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Extracellular Disulfide Bonds Support Scavenger Receptor Class B Type I-Mediated Cholesterol Transport

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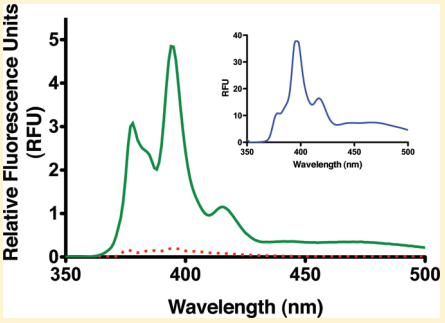
ABSTRACT: Scavenger receptor class B type I (SR-BI) binds high-density lipoprotein (HDL) and mediates the selective uptake of cholesteryl esters (CE). Although the extracellular domain of SR-BI is critical for function, the structural characteristics of this region remain elusive. Using sulfhydryl labeling strategies, we report the novel finding that all six cysteine (Cys) residues in the extracellular domain of SR-BI are involved in disulfide bond formation that is intramolecular by nature. We hypothesized that an SR-BI conformation stabilized by extracellular disulfide bonds is a prerequisite for SR-BI-mediated cholesterol transport. Thus, single-Cys mutant SR-BI receptors (C251S-, C280S-, C321S-, C323S-, C334S-, and C384S-SR-BI), as well as Cys-less SR-BI, a mutant SR-BI receptor void of all Cys residues, were created, and plasma membrane localization was confirmed. Functional assays revealed that C280S-, C321S-, C323S-, and C334S-SR-BI and Cys-less SR-BI mutant receptors displayed weakened HDL binding and subsequent selective uptake of HDL-CE. However, only C323S-SR-BI and Cys-less SR-BI were unable to mediate wild-type levels of efflux of free cholesterol (FC) to HDL. None of the Cys mutations disrupted SR-BI's ability to redistribute plasma membrane FC. Taken together, the intramolecular disulfide bonds in the extracellular domain of SR-BI appear to maintain the receptor in a conformation integral to its cholesterol transport functions.



Cysteine (Cys) is unique among the 20 common amino acids because of its ability to form covalently linked disulfide bonds. Intramolecular disulfide bonds, occurring between Cys residues on the same polypeptide chain, are the necessary reinforcements of protein architecture, aiding both in protein folding and in conformational stability of secondary and tertiary protein structure (reviewed in refs 1−5). This is especially true of secreted and large transmembrane proteins such as receptors, transporters, and channels, including ATP-binding cassette transporter A1,[6] $P2X_1$ receptors,[7] rhodopsin,[8,9] β-adrenergic receptors,[10,11] rat serotonin transporters,[12] cardiac Na^+-Ca^{2+} exchangers,[13] human transcobalamin II,[14] and human vesicle monoamine transporters,[15] all of which require one or more intramolecular disulfide bonds for proper folding, conformation, and biological function. On the other hand, intermolecular disulfide bonds formed between Cys residues on separate peptide chains provide a link between two protein molecules and contribute to the formation of dimers or oligomers in proteins such as the human prostacyclin receptor,[16] metabotropic glutamate receptors 1 and 5,[17−19] and CD36.[20,21]

Scavenger receptor class B type I (SR-BI) is an 82 kDa glycosylated cell surface receptor that functions in the selective uptake of HDL-CE into cells,[22] chiefly those of the liver and steroid-producing tissues.[23,24] The selective uptake process occurs in two steps: (i) binding of HDL to the extracellular domain of SR-BI and (ii) transfer of CE from HDL to the plasma membrane for hydrolysis,[25−28] without endocytosis of the HDL particle.[29,30] In addition to mediating the selective uptake of HDL-CE, SR-BI also functions in promoting efflux of free cholesterol (FC) to HDL,[31,32] as well as enlarging the pool of plasma membrane FC sensitive to exogenous cholesterol oxidase.[33,34]

The predicted topology model of SR-BI consists of a large extracellular domain anchored by a transmembrane domain at both the N- and C-termini (reviewed in ref 35). Although it is well-established that the extracellular domain is necessary for receptor function,[29,30,36−38] little is known about the structural organization of this domain at the plasma membrane, including the role of the six Cys residues located in the C-terminal half of this domain. As with CD36,[39] a scavenger receptor with a similar predicted topology, it is assumed that these six Cys residues are involved in disulfide bond formation, although there is no direct evidence to support this notion.

We hypothesized that a prerequisite of SR-BI-mediated cholesterol transport is an SR-BI conformation stabilized by the extracellular disulfide bonds. In this study, we provide the first evidence that all six extracellular Cys residues are indeed involved in the formation of intramolecular disulfide bonds. Our site-directed mutagenesis studies suggest that loss of at least one disulfide bond disrupts (i) binding of HDL, (ii) selective uptake of HDL-CE, and (iii) efflux of FC to HDL. However, disulfide

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bonds do not appear to be necessary for the ability of SR-BI to redistribute plasma membrane free cholesterol.

