air, at 37 °C. The COS-7 or CHO cells were seeded into 35 mm culture dishes at a density

of 6×10^5 cells/well. When cells reached 70−80% confluence, they were transiently transfected with 2 μg of native (wild-type) DNA or one of the various cysteine mutant SR-BI plasmid DNAs in 250 μL of Opti-MEM I and 6 μL of Lipofectamine Transfection Reagent in 200 μL of Opti-MEM I according to the manufacturer's instructions. After 4 h, the dishes were washed with complete culture medium, and following the addition of 2 mL of the same medium to each dish, the cells were allowed to grow for an additional 44 h. As a control, some cells were transfected with an empty vector (control). All experiments were conducted 48 h after transfection.

Fluorescence-Activated Cell Sorting (FACS) Analysis. Detection of native and various cysteine mutant forms of SR-BI protein on the cell surface of the transfected CHO cells was performed by flow cytometry using a rabbit polyclonal anti-SR-BI/SR-BII antibody. CHO cells transiently transfected with either native rat SR-BI-V5 plasmid or one of the various SR-BI-V5 cysteine mutant constructs were washed twice with phosphate-buffered saline (PBS), and subsequently, the cell suspension was incubated with an anti SR-BI/SR-BII antibody (1:100 dilution of the stock antibody) on ice for 30 min. After being washed twice with PBS, cells were incubated with Alexa Fluoro488-conjugated anti-rabbit IgG (BD-Pharmingen, San Jose, CA) (1:1000 dilution) for an additional 30 min on ice. Subsequently, cells were washed twice with PBS, resuspended in PBS supplemented with 1% bovine serum albumin (BSA), and analyzed by multicolor flow cytometry (BD FACSCalibur flow cytometer, BD-Pharmingen, Sparks, MD) at the VA Palo Alto Flow Cytometry Core Facility. The data were analyzed using the accompanying software.

Uptake and Internalization of HDL-Derived Cholesteryl Esters. Transiently transfected CHO or COS-7 cells were incubated with a [^{125}I]DLT/[^{3}H]COE-hHDL$_3$ mixture (100 μg of protein/mL) for 5 h at 37 °C. At the end of the incubation, the dishes were washed and then solubilized in 2 mL of 0.1 N NaOH. Aliquots (1 mL) were precipitated with an equal volume of 20% trichloroacetic acid (TCA) to determine acid soluble and insoluble ^{125}I radioactivity or extracted with organic solvents to determine ^{3}H radioactivity.[13] Endocytic uptake is calculated from the TCA soluble ^{125}I label only. The difference between total and TCA soluble radioactivity is taken as the surface-associated [^{125}I]hHDL$_3$. Because both ^{125}I and ^{3}H labels are on the same particle, the amount of surface-bound ^{125}I is also equal to the amount of surface-bound ^{3}H. Thus, the total amount of ^{3}H minus the amount of surface-bound ^{3}H equals the total amount of ^{3}H internalized. To calculate "selective" uptake of CE, soluble ^{125}I radioactivity is subtracted from soluble ^{3}H radioactivity. Finally, to calculate the mass of CE internalized, these values are divided by a protein:cholesterol ratio of hHDL$_3$ (i.e., 2.70).

For some studies, HDL-CE uptake was also measured using a fluorometric technique.[39] Transiently transfected CHO or COS-7 cells were incubated with *rec*-HDL-BODIPY-CE (10 μg of protein/mL) for 1−3 h at 37 °C, and following incubation, dishes were rapidly washed five times in phosphate-buffered saline and 0.1% bovine serum albumin at 0−4 °C and their contents extracted with a hexane/isopropyl alcohol mixture (3:2, v/v) as described previously.[39] In each case, a portion of hexane/isopropyl alcohol extract was transferred to a fluorescent plate and the fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 520 nm using a fluorescent plate reader (Spectra MAX Gemini, Molecular Devices). The results are expressed as arbitrary units per milligram of protein or microgram of DNA and represent total BODIPY-CE uptake by the transiently transfected cells.

PFO−, SDS−, or Triton X-100−PAGE/Western Blotting. We used a detergent−PAGE electrophoresis technique to determine the relative efficacy of three detergents, PFO, SDS, and Triton-X-100, on monomer, dimer, and oligomer forms of SR-BI. Among these, PFO, being a weak detergent, gently disrupts membranes while maintaining the oligomeric structure of membrane proteins before and during electrophoresis.[40] CHO cells transiently transfected with rat SR-BI-V5 for 36 h were lysed by sonication in lysis buffer [its common components being 125 mM Tris-HCl (pH 6.8), 5% glycerol, 10 μg/mL leupeptin, 1 mM PMSF, 20 μg/mL aprotenin, and 5 μg/mL pepstatin] containing 2% NaPFO, SDS, or Triton X-100 and clarified by centrifugation. Likewise, a highly purified double-membrane plasma membrane preparation from luteinized rat ovary[25] was solubilized in lysis buffer containing 2% SDS and centrifuged briefly to sediment insoluble material. In some cases, SDS extracts were treated with 50 mM dithiothreitol (DTT) at 95 °C for 2 min. Suitable aliquots (10−15 μg protein) of each sample were loaded on SDS-free gels, electrophoretically separated using Tris-glycine buffer without detergent, transferred to an Immobilon-P membrane (Millipore, Bedford, MA), and probed with rabbit antipeptide (C-terminal peptide) rat SR-BI antibody[20] followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma). The bands were visualized using ECL Plus Western blotting detection reagents (GE Healthcare, Piscataway, NJ).

Western Blotting Analysis. Expression levels of native (wild-type) and cysteine mutant SR-BI receptors were estimated by Western blot analysis of total cellular lysates. Transiently transfected cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and lysed in cell lysis buffer [125 mM Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, 10 μg/mL leupeptin, 1 mM PMSF, 20 μg/mL aprotenin, and 5 μg/mL pepstatin]. The protein content of cell lysates was determined using a modification[41] of the procedure of Lowry et al.[42] SDS−PAGE/Western blotting measurements were performed both under reducing and nonreducing conditions. Aliquots of samples (10 μg) were mixed with 0.01% bromophenol blue (nonreducing conditions), or 3 μg samples were mixed with 0.01% bromophenol blue and 50 mM DTT (reducing conditions). All samples were heated at 95 °C for 5 min, subjected to SDS−PAGE (4 to 20% premade protein gels, Thermo Fisher Scientific, Rockford, IL), and transblotted to nitrocellulose membranes. Expression of the V5 tag was detected using a 1:5000 dilution of anti-V5 tag monoclonal antibody (Invitrogen Life Technologies, Carlsbad, CA). The polyclonal rabbit antipeptide (C-terminal peptide) rat SR-BI antibody generated in house[20] was used at a dilution of 1:1000 to detect the expression of SR-BI protein itself. Gel loading of samples was detected using a 1:1000 dilution of the anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Following incubation of the membranes with the primary antibodies, detection of bands was performed using IRDye 680 goat anti-rabbit IgG (H + L) and IRDye goat anti-mouse IgG (H + L) secondary antibodies and the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Quantification of the infrared signals was performed using the Odyssey system software. The quantitative numbers shown under the 2% SDS plus (+) 50 mM DTT bands (Figure 7A,B) represent expression levels of SR-BI normalized to β-actin, i.e., SR-BI:β-actin ratio, where the native (wild-type) SR-BI:β-actin ratio equals 1.00.

The numbers shown under the 2% SDS and minus (−) 50 mM DTT bands represent the percent expression of SR-BI dimers. These numbers were derived by dividing the intensity of the SR-BI dimer band by the combined intensities of the SR-BI dimer and monomer bands, which equals the percent dimer

Confocal Immunofluorescence Microscopy. CHO cells were seeded into six-well tissue culture plates containing sterilized poly-D-lysine (Sigma)-treated 25 mm round glass coverslips and transiently transfected with 1.0 μg of native (wild-type) or mutant (C280S, C321S, C323S, C334S, and C280S/C334S) SR-BI-V5 DNA according to the aforementioned transfection protocol. After 48 h, transfected cells were washed with phosphate-buffered saline, fixed for 15 min in a freshly prepared solution of 4% paraformaldehyde in PBS, washed twice with PBS, and then permeabilized with 0.1% Triton X-100 in PBS for 10 min. After being incubated with blocking solution (5% normal goat serum and 5% nonfat dry milk in PBS) for 1 h, the cells were incubated with the anti-V5 mouse monoclonal antibody (SR-BI-V5 fusion protein) or anti-peptide SR-BI rabbit polyclonal antibody[20] together with the anti-calnexin rabbit polyclonal antibody (ER membrane marker) overnight at 4 °C. Cells were then washed and treated with goat anti-mouse IgG Alexa Fluor488 and goat anti-rabbit IgG Alexa Fluor568 in blocking solution for 45 min at room temperature, and after the cells had been washed, coverslips were mounted on microscope slides using Fluoromount G (Fisher Scientific, Pittsburgh, PA). All steps were conducted at room temperature. The labeled cells were viewed and photographed with a laser scanning confocal microscope (Zeiss LSM 510 Meta Confocal Microscope, Zeiss, Jena, Germany). Images were collected on the confocal microscope using LSM510 software and assembled into figures using Photoshop (Adobe, San Jose, CA).

Treatment of SR-BI-Expressing COS-7 Cells with Sulfhydryl Reagents, Methanethiosulfonate Compounds, β-Mercaptoethanol, and Dithiothreitol. To study the effect of MTS compounds on selective HDL-CE uptake, we treated COS-7 cells transiently overexpressing SR-BI (48 h post-transfection) with either vehicle (control), MTSEA (positively charged), MTSET (positively charged), MTSES (negatively charged), or MTSM (neutral) (final concentration of 1 mM) at 37 °C for 5 min. The reaction was stopped when the cells were washed with ice-cold PBS. Cells were then incubated in the culture medium containing 10 μg/mL HDL-BODIPY-CE at 37 °C for 2 h. After the cells had been washed with cold PBS, the total intracellular fluorescence was quantified.

Lipoprotein Preparation. Human apoE-free high-density lipoprotein$_3$ (hHDL$_3$) was isolated as described previously.[31] hHDL$_3$ was used exclusively because it is not recognized by the LDL (B/E) receptor-mediated endocytic pathway. For uptake and internalization studies, hHDL$_3$ preparations were conjugated with residualizing labels, i.e., ^{125}I-labeled dilactitol tyramine (DLT) and [^{3}H]cholesteryl oleolyl ether (COE).[13] Reconstituted HDL-BODIPY-cholesteryl ester particles (*rec*-HDL-BODIPY-CE) were used for the fluorometric determination of HDL-CE uptake and for confocal microscopic localization of HDL-derived cellular CEs. *rec*-HDL-BODIPY-CE was prepared as described previously.[39] The protein content of the HDL preparations was determined by a modification[43] of the procedure of Lowry et al.[42]

Statistical Analysis. Results are expressed as means ± the standard error of at least three independent experiments. Differences between two groups were evaluated using the Student's *t* test and GraphPad Prism version 4.0 for Windows (GraphPad Software Inc., San Diego, CA); differences among multiple groups were evaluated with a one-way analysis of variance followed by a post hoc Dunnett's test. *p* values of <0.05 were considered to be statistically significant.

RESULTS

Homo-Oligomeric SR-BI. Initially, we determined the effects of three different detergents (SDS, Triton X-100, and NaPFO) and DTT, a sulfhydryl reducing agent, on the stability and effective separation of multimeric forms of SR-BI by PAGE/Western blotting. Treatment of cells or isolated membranes with SDS results in efficient solubilization of membrane-bound proteins, and often solubilized proteins maintain their oligomeric forms following resolution by SDS–PAGE.[44−46] The poly(oxyethylene) series of nonionic detergents such as Triton X-100 is widely used for solubilization of membrane proteins because of its inoffensiveness toward membrane proteins with little or no undesirable effect on the stability of protein oligomers.[47] The weak detergent NaPFO is also known to gently disrupt the membranes while at the same time helps to maintain weak interactions between proteins as well as the oligomeric state of proteins before and during electrophoresis.[48] CHO cells transiently overexpressing rat SR-BI were solubilized in 2% SDS, 2% Triton X-100, or 2% NaPFO, and suitable aliquots were subjected to polyacrylamide gel electrophoresis as described above. Similarly, SR-BI dimer-enriched double-membrane luteinized plasma membranes were solubilized in 2% Triton X-100–SDS, exposed or not exposed to 50 mM DTT, and subjected to PAGE. As shown in Figure 2, Western

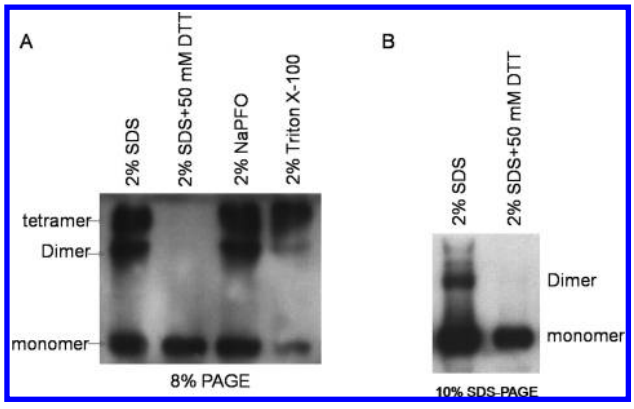

Figure 2. Analysis of multimerization of rat SR-BI. (A) CHO cells transiently transfected with rat SR-BI-V5 plasmid for 36 h were lysed in lysis buffer containing 2% SDS, NaPFO, or Triton X-100. After samples had been heated with or without 50 mM DTT at 95 °C for 2 min, suitable aliquots (15 μg of protein) were separated via 8% PAGE and immunoblotted with the rabbit anti-peptide (C-terminal peptide) rat SR-BI antibody as described in Experimental Procedures. (B) Highly purified double-membrane plasma membrane preparations from luteinized rat ovaries were solubilized in lysis buffer containing 2% SDS and heated at 95 °C for 2 min in the presence or absence of 50 mM DTT, and suitable aliquots were subjected to 10% PAGE. Following transfer, membranes were immunoblotted with the rabbit anti-peptide (C-terminal peptide) rat SR-BI antibody probed with the rabbit anti-peptide (C-terminal peptide) rat SR-BI antibody[20] followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma). The bands were visualized using ECL Plus Western blotting detection reagents (GE Healthcare) as described in Experimental Procedures. Arrows indicate the bands corresponding to monomeric, dimeric, and tetrameric forms of SR-BI. The blot shows that rat SR-BI dimers or oligomers are resistant to the types and presence of detergent but sensitive to the sulfhydryl reducing agent, dithiothreitol.

blot analysis of the detergent extracts from rat SR-BI-overexpressing CHO cells resolved via nonreducing SDS−PAGE indicates the presence of monomeric, dimeric, and tetrameric immunoreactive bands. Interestingly, all three detergents yielded almost identical results, suggesting that oligomeric forms of SR-BI are relatively resistant to the types and the presence of detergent (Figure 2A). In contrast, pretreatment of SDS extracts with DTT (reducing conditions) led to a complete dissociation of the large oligomeric forms of SR-BI to a single monomeric band, suggesting that the higher-order complexes potentially require disulfide bonding. Similar results were obtained using luteinized ovary plasma membranes; i.e., extract prepared in 2% SDS alone showed the presence of monomeric, dimeric, and oligomeric forms of SR-BI bands, whereas a single monomer band was visible when a DTT-treated SDS sample was used.

Effects of Disulfide Bond Reducing Agents and Sulfhydryl Recative Alkyl Methanethiosulfonate (MTS) Reagents on SR-BI-Mediated Selective HDL-CE Uptake. Rat SR-BI contains a total of eight cysteine residues, of which six conserved residues in the extracellular domain have the potential to form three interdisulfide bonds in the SR-BI monomer or intradisulfide bonds in the SR-BI dimers and higher-order oligomers (Table 2). To assess whether any disulfide (S−S) bond present in the extracellular domain is involved in SR-BI function, we examined the effects of chemical (β-mercaptoethanol or dithiothreitol) reduction of SR-BI on selective HDL-CE uptake.[36] We reasoned that if critical disulfide (S−S) bonds present within the extracellular domain of the SR-BI are required for selective CE transport, then chemical reduction of cell surface SR-BI should be associated with a defect in SR-BI function. Thus, selective HDL-CE uptake measurement was performed on SR-BI-overexpressing COS-7 cells pretreated with or without the optimal concentrations of β-mercaptoethanol or DTT, which breaks sulfhydryl (S−S) bonds and maintains cysteine residues in a reduced state. As shown in Figure 3, exposure of SR-BI-overexpressing COS-7 cells to 100 mM β-mercaptoethanol or 10 mM DTT for 30 min followed by quick washes modestly but significantly impaired the SR-BI-mediated selective uptake of HDL-BODIPY-CE. Under the experimental conditions employed, DTT was modestly more effective than β-mercaptoethanol in causing a reduction in the rate of selective HDL-BODIPY-CE uptake. Western blot analysis of cell lysates from β-mercaptoethanol- or DTT-pretreated cells demonstrated the near-complete absence of dimer and oligomer forms of SR-BI and an elevated pool of SR-BI monomer indicative of reduction of S−S bonds to SH. These results thus suggest that the optimal functioning of SR-BI-mediated selective HDL-CE uptake requires the presence of certain disulfide bonds.