EXPERIMENTAL PROCEDURES

Materials. The following antibodies were used: polyclonal anti-SR-BI specific for the C-terminal or extracellular domain (Novus Biologicals, Inc., Littleton, CO), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Millipore, Billerica, MA), peroxidase-conjugated goat anti-rabbit secondary IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), peroxidase-conjugated sheep anti-mouse secondary IgG (Jackson ImmunoResearch Laboratories), and FITC-conjugated goat anti-rabbit IgG (Fisher Scientific). Human HDL (1.063−1.21 g/mL) was purchased from Biomedical Technologies, Inc. [^{125}I]Iodine was from Perkin-Elmer, while [^{3}H]cholesterol and [^{3}H]cholesteryl oleoyl ether (COE) were from GE Healthcare (Piscataway, NJ). Cholesterol oxidase (*Streptomyces*), acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor (Sandoz 58-035), cholesterol, 4-cholesten-3-one, and cholesteryl oleate standards were purchased from Sigma. EZ-Link Sulfo-NHS-LC-biotin was purchased from Thermo Fisher Scientific (Rockford, IL). *N*-Biotinylaminoethyl methanethiosulfonate (MTSEA-biotin) was purchased from Toronto Research Chemicals, Inc. (North York, ON). *N*-(1-Pyrene)maleimide was purchased from Invitrogen. All other reagents were of analytical grade.

Plasmids and Sequencing. Site-directed mutations at C280, C321, and C334 were introduced into wild-type murine SR-BI [pSG5(SR-BI)][29] using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocols. The following oligonucleotide primers (Integrated DNA Technologies) were used for mutagenesis: C280S, 5′-GCC CGG AGG CAA GCA GGT CCA TGA AGC-3′ and 5′-GCT TCA TGG ACC TGC TTG CCT CCG GGC-3′; C321S, 5′-CCA CCC AAC GAA GGC TTC AGC CCA TGC CGA GAG TCT GGC-3′ and 5′-GCC AGA CTC TCG GCA TGG GCT GAA GCC TTC GTT GGG TGG-3′; C334S, 5′-GCA TTC AGA ATG TCA GCA CCA GCA GGT TTG GTG CGC C-3′ and 5′-GGC GCA CCA AAC CTG CTG GTG CTG ACA TTC TGA ATG C-3′. All plasmids were purified using endotoxin-free Qiagen Maxi-Prep kits and sequenced through the coding region to verify the correct substitution and the absence of undesired mutations generated during the amplification steps. DNA sequencing was performed on an ABI 3100 instrument at the Protein and Nucleic Acid Facility at the Medical College of Wisconsin. The C251S, C323S, C384S, and Cys-less mutant receptors were produced and sequenced by TOP Gene Technologies (Pointe-Claire, QC). Myc-SR-BI[36] and G420C-SR-BI[40] were previously described.

Cell Culture and Transfection. COS-7 cells were maintained in DMEM (Invitrogen), 10% calf serum (Invitrogen), 2 mM L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 1 mM sodium pyruvate and transfected as previously described.[29] Cells were assayed 48 h post-transfection, with the exception of efflux assays in which cells were assayed 72 h post-transfection.

Cell Lysis. COS-7 cells expressing either wild-type or mutant SR-BI were washed twice with PBS (pH 7.4) and lysed with 1% NP-40 cell lysis buffer[29] containing the following protease inhibitors: 1 μg/mL pepstatin, 0.2 mM phenylmethanesulfonyl fluoride, 1 μg/mL leupeptin, and 10 μg/mL aprotinin. Protein concentrations were determined by the Lowry method as previously described.[41]

PFO−PAGE. COS-7 cells expressing either wild-type or mutant SR-BI were washed twice with PBS (pH 7.4) and lysed with PBS containing the aforementioned protease inhibitors. Lysates were processed, and PFO−PAGE was completed as previously described[42] using 4 to 15% polyacrylamide gradient gels.

Immunoblot Analysis. Wild-type and mutant SR-BI, as well as GAPDH, were detected by immunoblot analysis as previously described.[42]

Detection of Free Sulfhydryls by Pyrene Labeling of SR-BI. Myc-tagged SR-BI was immunoprecipitated from transiently transfected COS-7 cells[43] and labeled with a 2-fold molar excess of *N*-(1-pyrene)maleimide for 3 h at 37 °C in the dark in the presence or absence of 10 mM tris(2-carboxylethyl)phosphine (TCEP). DTT was labeled as described above and served as a positive control. Pyrene fluorescence emission spectra (350−600 nm) were recorded on a Photon Technology International (PTI) fluorimeter following excitation at 345 nm.

Immunofluorescence. Wild-type or mutant SR-BI cell surface expression in transiently transfected COS-7 cells was visualized by immunofluorescence as previously described.[42,44] Nuclei were stained with ToPro3 (Invitrogen).[45]

Cell Surface Biotinylation. COS-7 cells transiently expressing either wild-type or mutant SR-BI were washed twice with PBS (pH 7.4) and labeled with 1 mg/mL EZ-Link Sulfo-NHS-LC-biotin as previously described.[42] To label free sulfhydryls at the cell surface, COS-7 cells expressing either wild-type or mutant SR-BI (in the presence and absence of 10 mM TCEP) were washed twice with PBS (pH 7.4) and labeled with 10 mM MTSEA-biotin for 15 min at room temperature in the dark, and the reaction was stopped with 50 mM ammonium chloride. Cells were lysed as described above, and biotinylated proteins were immunoprecipitated with streptavidin agarose. Proteins were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and visualized by immunoblot analysis.

Cell Surface Receptor Expression Levels Determined by Flow Cytometry. Forty-eight hours post-transfection, COS-7 cells transiently expressing empty vector, wild-type SR-BI, or mutant SR-BI receptors were assayed for cell surface expression via flow cytometry. Cells were washed, pelleted, resuspended in PBS and 0.5% BSA, and incubated with an antibody directed against the extracellular domain of SR-BI (1:200 dilution) for 20 min on ice. Cells were then washed, resuspended with PBS and 0.5% BSA, and incubated with FITC-conjugated goat anti-rabbit IgG (1:1000 dilution) on ice for an additional 20 min. Cells were washed with PBS and 0.5% BSA and resuspended in the same buffer, and fluorescence intensities were analyzed on a FACS Calibur (Flow Cytometry Core, Medical College of Wisconsin).