The contribution of cysteine (SH) residues in SR-BI-mediated selective HDL-CE uptake was also determined with the use of alkyl methanethiosulfonate (MTS) reagents that specifically react with the free sulfhydryl group of cysteine[49] and have been used widely to probe the role of cysteine residues in the structure and function of a number of proteins, including ion channels, transporters, and receptors.[50−56] We reasoned that if an endogenous cysteine residue in SR-BI is involved in ligand (HDL) binding, covalent modification with MTS reagents will potentially block the binding of HDL to SR-BI and, consequently, inhibition of selective delivery of HDL-CE. Here we utilized four cysteine reactive MTS reagents, MTSEA, MTSET, MTSES, and MMTS, to determine their

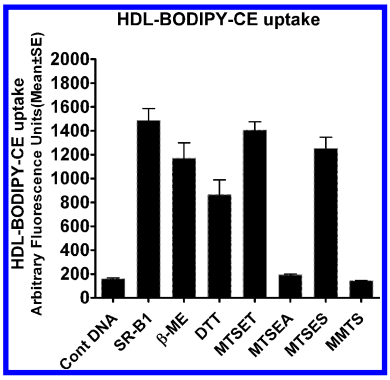

Figure 3. Effects of reducing agents and MTS reagents on the ability of SR-BI to mediate selective HDL-CE transport. Intact COS-7 cells transiently expressing rat SR-BI were preincubated with freshly prepared DTT (10 mM) or β-mercaptoethanol (100 mM) at 37 °C for 30 min. Likewise, some SR-BI-overexpressing COS-7 cell dishes were pretreated with freshly prepared MTSEA, MTSET, MTSES, or MMTS (1 mM) for 5 min. After a few washes, the intact cells were then incubated for 5 min at 37 °C with HDL-BODIPY-CE, and selective HDL-BODIPY-CE uptake by the COS-7 cells was quantified by the measurement of fluorescence as described in Experimental Procedures. Each bar represents the mean ± the standard error of data obtained from at least three independent experiments. As one can see, no significant change in HDL-BODIPY-CE uptake was observed when SR-BI-expressing COS-7 cells were treated with disulfide (S−S) bond reducing agent β-mercaptoethanol or DTT. In contrast, treatment of SR-BI-expressing cells with cysteine (SH) reactive alkyl methanethiosulfonate (MTS) reagents, MTSEA, MTSET, and MMTS, completely blocked the SR-BI-mediated selective HDL-BODIPY-CE uptake, whereas cells were relatively insensitive to treatment with the negatively charged MTSES. For SR-BI-V5 alone vs that with β-mercaptoethanol (β-ME), p = 0.00715. For SR-BI-V5 alone vs that with dithiothreitol (DTT), p = 0.00458. For SR-BI-V5 alone vs that with MTSEA, p = 0.00017. For SR-BI-V5 alone vs that with MTSES, p = 0.08908 (NS). For SR-BI-V5 alone vs that with MMTS, p = 0.0.00014. For SR-BI-V5 alone vs that with MTSET, p = 0.316155 (NS).

effects on SR-BI-mediated selective HDL-CE uptake in SR-BI-overexpressing COS-7 cells. Among these, the water-soluble and largely positively charged MTSEA is the most commonly used MTS reagent, which converts the neutral cysteine side chain to a positively charged group resembling a lysine side chain. At the extracellular pH of 7.4 in the incubation medium, a significant fraction of MTSEA is unprotonated and membrane permeable.[57] MTSET, like MTSEA although it carries a fixed positive charge, is not expected to cross the cell membrane. MTSES, being negatively charged, does not enter into the cell interior. Thus, both MTSET and MTSES are membrane-impermeant. MMTS is a small and uncharged (neutral) MTS compound, which crosses the cell membrane readily and, thus, could potentially interact with accessible residues on either the cytoplasmic or extracellular domain of the SR-BI. Figure 3 shows that treatment of SR-BI-overexpressing COS-7 cells with optimal doses of MTSEA and MMTS completely blocked SR-BI-mediated selective HDL-BODIPY-CE uptake, indicating that free cysteine residues are critically involved in the selective cholesterol transport process. Treatment with a negatively charged MTSES, however, had no significant effect on the selective delivery of HDL-BODIPY-CE to the cell interior of SR-BI-expressing COS-7 cells. Likewise, the positively charged but membrane impermeable MTSET also showed no effect on

selective uptake of HDL-BODIPY-CE in SR-BI-expressing COS-7 cells.

Construction of SR-BI Forms Having Single, Paired, and Triple Cysteine Mutations or Introduced Cysteine Residues. Given that sulfhydryl reducing agents, β-mercaptoethanol, DTT, and three MTS reagents, had a significant effect on selective HDL-CE transport function, we directly and individually tested the role of eight cysteine residues, including six highly conserved residues in the extracellular domain, in SR-BI function. To accomplish this, we constructed SR-BI mutant receptors, each of which contained a serine substituted for one of the eight cysteines (C21, C251, C280, C321, C323, C334, C384, and C470) in rat SR-BI, and assessed the functional implications of the loss of each individual cysteine. In addition, we examined the effects of replacing certain pairs of cysteine residues on the expression and function of SR-BI (Table 3). We used serine instead of alanine substitution because of the better structural match between the alcohol and sulfhydryl side chains of serine and cysteine, respectively.[58] To establish specificity, some key observations were also confirmed by employing alanine substitution of certain cysteine residues. We also tested the function of artificially creating one of the three cysteine residues in rat SR-BI that additionally exist in human SR-BI/CLA-I (Table 2). Finally, two triple mutants were created in which Gly453 was mutated to cysteine, Tyr462 to Cys, and Cys470 to Ala (first mutant) or Ser (second mutant).

Characterization of Cysteine Mutants. Initially, fluorescence-activated cell scanning (FACS) analysis using a polyclonal anti-SR-BI/SR-BII extracellular domain-specific antibody was used to assess the cell surface expression of native and mutant SR-BI proteins. As shown in Figure 4, several single and paired mutations behaved differently with respect to cell surface expression. Single-cysteine mutants C280S, C321S, C323S, and C334S and paired cysteine mutants C280S/C321S, C280S/C334S, C321S/C323S, and C323S/C334S of the extracellular domain exhibited decreases in the level of cell surface expression of SR-BI ranging from ~20 to ~80%, when expressed in CHO cells. Likewise, CHO cells transfected with double-cysteine mutants, C251S/C321S, C280S/C323S, and C334S/C384S, showed only a 30−50% cell surface expression of the mutant SR-BI as compared to the expression of native (wild-type) SR-BI (data not shown). Two of the single-cysteine mutants (C321S and C323S) showed ~40% cell surface expression of wild-type SR-BI, while C280S and C334S exhibited ~50 and ~60% of the expression of the wild type, respectively (Figure 4A). A comparison of various double-cysteine mutants indicated that two double mutants, C280S/C312S and C323S/C334S, gave the lowest level of cell surface expression of SR-BI (~20% of that of the wild type), suggesting that these four cysteine residues are critically involved in the translocation of SR-BI from the cell interior to the cell surface. Interestingly, the use of C280S/C334S and C321S/C323S double mutants showed somewhat improved cell surface expression of SR-BI compared to the expression noted with the use of C321S and C323S mutants alone. Additional mutations targeting Cys21 (C21S), Cys251 (C251S), Cys384 (C384S), and Cys470 (C470S or C470A) or introduction of new cysteine residues (V3C, G453C, or Y462C) did not significantly alter the cell surface expression of SR-BI. Likewise, various combinations of other double- and triple-cysteine mutants also had no effect on the cell surface localization of SR-BI (Figure 4). To further confirm the reduced level of cell surface expression of representative single (C280S) and double (C280S/C321S) mutants of SR-BI, we also examined

Table 3. Effects of Cysteine Mutations and Substitutions of SR-BI on Selective HDL-CE Uptake by COS-7 or CHO Cells[a]

SR-BI	selective HDL-[^{3}H]COE uptake in COS-7 cells (ng of HDL/mg of protein)	selective HDL-BODIPY-CE uptake in CHO cells (fluorescence units)
vector alone[b]	105.25 ± 14.77	106.23 ± 3.53
wild type[c]	500.24 ± 36.92	363.22 ± 37.38
V3C	617.49 ± 40.24	293.57 ± 31.84
C21S	591.71 ± 58.48	267.60 ± 15.37
C251S	525.25 ± 74.50	310.02 ± 43.30
C280S	370.41 ± 55.11	123.60 ± 3.49
C321S	344.61 ± 16.58	111.14 ± 15.60
C323S	240.53 ± 53.00	92.82 ± 5.00
C334S	340.20 ± 60.46	176.78 ± 16.34
C384S	675.55 ± 106.20	242.00 ± 18.23
G453C	550.21 ± 28.62	324.33 ± 32.86
Y462C	605.00 ± 67.23	316.67 ± 36.39
C470S	565.20 ± 16.16	243.08 ± 24.01
C470A	663.85 ± 65.27	250.63 ± 14.11
C21S/C251S	643.79 ± 43.25	246.80 ± 8.00
C251S/C384S	320.10 ± 39.23	229.15 ± 17.33
C280S-C323S	275.32 ± 60.20	83.75 ± 1.72
C280S/C334S	365.00 ± 50.20	121.73 ± 8.06
C321S/C323S	216.88 ± 58.25	132.76 ± 21.00
C323S/C334S	547.29 ± 36.64	82.84 ± 1.04
G453C/Y462C	519.13 ± 54.58	184.24 ± 4.42
G453C/Y462C/C470S	530.20 ± 75.21	185.18 ± 6.57
G453C/Y462C/C470A	375.45 ± 46.10	204.85 ± 5.42

[a]COS-7 cells or CHO cells were transiently transfected with vector alone, V5 rat SR-BI, or the indicated mutants. Thirty-six hours post-transfection, COS-7 cells were incubated with culture medium containing [^{125}I-DLT]-[^{3}H]COE-labeled hHDL$_3$ (50 μg of protein/mL) for 5 h. Following incubation, the cells were processed for the quantification of selectively internalized HDL-[^{3}H]COE as described in Experimental Procedures. Likewise, 36 h after transfection, CHO cells were incubated with *rec*-HDL-BODIPY-CE (10 μg of protein/mL) for 2 h and, subsequently, processed for the fluorometric determination of selectively internalized HDL-BODIPY-CE as detailed in Experimental Procedures. The results are means ± the standard error of at least three or four independent experiments. [b]PcDNA6-V5HisB. [c]SR-BI-V5.

the surface expression of these mutants by confocal immunofluorescence microscopy. Figure 5A shows that, as expected, the level of cell surface localization of the C280S and C280S/C321S SR-BI proteins monitored by confocal microscopy was significantly reduced compared to that of wild type SR-BI. These results complement the FACS data (Figure 4A) and provide additional confirmation for the reduced level of cell surface expression of these two cysteine mutant SR-BIs.

Because four single (C280S, C321S, C323S, and C334S) and four double (C280S/C321S, C280S/C334S, C321S/C323S, and C323S/C334S) mutants showed significant decreases in their levels of cell surface expression (Figures 4 and 5), we

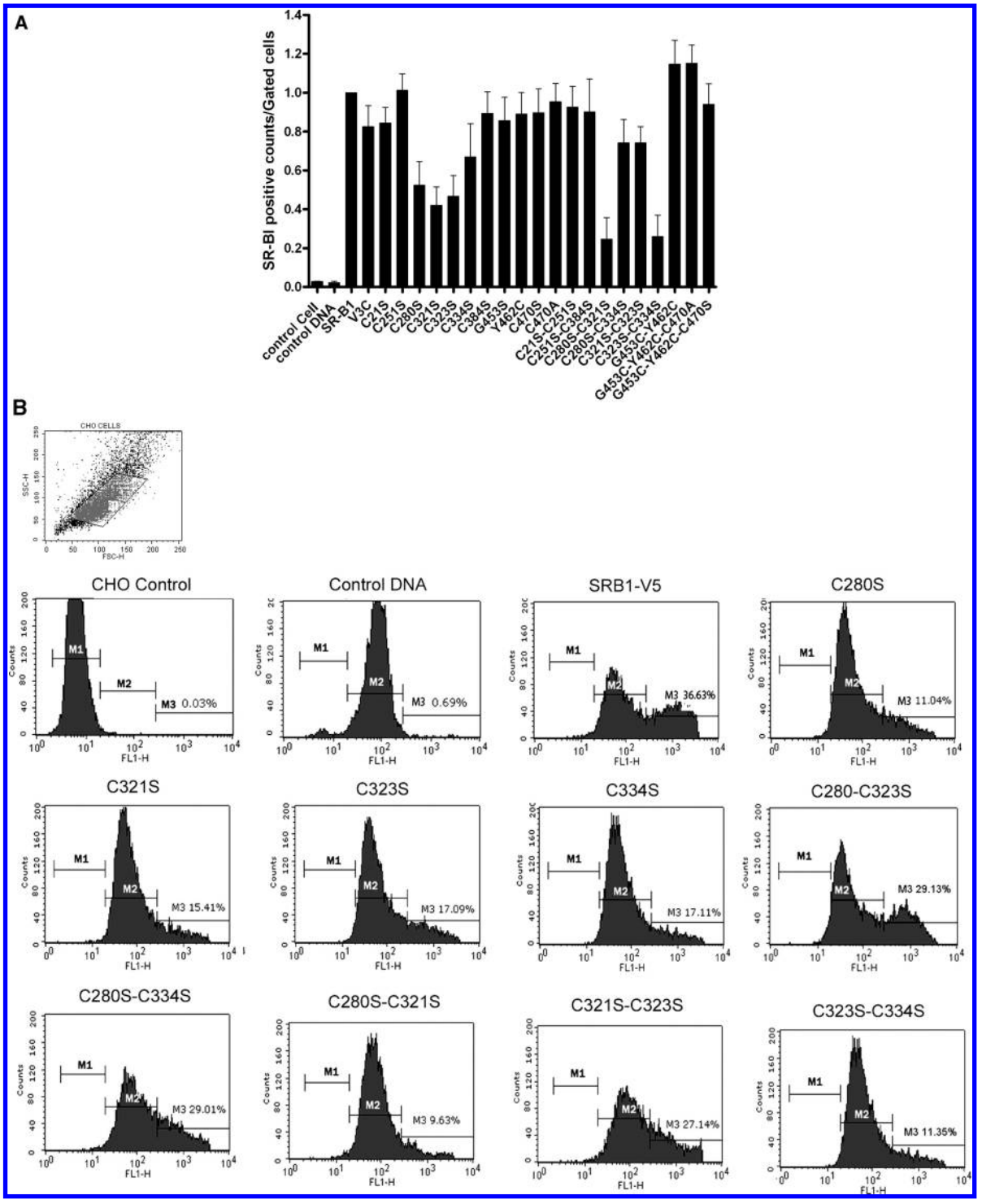

Figure 4. FACS analysis of CHO cells transiently overexpressing wild-type rat SR-BI or its cysteine mutants. (A) Cell surface expression of rat SR-BI and its cysteine mutants or rat SR-BI with introduced cysteines. CHO cells were transiently transfected with empty vector (mock), V5-tagged rat SR-BI, or the indicated mutants. The cells were then incubated with a primary polyclonal SR-BI (ECD) antibody on ice for 30 min. After being washed with PBS, cells were incubated with Alexa Fluoro488-conjugated anti-rabbit IgG for 30 min on ice. The level of cell surface expression of each construct was estimated by flow cytometry. The results are the mean ± the standard error of three independent experiments. (B) Representative results showing expression of wild-type rat SR-BI or its various cysteine mutants as indicated. The number of cells was estimated by flow cytometry.

wanted to determine whether such decreases were due to the defective transport of proteins to the cell surface or simply due to differences in their protein expression. To address these two possibilities, we first examined the total intracellular expression and localization of some of these SR-BI mutants (C280S, C321S, or C280S/C321S) as V5-tagged fusion proteins by confocal immunofluorescence microscopy. Transiently transfected CHO cells were permeabilized and assayed for SR-BI expression using an anti-V5 antibody (Sr-BI-V5 fusion protein). As shown in Figure 6, a very low level of diffuse fluorescence was observed when either mock or control DNA-transfected CHO cells were incubated with monoclonal mouse anti-V5 IgG