HDL Labeling. HDL was doubly labeled with [^{125}I]dilactitol tyramine and nonhydrolyzable [^{3}H]COE as previously described.[29] For the various preparations of radiolabeled HDL, average specific activities were 168.3 dpm/ng of protein for ^{3}H and 204.1 dpm/ng of protein for ^{125}I.

Cell Association of [^{125}I]HDL and Selective Uptake of [^{3}H]HDL-COE. COS-7 cells were transiently transfected with empty pSG5 vector, wild-type SR-BI, or mutant SR-BI plasmid. Cell association of [^{125}I]HDL and selective uptake of nonhydrolyzable [^{3}H]COE were assayed as previously described.[29] Data presented are the average of two independent experiments and are representative of eight separate experiments, all performed in triplicate. Data were calculated as nanograms of HDL per milligram of cell protein or nanograms of HDL-COE per milligram of cell protein for binding and selective uptake experiments,

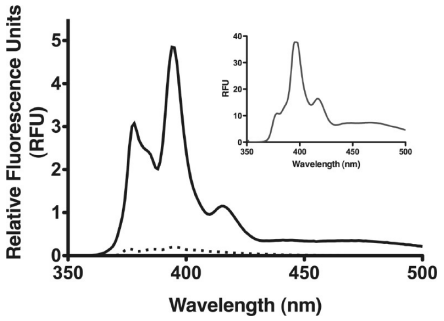

Figure 1. Pyrene labeling of free Cys residues. Myc-SR-BI was immunoprecipitated from COS-7 cell lysates and labeled with pyrenemaleimide for 3 h at 37 °C in the dark in the presence (solid line, reducing conditions) or absence (dotted line, oxidizing conditions) of 10 mM TCEP. DTT served as a positive control for pyrene labeling (inset). Fluorescence emission spectra were recorded following excitation at 345 nm.

respectively. The data were then normalized to cell surface expression as determined by flow cytometry described above. Vector (pSG5) values were subtracted from all wild-type and mutant values. The resulting values were expressed relative to wild-type SR-BI values, which were set at 100%. Statistical comparisons were determined using one-way ANOVA with Bonferroni post-tests for all groups.

Free Cholesterol Efflux and Cholesterol Oxidase Sensitivity Assays. Assays were performed as previously described,[37] unless otherwise specified. Data represent the average of at least four separate experiments performed in quadruplicate. Statistical comparisons were determined using one-way ANOVA with Bonferroni post-tests for all groups.

RESULTS

All Extracellular Cysteine Residues Are Involved in Disulfide Bond Formation. The SR-BI receptor possesses a total of eight Cys residues in its amino acid sequence. Two of these residues, C462 and C470, are located at the junction of the C-terminal transmembrane domain and the C-terminal cytoplasmic domain.[35] Both residues are postulated to be fatty acylated,[46] yet they are dispensable for SR-BI function.[36] The six remaining Cys residues (at positions 251, 280, 321, 323, 334, and 384) are located in the C-terminal half of the extracellular domain of SR-BI and are conserved among the human, mouse, rat, pig, bovine, dog, rabbit, hamster, horse, and chicken species. While it is assumed that these six extracellular Cys residues in SR-BI are involved in disulfide bond formation, direct evidence to support this notion in the literature is not available. Therefore, we used two different strategies to ascertain whether the sulfhydryl groups of these extracellular Cys residues were free or involved in disulfide bond formation.

In the first strategy, myc-SR-BI was immunoprecipitated from transiently transfected COS-7 cell lysates and labeled with *N*-(1-pyrene)maleimide, a sulfhydryl-specific reagent that fluoresces only when bound to a free sulfhydryl group.[47] Pyrene labeling was performed in the presence (reducing conditions) or absence (oxidizing conditions) of TCEP.[48] As shown in Figure 1, pyrene was unable to label SR-BI in the absence of TCEP, suggesting the lack of free sulfhydryl groups available for pyrene labeling under oxidizing conditions. However, the typical pyrene fluorescence spectrum, with emission peaks at 375 and 395 nm,

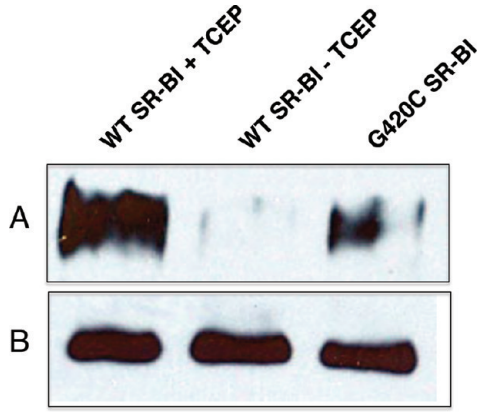

Figure 2. MTSEA-biotin labeling of free Cys residues. COS-7 cells expressing wild-type or Cys-less SR-BI were labeled with MTSEA-biotin for 15 min at room temperature under reducing (+TCEP) and nonreducing (−TCEP) conditions. G420C-SR-BI served as a positive control for MTSEA labeling. Biotinylated complexes were immunoprecipitated from cell lysates using streptavidin agarose, and samples were subjected to SDS−PAGE. Protein bands on the immunoblot were detected using an antibody directed against the C-terminal cytoplasmic tail of SR-BI: (A) biotinylated SR-BI (from ∼200 μg of total lysate) and (B) total SR-BI expression in 20 μg of total cell lysates.

was observed for SR-BI under reducing conditions in the presence of TCEP. DTT served as a positive control for pyrene labeling (Figure 1, inset).