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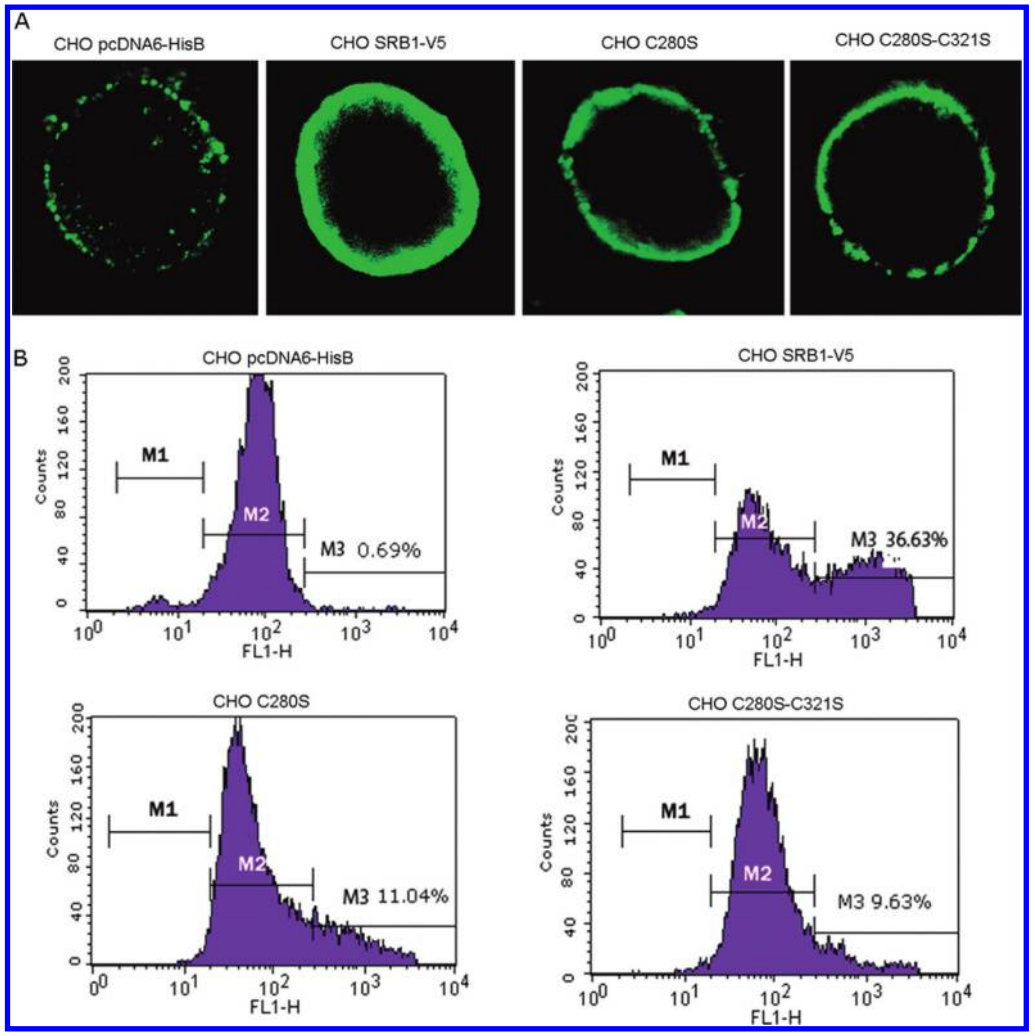

Figure 5. Comparison of the surface expression of the wild type and SR-BI with a single cysteine mutation at position 280 (C280S) or double mutations at positions 280 and 321 (C280S/C321S) by confocal immunofluorescence microscopy and FACS analysis. (A) Cell surface localization of wild-type and single-cysteine (C280S) and double-cysteine (C280S/C321S) mutant SR-BI by confocal immunofluorescence microscopy. CHO cells were transiently transfected with V5-tagged SR-BI, C280S SR-BI, or C280S/C321S SR-BI, and 36 h after being transfected, the cells were fixed, treated with primary and secondary antibodies, mounted, and analyzed by confocal microscopy as described in Experimental Procedures. (B) Cell surface expression of wild-type and single-cysteine (C280S) and double-cysteine (C280S/C321S) mutant SR-BI analyzed by flow cytometry. CHO cells were transiently transfected with either wild-type SR-BI, C280S SR-BI, or C280S/C321S SR-BI for 36 h and subsequently analyzed for cell surface expression by flow cytometry as described in Experimental Procedures.

antibodies. In contrast, staining of wild-type, C280S, C321S, or C280S/C321S SR-BI-transfected cells with anti-V5 antibodies resulted in fluorescence next to and surrounding part of the nucleus, a pattern typical of ER localization. As further evidence that overexpressed SR-BI proteins preferentially accumulate in the ER, SR-BI-transfected cells express either native or mutant protein with an antibody specific for the ER membrane protein, calnexin, the calcium binding protein. As shown in Figure 6, there was a colocalization of the SR-BI protein with calnexin in cells transfected with either the native form or one of the three mutant SR-BI constructs. Likewise, the total level of intracellular SR-BI expression (as measured by the intensity of immunofluorescence) was not affected by the C280S mutation, the C321S mutation, or both (Figure 6). These results were further complemented by Western blotting analysis that showed equivalent expression of total SR-BI protein in cells transfected for wild-type or various mutant SR-BIs under both reducing and nonreducing conditions (Figure 7A,B). Thus, the loss of cell surface expression seen with C280S, C321S, and C280S/C321S mutants or other cysteine mutants (C323S, C334S, C280S/C334S, C321S/C323S, and C323S/C334S) cannot be attributed to the decreased level of SR-BI protein expression or increased level of intracellular degradation. Because we were dealing with an overexpressing system, most of the expressed SR-BI protein was localized intracellularly with only a small fraction of even native (wild-type) SR-BI transported to the cell surface. When considered together, even >80% inhibition of cell surface localization of SR-BI should not be expected to alter intracellular levels in any significant way.

Effects of Cysteine Mutations and Substitutions on HDL Binding and Selective HDL-CE Uptake. To examine the role of cysteine residues in SR-BI-mediated HDL binding and selective HDL-CE uptake, we again employed cysteine mutation and substitution and overexpression strategies. As before, CHO cells were transiently transfected with the wild type or various SR-BI mutants and subsequently analyzed for HDL binding and/or selective HDL-CE using either radioactive ([^{125}I-DLT]-[^{3}H]COE-labeled hHDL$_3$) or fluorescent (*rec*-HDL-BODIPY-CE)

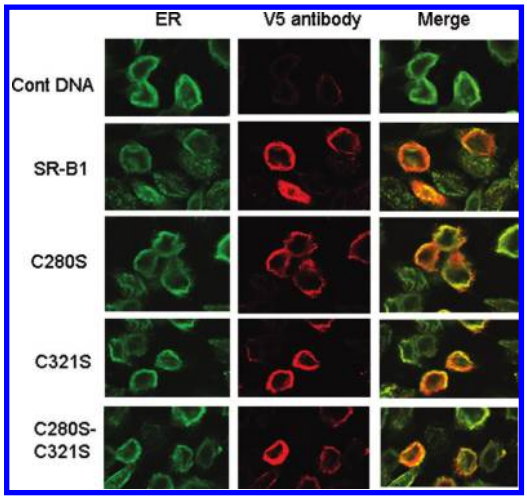

Figure 6. Comparison of the cellular distribution of wild-type SR-BI and SR-BIs with cysteine mutations at position 280 or positions 280 and 321. CHO cells transiently expressing wild-type V5-SR-BI, C280S V5-SR-BI, C321S V5-SR-BI, or C280S/C321S V5-SR-BI were fixed, permeabilized, and simultaneously stained with anti-V5 (to detect the V5 epitope-tagged SR-BI) and anti-calnexin (ER marker) antibodies followed by appropriate fluorescence-linked secondary antibodies as summarized in Experimental Procedures and, subsequently, analyzed by confocal microscopy: green for V5 fluorescence from SR-BI (left), red for calnexin (middle) fluorescence, and yellow for colocalization of SR-BI and calnexin. Images are representative of three or four independent transient transfections.

HDL preparations as a tracer ligand for SR-BI. Expression of C280S, C321S, C323S, and C334S single mutants and C280S/C321S, C280S/C334S, C321S/C323S, and C323S/C334S double mutants significantly reduced the level of HDL binding compared to that seen with wild-type SR-BI (Figure 8B), consistent with their level of presentation at the cell surface (Figure 4A,B). Similarly, expression of double-cysteine mutants C251S/C321S, C280S/C323S, and C334S/C384S caused a 50−60% reduction in the level of cell surface binding of HDL (i.e., association of HDL with SR-BI) (data not shown). Expression of the remaining mutants or constructs with cysteine substitutions, including C251S and C384S, exhibited HDL binding that was comparable to the binding seen with the CHO cells overexpressing wild-type SR-BI.

We then assessed SR-BI-mediated selective HDL-CE uptake in CHO cells expressing the wild type or one of the cysteine mutant or cysteine substitution constructs. Consistent with HDL binding, expression of C280S, C321S, C323S, and C334S single mutants or C280S/C321S, C280S/C334S, C321S/C323S, C323S/C334S, C251S/C321S, C280S/C323S, and C334S/C384S double mutants resulted in a significant reduction in the level of HDL-CE uptake as measured by the internalization of the HDL-derived tracer [^{3}H]COE (Figure 8A). Also, as with HDL binding, the remaining mutants did not significantly impact SR-BI-mediated selective HDL-CE uptake. Complementary results were obtained when the relative efficacy of these various mutants was assessed in COS-7 and CHO cells using [^{125}I-DLT]-[^{3}H]COE-labeled $hHDL_3$ and HDL-BODIPY-CE as ligands, respectively (Table 3).

Effects of Cysteine Mutations and Substitutions on the Relative Expression of SR-BI Monomers, Dimers, and Oligomers. Our recently published data provide evidence that the physical state of the SR-BI protein (i.e., monomeric vs dimeric and higher-order oligomeric forms of SR-BI) and architechtural changes in the cell surface induced by the expression of SR-BI also play major roles in the functional efficiency of the selective pathway.[12,32,33] To determine whether cysteine residues are important for SR-BI oligomerization, various cysteine mutants described above were tested by Western blotting for their ability to form SR-BI dimers and oligomers. Figure 7A indicates that expression of single-cysteine mutants C21S, C251S, and C384S in CHO cells inhibited while C280S, C321S, C323S, and C334S promoted the formation of the SR-BI dimer. The C470S mutation or introduction of a new cysteine residue, V3C or Y462C, however, had no effect on SR-BI dimerization (Figure 7A). Likewise, expression of double mutants C251S/C384S, C280S/C321S, C280S/C334S, and C321S/C323S and triple mutant G453C/Y462C/C470S caused increased levels of formation of SR-BI dimers (Figure 7B). The remaining double and triple mutants exhibited either no or very little modulatory effect on SR-BI dimerization. It is noteworthy that expression of four mutants (C280S, C321S, C323S, and C334S) or their various paired combinations, which lead to a loss of selective HDL-CE uptake, strongly favors formation of the SR-BI dimer. Thus, a complex but unresolved relationship exists between the formation of the SR-BI dimer or oligomer and SR-BI-mediated HDL binding and associated selective HDL-CE uptake. Moreover, although C280, C321, C323, and C334 strongly favor dimer formation, they are rendered nonfunctional. However, they are rendered nonfunctional presumably because of the formation of aberrant disulfide linkages resulting in inhibition of optimal HDL binding and, hence, selective HDL-CE uptake.

■ DISCUSSION

The SR-BI, an HDL receptor, mediates the bulk delivery of plasma lipoprotein-cholesteryl esters (CEs) to steroidogenic and liver tissues for product formation (steroid hormones and bile acids) or their elimination by a process known as selective transport.[2,3,5−7] This process differs from the classic endocytic, LDL receptor pathway in that exogenous circulating lipoproteins such as HDL and LDL contribute their CEs to cells without internalization of the intact lipoprotein particle.[7,13,15,59] Thus, in the selective cholesterol uptake process, lipoprotein lipids enter cells unaccompanied by apolipoproteins. However, the information about the cellular events connected with and underlying mechanisms involved in selective CE transport remains poorly defined. In this study, we evaluated the importance of eight cysteine residues in the extracellular, transmembrane, and C-terminal domains of the rat SR-BI. Because seven of eight cysteine residues (C251, C280, C321, C323, C334, C384, and C470) of SR-BI are well-conserved across the species (Table 2), we suspected that they might play important roles in SR-BI expression, dimerization and oligomerization, and/or function (binding of HDL and SR-BI-mediated selective HDL-CE uptake). In addition, we determined the effects of artificially introduced cysteine residues on the structure and function of SR-BI. All of these studies were accomplished using a combination of site-directed mutagenesis and chemical modification of cysteine residues of the SR-BI. The latter employed disulfide bond reducing agents β-ME and DTT and sulfhydryl alkylating agents, like MTS reagents.

In the first set of studies, we found that treatment of SR-BI-overexpressing cells with reducing agents (β-ME and DTT) caused small but significant reductions in the level of selective HDL-CE uptake, although DTT was somewhat more effective than β-ME (Figure 3). Such a modest effect of β-ME and DTT on SR-BI function suggests that disulfide bonds either exist at low levels or contribute minimally to the maintenance of the

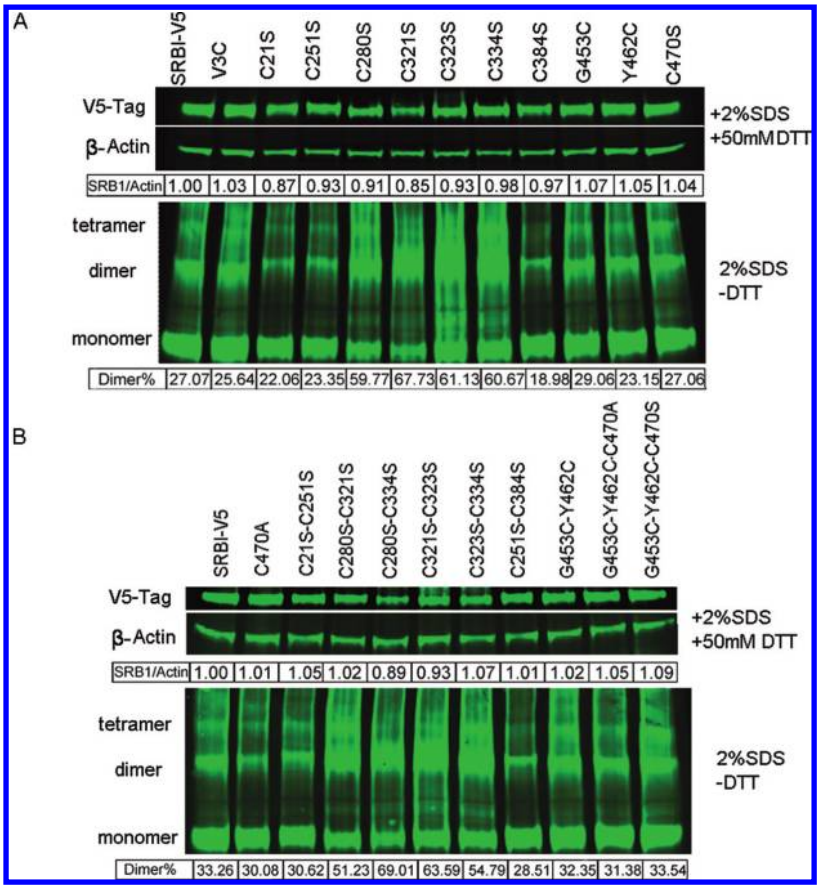

Figure 7. Western analysis of wild-type and mutant oligomeric forms of SR-BI proteins. CHO cells were transiently transfected with an empty vector (mock), V5-tagged rat SR-BI, or the indicated mutant. After being transfected for 36 h, cell lysates were prepared, and aliquots of 10 μg samples were mixed with 0.01% bromophenol blue (nonreducing conditions) or 3 μg samples with 0.01% bromophenol blue and 50 mM DTT (reducing conditions) and subjected to Western blot analysis as described in Experimental Procedures. Expression of V5-tagged rat SR-BI was detected using a 1:5000 dilution of anti-V5 tag monoclonal antibody (Invitrogen Life Technologies). Gel loading of samples was detected using a 1:1000 dilution of the anti-β-actin monoclonal antibody (Santa Cruz Biotechnology). Following incubation of the membranes with the primary antibodies, detection of bands was performed using IRDye 680 goat anti-rabbit IgG (H + L) and IRDye goat anti-mouse IgG (H + L) secondary antibodies and the Odyssey Infrared Imaging System. (A) Blots showing the total protein levels of the wild type, single-cysteine mutants, and introduced cysteine mutants under reducing (SR-BI monomer) and nonreducing conditions (SR-BI dimer and oligomers). (B) Blots showing the total protein levels of the wild type and various double- and triple-cysteine mutant SR-BIs under reducing (SR-BI monomer) and nonreducing (SR-BI dimer and oligomers) conditions. The blots shown are representative of three independent experiments. Note that the amount of total cell lysate protein applied in each case under reducing conditions was only one-third of that of the respective amount applied to gels under nonreducing conditions. This was in an effort to prevent overloading of monomeric SR-BI protein from the DTT-treated samples. Detection and quantification of the infrared signals were performed using the Odyssey system software. Quantification of the infrared signals was performed using the Odyssey system software. The quantitative numbers shown under the 2% SDS plus (+) 50 mM DTT bands (panels A and B) represent expression levels of SR-BI normalized to β-actin, i.e., SR-BI:β-actin ratio, where the native (wild-type) SR-BI:β-actin ratio equals 1.00. The numbers shown under the 2% SDS and minus (−) 50 mM DTT bands represent the percent expression of SR-BI dimers. These numbers were derived by dividing the intensity of the SR-BI dimer band by the combined intensities of the SR-BI dimer and monomer bands, which equals the percent dimer.