In the second strategy, we further confirmed the involvement of all extracellular Cys residues in disulfide bond formation by cell surface biotinylation experiments using sulfhydryl-specific, membrane-impermeable MTSEA-biotin.[7,49,50] COS-7 cells transiently transfected with wild-type SR-BI were labeled with MTSEA-biotin in the presence and absence of TCEP. MTSEA-biotinylated SR-BI complexes were immunoprecipitated from cell lysates and analyzed by protein immunoblotting. Wild-type SR-BI was present in total lysates (Figure 2B) but was only biotinylated under reducing conditions (Figure 2A, lane 1). The inability to detect SR-BI under oxidizing conditions suggests that there were no free sulfhydryl groups available for MTSEA biotinylation. G420C-SR-BI harbors an extra Cys residue in the extracellular domain and therefore served as a positive control for MTSEA biotinylation. Together, these data confirm that all of the extracellular Cys residues are involved in disulfide bond formation. The inability of 10 mM iodoacetamide, a sulfhydryl alkylating agent,[51] to react with SR-BI in cells prior to cell lysis confirmed that these bonds were preexisting in live cells and not nonspecific due to oxidation of SH groups during the lysis procedure (data not shown).

SR-BI Possesses Intramolecular Disulfide Bonds. Next, we sought to determine whether the extracellular disulfide bonds within SR-BI were intramolecular or intermolecular by nature. Because intramolecular disulfide bonds can affect protein conformation due to their role in protein folding and structural stability, polyacrylamide gel analysis often reveals a difference in migration between the oxidized and reduced forms of a protein.[52−55] We compared the migration of SR-BI by 10% SDS−PAGE under oxidizing and reducing conditions. Our analysis revealed a slower electrophoretic mobility for SR-BI reduced with either 100 mM DTT or 10% β-mercaptoethanol (Figure 3, left panel). This observation (i) indicates the presence of intramolecular disulfide bonds and (ii) suggests that intramolecular

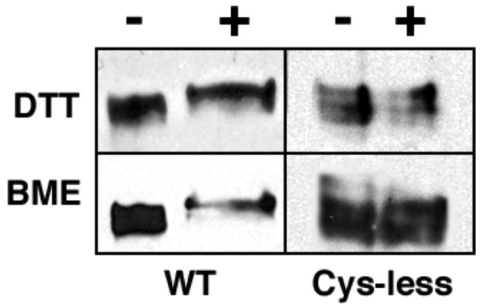

Figure 3. Reduction of intramolecular disulfide bonds by DTT and βME. COS-7 cells expressing wild-type (left) or Cys-less SR-BI (right) receptors were lysed and subjected to 10% SDS−PAGE in the presence and absence of either 10 mM DTT or 10% βME. Protein bands were detected using an antibody directed against the C-terminal cytoplasmic tail of SR-BI.

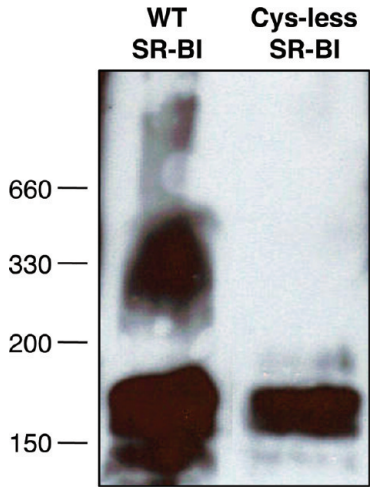

Figure 4. Oligomerization patterns of wild-type and Cys-less SR-BI. COS-7 cell lysates expressing either wild-type or Cys-less SR-BI were examined by PFO−PAGE as previously described[42] using 4 to 15% polyacrylamide gradient gels. SR-BI was detected using an antibody directed against the C-terminal cytoplasmic tail of SR-BI.

disulfide bonding correlates with conformational changes within SR-BI's secondary structure.[18,54−56]

SR-BI is known to form dimers and higher-order oligomers.[43,57−59] Because disulfide bonds can contribute to the formation of multimeric complexes through the linkage of Cys residues on two separate peptide chains, we wished to determine whether any of the proposed disulfide bonds in the extracellular domain of SR-BI were intermolecular in nature. To this end, we created an SR-BI receptor void of Cys residues, herein termed Cys-less SR-BI. As expected, there were no differences in electrophoretic mobility of Cys-less SR-BI under reducing and oxidizing conditions (Figure 3, right panel). Protein complexes from COS-7 cell lysates transiently expressing either wild-type or Cys-less SR-BI were resolved by PFO−PAGE, an electrophoretic technique used to stabilize and separate existing oligomeric complexes.[60] As shown in Figure 4, Cys-less SR-BI is still able to form dimers, despite the absence of Cys residues. These data support our initial findings that the disulfide bonds in the extracellular domain of SR-BI are most likely intramolecular by nature and that SR-BI oligomerization probably occurs via noncovalent interactions, as further evidenced by the presence of SR-BI dimers under reducing conditions.[58] Further, it appears that the loss of disulfide bonds in Cys-less SR-BI has resulted in an extracellular domain with a conformation that can no longer support higher-order oligomer formation as observed for wild-type SR-BI.