secondary structure of SR-BI. Another possibility could be that the structural changes that result from disrupting the accessible disulfide bonds in SR-BI may not be severe enough to cause a much greater impact on its function. Furthermore, one cannot rule out the possibility that potential SR-BI disulfide bonds are inaccessible to DTT and β-ME. A similar inaccessibility of DTT has been suggested for the disulfide bonds in channels and receptors such as the inwardly rectifying K^+ channel, Kir 2.1, and $P2X_1$ and $P2X_2$ receptors.[60−62] Although it has been clearly established that disulfide bonds are essential for the proper functioning of the Kir2.1 channel and $P2X_1$ and $P2X_2$ receptors, these channel and receptor proteins, however, are unaffected by reducing agents. This is also consistent with the findings that disulfide bonds of the mature proteins are often inaccessible to reducing agents unless the protein is rendered denatured.[63,64]

We next examined the potential contribution of endogenous free cysteines of the native SR-BI in selective HDL-CE transport. This was accomplished using free cysteine (sulfhydryl) reactive neutral (MMTS), positively charged (MTSEA and MTSET), and negatively charged (MTSES) MTS reagents. Our studies show that neutral MMTS and positively charged MTSEA reagents greatly reduced the level of SR-BI-mediated selective HDL-CE uptake in SR-BI-overexpressing COS-7 cells (Figure 3). On the basis of these findings, it would appear that certain endogenous cysteine residues participate in SR-BI-mediated selective HDL-CE uptake. Given that MTS reagents, including MMTS and MTSEA, primarily react with ionized thioles present in water-accessible regions,[64−67] these results indicate that critical endogenous cysteine residues involved in the selective transport process must exist in a water-accessible environment. Because MMTS and MTSEA (in the

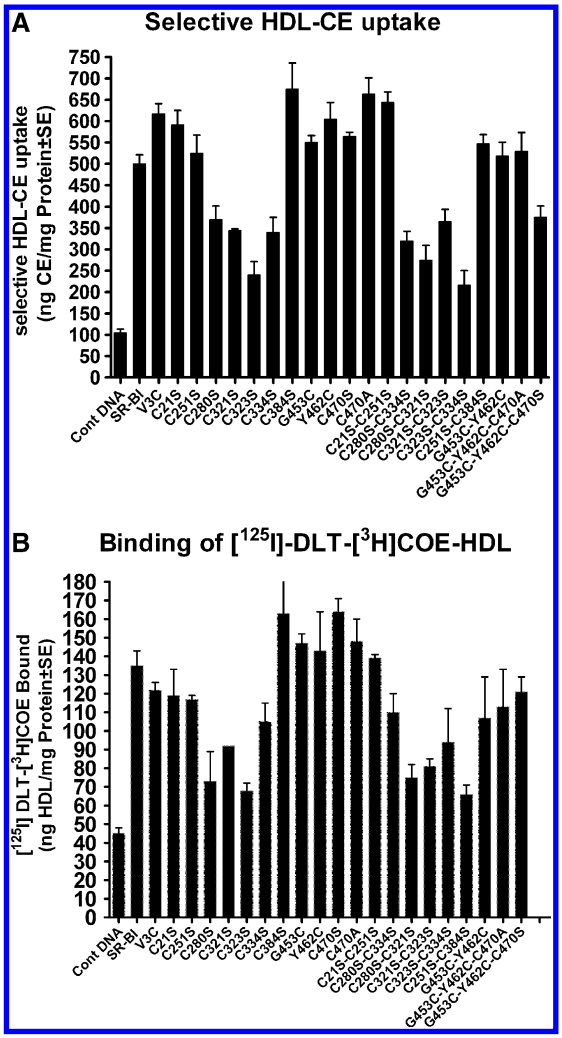

Figure 8. Effects of cysteine mutations and substitutions of SR-BI on HDL binding activity and selective HDL-CE uptake by CHO cells. CHO cells were transiently transfected with vector alone, V5 rat SR-BI, or the indicated mutants. Thirty-six hours post-transfection, cells were incubated with a culture medium containing [^{125}I-DLT]-[^{3}H]COE-labeled hHDL$_3$ (50 μg of protein/mL) for 5 h. At the end of the incubation, the cells were processed for the determination of [^{125}I-DLT]hHDL$_3$ binding activity and the quantification of selectively internalized HDL-[^{3}H]COE as described in Experimental Procedures. The results are means ± the standard error at least three independent experiments and normalized per unit of total expressed SR-BI: (A) selective HDL-CE uptake and (B) HDL binding activity.

pH range of the functional assay, a significant fraction of MTSEA is neutral and able to permeate the lipid bilayer) can enter the cell quite readily, it is possible that they merely interact with the N-terminal transmembrane membrane domain cysteine 21 and/or C-terminal domain cysteine 470 and produce an inhibitory effect. However, using mutagenesis, we have shown that the functional behaviors of C21S and C470S were, in fact, very similar to that exhibited by wild-type SR-BI and, thus, are unlikely to be involved in selective CE transport. In contrast, the insensitivity to membrane-impermeant positively and negatively charged MTSET and MTSES reagents, respectively, suggests either endogenous cysteine residues are inaccessible to these MTS reagents in the aqueous environment or their alkylation does not hamper the SR-BI-mediated selective HDL-CE uptake. A second possibility is that MTSET or MTSES might "bypass" the crucial cysteine residues involved in the selective CE transport process. Similar differential sensitivity to various MTS reagents has also been described for several other systems. For example, Zhu et al.[68] reported that two introduced cysteine mutants (A435C and T442C) in the TM1 region of human NBCel-A were strongly inhibited by three MTS reagents, MTSES, MTSET, and MTSEA. Furthermore, the I441C mutant was significantly inhibited by MTSET and MTSEA and A428 by MTSEA alone.[68] Likewise, it was reported that treatment of COS-1 cells expressing human cannabinoid receptor (CB1) with MMTS or MTSEA leads to a dramatic reduction in the level of binding of agonist SR141716A to the receptor; such treatment, however, had no significant effect on the binding of agonist CP55940 to the CB1 receptor.[67] Human AT$_1$ receptors[69] and P2X$_2$[61] are shown to be insensitive to MTSEA and MTSET, respectively. On the other hand, it was reported that treatment of HEK cells transiently overexpressing the glucagon receptor with MTSET enhanced (instead of weakened) the glucagon potency for cAMP stimulation by 2−3-fold.[70]

Our data for the use of sulfhydryl reactive reagents, such as cell-impermeant DTT, raised the possibility that disulfide bonds exist in SR-BI and contribute to selective HDL-CE uptake (Figure 3). In addition, the reduction of higher-order complexes by DTT suggested that the formation of ectodomain (or ECD) dimers or oligomers may be mediated, at least in part, by disulfide bond formation via the six conserved cysteine residues found on the ECD of the SR-BI. To study directly the role of disulfide bonds and other cysteine residues, we employed single- and paired-cysteine mutagenesis coupled with Western blotting, confocal microscopy, and functional studies to show that certain cysteine residues in the ECD of rat SR-BI form disulfide bonds, participate in SR-BI dimerization and oligomerization, facilitate normal trafficking of SR-BI to the cell surface, and contribute to the binding of HDL to SR-BI and SR-BI-mediated selective HDL-CE uptake.

As noted above and in Results, rat SR-BI, the prototype used here, contains eight cysteine residues; while these residues are dispersed throughout the protein, they are mainly concentrated in the ECD. Because seven of eight Cys residues, including six in the ECD (C251, C280, C321, C323, C334, and C384), of SR-BI are well-conserved across the species (Table 1), we reasoned that they might play an essential role in SR-BI expression and function as well as in its dimerization and/or oligomerization. Substitution of cysteine with serine at positions 280, 321, 323, and 334 in the extracellular domain caused a significant reduction in the level of binding of ligand HDL to SR-BI and the ability of SR-BI to mediate the selective delivery of HDL-CE into the cell (Table 3 and Figure 8A,B). The data suggest that these residues may directly participate in SR-BI−HDL interaction and SR-BI-mediated selective HDL-CE uptake and may also be involved in disulfide bond formation. Interestingly, despite the absolute conservation and occurrence of C384 in the ECD, the C384 mutant (C384S) did not affect the function of SR-BI (Table 3 and Figure 8A,B). Likewise, mutation of the N-terminal domain C21 (C21S) and the C-terminal domain C470 (C470S) to serine or mutation of the N-terminal domain V3 (V3C) and the C-terminal transmembrane domain G453 (G453C) and Y462 (Y462C) to cysteine also was without effect, suggesting that they are not required for optimal binding of HDL to the SR-BI or SR-BI-mediated selective HDL-CE uptake (Table 3 and Figure 8A,B).