Wild-Type and Mutant SR-BI Receptors Are Expressed at the Cell Surface. To determine the functional role of the Cys residues in the extracellular domain of SR-BI, in addition to Cys-less SR-BI, we generated a panel of single-point mutations in which each Cys residue was mutated to serine (Ser, S) to generate the following receptors: C251S-, C280S-, C321S-, C323S-, C334S-, and C384S-SR-BI. Because the loss of Cys residues can lead to impaired receptor trafficking to the plasma membrane,[61,62] COS-7 cells transiently expressing wild-type or mutant SR-BI receptors were analyzed for plasma membrane localization by fluorescence microscopy. All mutant receptors exhibited similar staining patterns at the cell surface and cell extensions as wild-type SR-BI[44] (Figure 5). No staining was observed in cells transfected with empty vector or in nontransfected cells. In conjunction with these studies, immunoblot analysis from MTSEA biotinylation of single-Cys SR-BI mutant receptors supported the cell surface localization of these receptors and further confirmed the presence of a free sulfhydryl group (Figure 6). Densitometry analyses did not reveal statistically significant differences in cell surface expression for all receptors tested [ANOVA, $p > 0.05$ (data not shown)]. Sulfo-NHS-LC biotinylation also verified the cell surface localization of Cys-less SR-BI (Figure 6, right panels), although it tended to be expressed more weakly than wild-type SR-BI. Expression of all mutant SR-BI receptors in total cell lysates is also shown (Figure 6B). Therefore, loss of a single extracellular Cys residue, and the corresponding loss of a single disulfide bond, did not affect trafficking of SR-BI to the cell surface.

Several Mutant Receptors Exhibit a Weakened Ability To Bind HDL and Mediate the Selective Uptake of HDL-COE. To determine whether the loss of Cys residues affected SR-BI function, wild-type and mutant SR-BI receptors were tested for their ability to bind HDL and mediate selective uptake of HDL-COE following transient transfection in COS-7 cells. Flow cytometry using antibodies directed against the extracellular domain of SR-BI revealed that all receptors were expressed at similar levels at the cell surface [$p > 0.05$ (data not shown)]. Upon normalization to cell surface expression, our analysis revealed that C251S- and C384S-SR-BI mediated wild-type levels of HDL binding and selective uptake of HDL-COE. However, for C280S-, C321S-, C323S-, and C334S-SR-BI mutant receptors, their weakened ability to bind HDL [28, 48, 41, and 43% of that of the wild type, respectively (Figure 7A)] was accompanied by an impaired ability to mediate the selective uptake of HDL-COE [35, 45, 40, and 46% of that of the wild type, respectively (Figure 7B)]. Cys-less SR-BI displayed an even greater reduction in its level of binding of HDL and selective uptake of HDL-COE (11 and 4% of that of the wild type, respectively). These data demonstrate the requirement of disulfide bonds associated with residues C280, C321, C323, and C334 for efficient CE uptake.

Mutant Receptors Do Not Display Major Changes in Cholesterol Efflux and Oxidase Sensitivity of Membrane Free Cholesterol. SR-BI not only mediates flux of CE from HDL into cells but also can stimulate the flux of FC out of cells to HDL and other acceptor particles.[31−33] To establish whether this cholesterol transport function was affected by our SR-BI mutations,

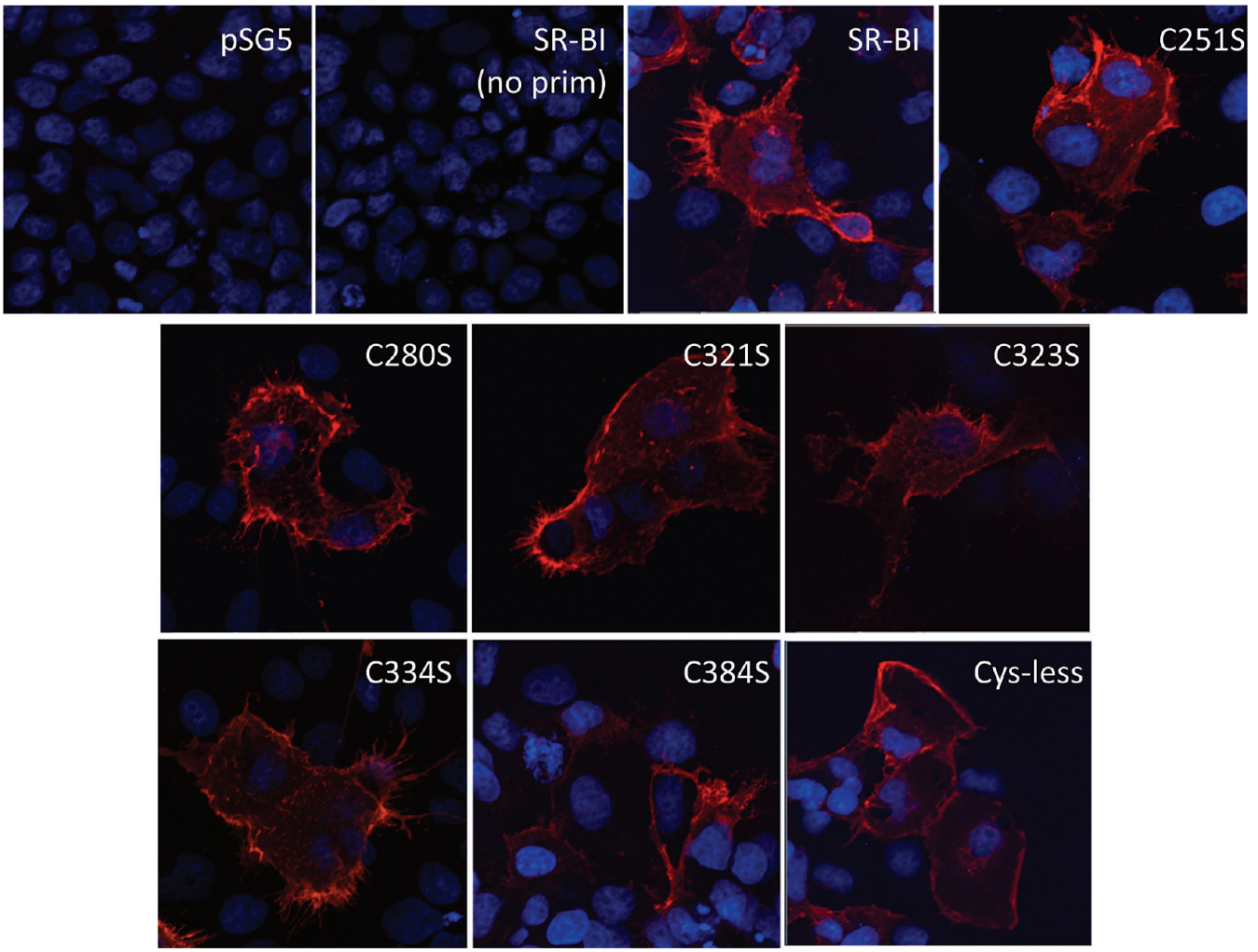

Figure 5. Cell surface staining patterns of wild-type and mutant SR-BI receptors. Twenty-four hours post-transfection, COS-7 cells expressing wild-type or mutant SR-BI receptors were plated onto glass coverslips. Cells were fixed and stained with an antibody directed against the extracellular domain of SR-BI followed by an Alexa 568-conjugated secondary antibody. Cells expressing empty vector stained with primary antibody (pSG5) or SR-BI without primary antibody staining (SR-BI, no prim) are also shown. Nuclei were stained with ToPro.

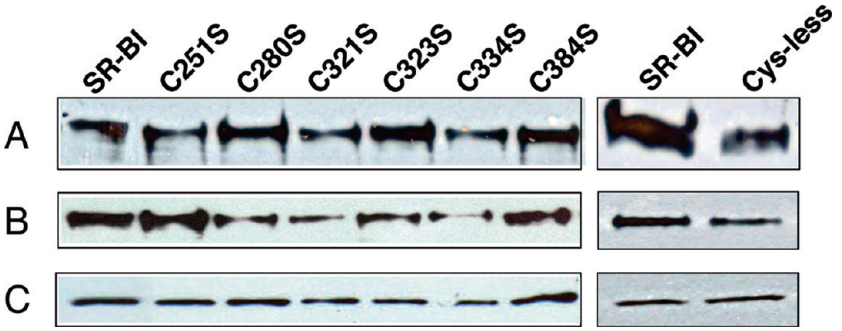

Figure 6. Cell surface expression of mutant SR-BI receptors. COS-7 cells expressing wild-type (in the presence of reducing TCEP) or single-mutant SR-BI receptors were incubated with MTSEA-biotin. In a separate experiment, cells expressing wild-type and Cys-less SR-BI receptors were incubated with Sulfo-NHS-LC-biotin. All biotinylated proteins were immunoprecipitated as previously described.[42] Immunoblot analysis of SR-BI expression (A) at the cell surface by biotinylation (from ~200 μg of total lysate) and (B) in 20 μg of total cell lysate is shown. Protein bands were detected at ~82 kDa using an antibody directed against the C-terminal cytoplasmic tail of SR-BI. (C) GAPDH was detected at ~37 kDa as a loading control. Data are representative of three independent transfections.

COS-7 cells expressing either wild-type or mutant SR-BI receptors were assayed for their ability to stimulate efflux of FC to HDL. Interestingly, with the exception of C323S-SR-BI, all single-Cys mutant receptors displayed wild-type levels of cholesterol