The parallel decrease in the level of cell surface expression (Figures 4 and 5) and the lack of an effect of the mutations on the intrinsic SR-BI properties indicate that the primary cause of

impaired SR-BI expression and function for the C280S, C321S, C323S, and C334S mutants is the routing of the SR-BI to the cell surface. Although at present we are unable to provide an exact explanation, two possibilities may be considered to explain the decreased level of localization of mutant SR-BI proteins at the cell surface: (a) introduction of cysteine mutations resulted in misfolding of SR-BI proteins, which interfered with protein trafficking, and (2) reduced levels of cysteine mutant SR-BI proteins because of their enhanced susceptibility to degradation. The latter possibility, however, seems unlikely given that both Western blotting and immunofluorescence quantification of total SR-BI protein showed equal expression of SR-BI in cells transfected with native (wild-type) or various cysteine mutant SR-BIs (Figures 6 and 7). Therefore, the loss of HDL binding and selective cholesterol transport function seen with C280S, C321S, C323S, and C334S (Table 3 and Figure 8) cannot be attributed to a decreased level of SR-BI protein expression (synthesis) or an increased level of intracellular degradation. Considering the fact that these four cysteine residues (i.e., C280, C321, C323, and C334) are clustered within the ECD and that mutants of these cysteines were quite similar in their level of cell surface expression and failure to bind HDL and mediate selective HDL-CE uptake, it is most likely that they participate in interdisulfide bond formation. Moreover, it is possible that mutation of these cysteine residues affects disulfide formation, in turn affecting folding, ultimately intracellular SR-BI trafficking, and therefore ligand (HDL) binding and function (selective HDL-CE uptake). Previous studies have shown that the extracellular domain of the CD36 scavenger receptor, a defining member of the SR-BI gene family, contains three disulfide bridges (C313−C322, C243−C311, and C280−C333).[71] Given that ECDs of CD36 and SR-BI show a very high level of sequence homology, it would appear that SR-BI may also contain three disulfide bonds (C323−C334, C251−C321, and C280−C384). Additional experimental studies are underway to explore this likely possibility.

We further examined the possibility that paired cysteine mutations may rescue the loss of expression and function of SR-BI. We reasoned that simultaneous elimination of both cysteine partners (which form disulfide bonds) in any specific pair combination would minimize the structural alterations in SR-BI and restore the functional expression of SR-BI. However, this turned out not to be the case, and double mutations of SR-BI (C280S/C321S, C280S/C334S, C321S/C323S, C323S/C334S, C251S/C321S, C280S/C323S, and C334S/C384S) also showed impaired cell surface expression, HDL binding, and selective CE transport function (Figures 4, 5, and 8 and Table 3). A comparison of these various double-cysteine mutants indicated that two double mutants, C280S/C321S and C323S/C334S, gave the lowest levels of cell surface expression of SR-BI (~20% of that of the wild type), suggesting that these four cysteine residues are not only involved in the maintenance of SR-BI structure but also important for optimal cell surface expression of SR-BI, and the low level of cell surface expression is the primary cause of the impaired SR-BI function (Figures 4 and 5).

We also studied the potential involvement of disulfide bonds in SR-BI dimer formation. To do this, we expressed the mutated single- and paired-cysteine residue constructs as described above and analyzed them for the presence of SR-BI monomer and dimer by SDS−PAGE under nonreducing conditions following Western blotting. Expression of single-cysteine mutants (C21S, C251S, and C384S) in CHO cells inhibited while C280S, C321S, C334S, and C323S and other double (C251S/C384S, C280S/C321S, C280S/C334S, and C321S/C323S) and triple (G453C/Y462C/C470S) mutants promoted SR-BI dimer formation (Figure 7A,B). Use of other double- and triple-cysteine mutants exhibited either no or very little influence on SR-BI dimer formation. Introduction of a new cysteine residue had no effect (V3C) or inhibited (Y462C) SR-BI dimerization. It is noteworthy that expression of four single mutants (C280S, C321S, C323S, and C334S) or their various paired combinations, which lead to a loss of selective HDL-CE uptake, strongly favors the formation of SR-BI dimers. The dysfunctional SR-BI dimers observed with the use of these single and double mutants could potentially result from the formation of aberrant disulfide linkages. In this regard, mutations of one or more of these cysteine residues that are essential for intradisulfide bond formation and SR-BI dimerization could disrupt these bonds, but at the same time, other free cysteine residues may rearrange to form new disulfide bonds and cause enhanced SR-BI dimerization. However, they are rendered nonfunctional because these residues may also be important for optimal HDL binding and, hence, selective HDL-CE uptake.

In conclusion, the results of this study provide evidence that four extracellular domain cysteine residues of SR-BI (C280, C321, C223, and C334) are essential for SR-BI structure, including dimerization, its optimal expression at the cell surface, and HDL binding and selective CE transport function. In addition, on the basis of MTS studies, certain free cysteine residues, especially those localized in the transmembrane and C-terminal domains, also play an important role in SR-BI function. These results provide important insights into the functional role of cysteines in SR-BI dimerization, cell surface expression, and function. While this work was being finalized, a report by Papale et al.[72] providing experimental evidence of the potential role of the intramolecular disulfide bonds in the extracellular domain of SR-BI in SR-BI-mediated selective cholesterol transport appeared. Although this publication also emphasizes the importance of four ECD cysteines in SR-BI function, some notable differences exist between the results described in ref 72 and the results of our study. For example, the major finding of Papale et al.[72] is that mutation of any of the four cysteine residues (C280S, C321S, C323S, or C334S) of the extracellular domain of SR-BI leads to impaired HDL binding and subsequent inhibition of selective HDL-CE uptake. Furthermore, such inhibition of SR-BI binding activity and cholesterol transport occurred without an impact on the cell surface expression of mutant SR-BI proteins. While our studies reached the same conclusion, we provide extensive evidence to suggest that mutation of any of the four cysteine residues (C280S, C321S, C323S, or C334S) results in a mutant SR-BI protein whose level of surface expression is greatly reduced and as a consequence results in inhibition of both HDL binding and selective HDL-CE uptake. This inhibition of surface transport occurs because mutations likely result in misfolding of proteins, which interferes with SR-BI trafficking. Interestingly, Western blotting and immunofluorescent measurement of the total SR-BI protein (Figures 6 and 7) showed equivalent expression of SR-BI in cells transfected with native or various cysteine mutant SR-BIs. Thus, as discussed above, loss of SR-BI HDL binding activity and selective cholesterol transport function is not due to a decreased level of SR-BI protein expression or an increased level of intracellular degradation but results from a reduced level of cell surface expression of the mutant SR-BI proteins. At present, we are unable to provide an exact explanation for the

observed discrepancy between our results and those of Papale et al.[72] However, a close visual inspection of Western blot data shown in Figure 6 of ref 72 revealed that total expression of the C280S, C321S, C323S, or C334S mutant may be less than 50% of that of wild-type SR-BI, although no quantitative data accompanied such blots. To what extent such potential reduction in total mutant SR-BI protein levels may have contributed to the observed loss of SR-BI ligand binding activity and selective cholesterol transport was not apparent. Our studies further demonstrate that introduction of cysteine mutations can result in aberrant SR-BI dimer formation, which is also likely to interfere with selective HDL-CE uptake function [SR-BI dimers formed from native (wild-type) SR-BI are the ones that mediate selective cholesterol transport]. Finally, with the use of various charge-specific MTS reagents, we provide evidence that free cysteine residues also play a role in SR-BI function.

AUTHOR INFORMATION

Corresponding Author

*GRECC-182B, VA Palo Alto Health Care System, 3801 Miranda Ave., Palo Alto, CA 94304. Telephone: (650) 858-3933. Fax: (650) 496-2505. E-mail: salman.azhar@va.gov.

Author Contributions

J.H. and Z.Z. contributed equally to this work.

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ABBREVIATIONS

CE, cholesteryl ester; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; COE, cholesteryl oleolyl ether; COS-7, African green monkey kidney; CTD, C-terminal domain; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; DLT, dilactitol tyramine; ECD, extracellular domain; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; HDL, high-density lipoprotein; β-ME, β-mercaptoethanol; MMTS, methyl methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; MTS, methanethiosulfonate; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; NHERF3, Na^+/H^+ exchanger regulatory factor 3; PAGE, polyacrylamide gel electrophoresis; PDZ, postsynaptic density protein PSD95-*Drosophila* homologue Discs-large and tight junction protein Zona occludens-1; PDZK1, PDZ domain-containing protein kidney 1; *rec*-HDL-BODIPY-CE, recombinant HDL-BODIPY-CE; SDS, sodium dodecyl sulfate; NaPFO, sodium perfluorooctanoate; SR-BI, scavenger receptor, class B, type I; TMD, transmembrane domain.

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