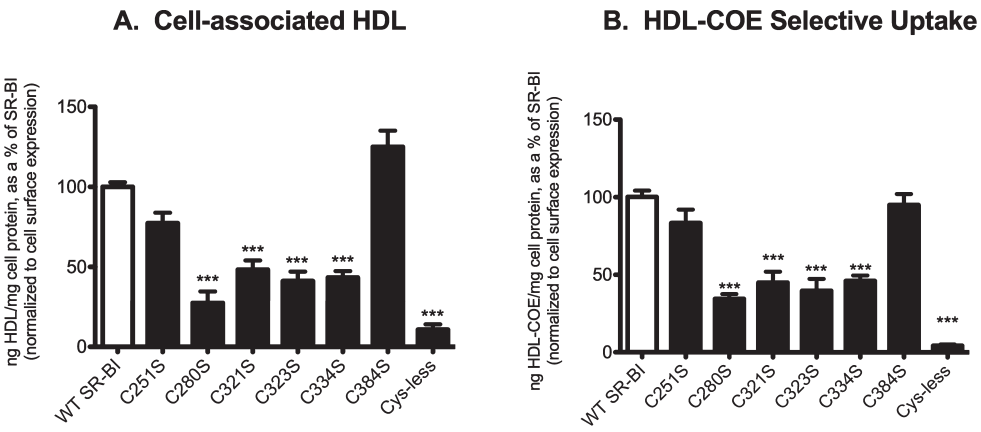

Figure 7. HDL binding and selective uptake of HDL-COE in cells expressing wild-type and mutant SR-BI receptors. COS-7 cells transiently expressing wild-type or mutant SR-BI receptors were incubated at 37 °C for 1.5 h with doubly labeled [^{125}I]DLT and [^{3}H]COE-labeled HDL (10 μg/mL). Cells were then processed and analyzed as previously described.[29] Cell-associated HDL (A) and selective uptake of HDL-COE (B) are shown. For both panels, data are normalized to cell surface receptor expression (as determined by flow cytometry of cells from parallel wells) and expressed relative to wild-type SR-BI values, which were set at 100%, following subtraction of empty vector values. Flow cytometry did not reveal any significant differences in cell surface expression among all receptors tested [ANOVA, $p > 0.05$ (data not shown)]. Values represent means ± the standard deviation of six replicates from two independent experiments and are representative of eight independent experiments overall. Asterisks denote $p < 0.001$ as determined by one-way ANOVA.

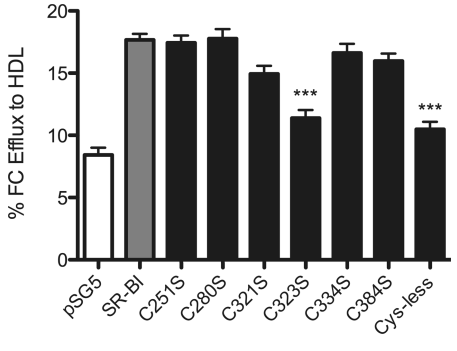

Figure 8. Efflux of [^{3}H]cholesterol to HDL by wild-type and mutant SR-BI receptors. COS-7 cells transiently expressing wild-type or mutant SR-BI receptors were prelabeled with [^{3}H]cholesterol and incubated with 50 μg/mL HDL for 4 h to measure the efflux of [^{3}H]cholesterol to the HDL acceptor. Values represent means ± the standard deviation of six independent experiments, each performed in quadruplicate. Asterisks denote $p < 0.001$ as determined by one-way ANOVA.

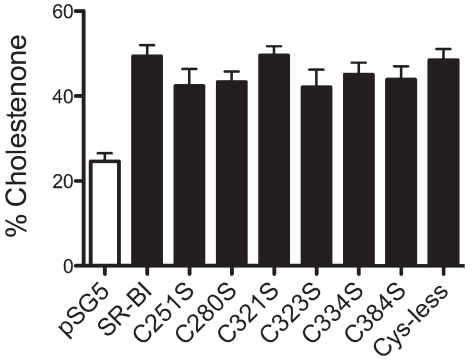

Figure 9. Sensitivity of cells expressing wild-type and mutant SR-BI receptors to cholesterol oxidase. COS-7 cells transiently expressing wild-type or mutant SR-BI receptors were prelabeled with [^{3}H]cholesterol and incubated with exogenous cholesterol oxidase (0.5 unit/mL) for 4 h to measure the production of cholestenone from free cholesterol. Values represent means ± the standard deviation of five independent experiments, each performed in quadruplicate.

efflux (Figure 8). Cys-less SR-BI exhibited a similar muted ability to efflux FC to HDL as C323S-SR-BI. Control FC efflux to 0.5% BSA was minimal (1.0−1.8%) and did not differ between wild-type SR-BI and mutant receptors (data not shown).

SR-BI also increases the size of the pool of plasma membrane FC available for oxidation by exogenous cholesterol oxidase, as judged by a higher membrane content of cholestenone.[34] To determine whether our mutations affected the distribution of FC in the plasma membrane, COS-7 cells expressing either wild-type or mutant SR-BI receptors were treated with exogenous cholesterol oxidase 48 h post-transfection. Lipids were extracted, separated by thin layer chromatography, and assessed for cholestenone production. Surprisingly, none of the mutant receptors, including Cys-less SR-BI, exhibited a decrease in FC accessibility, as indicated by wild-type levels of cholestenone production (Figure 9). This observation suggests that the sensitivity of plasma membrane FC pools to exogenous oxidase is not dependent on Cys residues, nor perhaps a specific conformation of SR-BI.

DISCUSSION

The absence of a high-resolution structure of SR-BI remains an obstacle in understanding the molecular mechanisms of SR-BI-mediated cholesterol transport and how the molecular architecture of the extracellular domain contributes to this process.[29,30,36−38] In this study, we sought to understand the role of the six evolutionarily conserved Cys residues that are located in the extracellular domain of SR-BI. We provide the first known confirmation of the involvement of these Cys residues in disulfide bond formation and show that these bonds are most likely intramolecular. We also investigated the impact of these disulfide bonds on SR-BI function using in vitro assays. Our data revealed that Cys-less SR-BI, as well as four single-Cys mutant receptors (C280S-, C321S-, C323S-, and C334S-SR-BI), displayed a reduced ability to bind HDL and mediate the selective uptake of HDL-CE. Further, C323S-SR-BI and Cys-less SR-BI

displayed a defect in efflux of FC to HDL. Taken together, we conclude that a specific disulfide bonding pattern is required to maintain SR-BI in a conformation that supports these cholesterol transport functions.

An interesting finding of our experiments is that Cys 323 is critical for SR-BI function, as it not only disrupted HDL-CE selective uptake but also was the only single-Cys mutant receptor that revealed a deficiency in FC efflux to HDL. Cys-less SR-BI displayed a similarly blunted level of efflux capacity. It is likely that the lack of the C323 disulfide bond negatively affects the conformation of the extracellular domain, thus supporting the notion that SR-BI function is compromised if proper protein folding is not maintained. Alternatively, it is also possible that mutation of C323 may have caused disulfide shuffling or reorganization to form new "promiscuous" disulfide bonds, as reported for mutants of the $P2X_1$ receptor.[7] The resulting "alternate" protein conformation may be unfavorable for efflux of FC to HDL.

An unexpected finding of our studies was the fact that sensitivity of plasma membrane FC to exogenous cholesterol oxidase was not affected by any of the Cys mutations. The various functions of SR-BI (e.g., FC efflux vs oxidase sensitivity) are considered to be separable and dependent on individual subdomains within the extracellular domain.[38,63,64] Therefore, it is possible that the region of the extracellular domain that spans Cys 251−Cys 384 is located within a subdomain that is not responsible for mediating the redistribution of plasma membrane FC. Indeed, we recently published data that demonstrate the importance of several hydrophobic regions within the N-terminal half of the extracellular domain of SR-BI that influence the cholesterol oxidase sensitivity of plasma membrane FC.[42]

We took advantage of the known changes in electrophoretic migration of proteins based on oxidation and reduction states of the sulfhydryls[52−55] to demonstrate that the extracellular Cys residues, all of which are located in the C-terminal half of the receptor, are most likely involved in intramolecular disulfide bonding. This result was not surprising because the presence of a monomeric SR-BI population in cell lysates is indicative of intramolecular bonding.[43] This bonding pattern has a considerable impact on the structural organization of the region spanning residues 240−400 of the extracellular domain of SR-BI and most likely maintains this region in a conformation that supports productive complex formation,[65] where both the HDL ligand and the receptor are precisely aligned and/or have the capacity to undergo appropriate conformational changes for efficient lipid transport to occur. On the basis of the data obtained from the single-Cys mutant SR-BI receptors, mutation of C280, C321, or C334 is still able to mediate between 35 and 46% of HDL-CE selective uptake, suggesting that loss of a single disulfide bond may not have major effects on disruption of a functional conformation. However, mutation of C323 only, or all the Cys residues in Cys-less SR-BI, results in much greater decreases in the ability of SR-BI to mediate selective uptake of HDL-CE and free cholesterol efflux to HDL, thus highlighting the important contribution of all the disulfide bonds, in particular the bond linked to C323, to the maintenance of SR-BI's extracellular domain in a conformation that supports its functions.

As our previous studies demonstrated that SR-BI dimerizes via regions within the C-terminal half of its extracellular domain,[59] it is possible that the extracellular Cys residues could contribute to SR-BI oligomerization by mediating receptor−receptor interactions via intermolecular disulfide bonds. However, as shown in Figure 4, PFO−PAGE analysis of Cys-less SR-BI eliminated this possibility, as this mutant receptor was still able to form dimers, similar to wild-type SR-BI. Our data suggest that self-association between C-terminal regions of SR-BI is most likely mediated via noncovalent interactions between SR-BI monomers (P. J. Hanson and D. Sahoo, unpublished data). More recently, a glycine dimerization motif was also identified in the N-terminal transmembrane domain of SR-BI.[66]

In addition to the maintenance of protein structure, disulfide bond formation has been reported to be important for proper protein targeting to the plasma membrane.[61,62] Because immunofluorescence and biotinylation studies confirmed the cell surface localization of all single-Cys mutant SR-BI receptors, as well as Cys-less SR-BI, we are confident that Cys residues in SR-BI are not required for protein trafficking to the plasma membrane. It should be noted, however, that as the level of Cys-less SR-BI expression in total cell lysates (by immunoblotting) was generally slightly lower than the level of wild-type SR-BI expression, loss of all disulfide bonds may have minor effects on protein stability or rates of receptor trafficking to the cell surface. Therefore, unlike other transporters such as sucrose permease,[67] ACAT1,[68] mouse organic anion transporter 1,[61] and sulfate transporter SHST1[69] that display normal membrane trafficking and function despite the loss of all Cys residues, we believe the loss of function in our mutant receptors correlates with the requirement of Cys residues for the structural integrity of the extracellular domain of SR-BI.

As the disruption of selective uptake of HDL-CE correlates with the loss of disulfide bonds from SR-BI's extracellular domain, assigning a bonding pattern to each pair of Cys residues will be critical to gaining insight into how the structural organization of the C-terminal half of the extracellular domain influences SR-BI function. Our data suggest that a single substitution of Cys residues at positions 251 and 384 had no effect on SR-BI function. These results imply that a disulfide bond most likely exists between C251 and C384, and this bond is not necessary to support a receptor conformation that supports the cholesterol transport-related functions of SR-BI. It is also possible that these residues may be in separate disulfide bonds but are able to substitute for one another if one of the residues is absent, as reported for other proteins.[6,14] However, this is an unlikely scenario as Ser substitution of Cys residues at positions 280, 321, 323, and 334 causes a significant reduction in SR-BI function, suggesting that these four Cys residues are combined in such a way to form two disulfide bonds. Experiments are currently underway to map each extracellular disulfide bond in SR-BI. These data will be valuable in understanding how the conformational restraints afforded by these covalent bonds impact the molecular architecture of this portion of the extracellular domain that is critical for SR-BI function.

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■ ABBREVIATIONS

ACAT, acyl-CoA:cholesterol acyltransferase; BME, β-mercaptoethanol; CE, cholesteryl ester; COE, cholesteryl oleyl ether; FC, free cholesterol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high-density lipoprotein; MTSEA, *N*-biotinylaminoethyl methanethiosulfonate; PFO, perfluorooctanoic acid; SR-BI, scavenger receptor class B type I; TCEP, tris(2-carboxyethyl)phosphine.

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