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Contributions of a Disulfide Bond and a Reduced Cysteine Side Chain to the Intrinsic Activity of the High-Density Lipoprotein Receptor SR-BI

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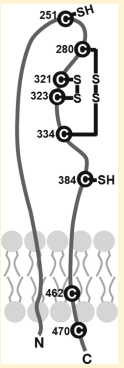
Supporting Information

ABSTRACT: The high-density lipoprotein (HDL) receptor scavenger receptor class B, type I (SR-BI), binds HDL and mediates selective cholesteryl ester uptake. SR-BI's structure and mechanism are poorly understood. We used mass spectrometry to assign the two disulfide bonds in SR-BI that connect cysteines within the conserved Cys^{321}-Pro^{322}-Cys^{323} (CPC) motif and connect Cys^{280} to Cys^{334}. We used site-specific mutagenesis to evaluate the contributions of the CPC motif and the side chain of extracellular Cys^{384} to HDL binding and lipid uptake. The effects of CPC mutations on activity were context-dependent. Full wild-type (WT) activity required Pro^{322} and Cys^{323} only when Cys^{321} was present. Reduced intrinsic activities were observed for CXC and CPX, but not XXC, XPX, or XXX mutants (X ≠ WT residue). Apparently, a free thiol side chain at position 321 that cannot form an intra-CPC disulfide bond with Cys^{323} is deleterious, perhaps because of aberrant disulfide bond formation. Pro^{322} may stabilize an otherwise strained CPC disulfide bond, thus supporting WT activity, but this disulfide bond is not absolutely required for normal activity. $C^{384}X$ (X = S, T, L, Y, G, or A) mutants exhibited altered activities that varied with the side chain's size: larger side chains phenocopied WT SR-BI treated with its thiosemicarbazone inhibitor BLT-1 (enhanced binding, weakened uptake); smaller side chains produced almost inverse effects (increased uptake:binding ratio). $C^{384}X$ mutants were BLT-1-resistant, supporting the proposal that Cys^{384}'s thiol interacts with BLT-1. We discuss the implications of our findings on the functions of the extracellular loop cysteines in SR-BI and compare our results to those presented by other laboratories.



The high-density lipoprotein (HDL) receptor scavenger receptor class B, type I (SR-BI), is a cell surface receptor that plays an important role in controlling the structure and metabolism of HDL in mice and humans.[1−3] Studies in mice have established the importance of SR-BI in mediating cholesterol transport, as well as gastrointestinal, endocrine, reproductive, and cardiovascular physiology (reviewed in ref 1), and SR-BI's role in protecting against atherosclerosis and coronary heart disease (reviewed in ref 1). In humans, SR-BI influences HDL metabolism. There is considerable interest in HDL metabolism because the risk for atherosclerotic disease is inversely proportional to the levels of HDL cholesterol.[4,5]

SR-BI controls HDL metabolism by mediating the transfer of cholesteryl esters in HDL particles to cells via a mechanism called selective lipid uptake.[6−8] Selective lipid uptake is fundamentally different from classic receptor-mediated endocytosis via coated pits.[9] After HDL binds to the receptor, SR-BI selectively transfers the cholesteryl esters from the HDL particle into the cells. The lipid-depleted HDL subsequently dissociates from the receptor and re-enters the circulation, and the receptor is free to mediate additional rounds of selective lipid uptake. During this process, there is no requirement for cellular internalization of the SR-BI−HDL complex or degradation or hydrolysis of the HDL particles.[10] The precise mechanisms underlying HDL binding and lipid uptake are not well understood.

SR-BI is a member of the large CD36 superfamily of proteins. In mammals, there are three members of the CD36 superfamily: SR-BI, CD36, and LIMPII. CD36 is a multifunctional, cell surface protein that binds many diverse ligands, participates in fatty acid transport, and is a candidate lipid sensor (reviewed in refs 11 and 12). LIMPII (lysosomal integral membrane protein II) is located primarily in the membranes of endosomes and lysosomes. It functions as an intracellular sorting receptor for the lysosomal enzyme β-glucocerebrosidase[13] and is involved in the pathogensis of a subset of human enterovirus species A viruses (e.g., enterovirus 71 that causes hand, foot, and mouth disease).[14]

Members of the CD36 superfamily share a common topology with a large, glycosylated extracellular loop, and N- and C-terminal transmembrane domains, each with short cytoplasmic extensions.[15] Relatively little is known about the detailed structures of these receptors or how their structures contribute to their functions. For example, only relatively recently has there been analysis of the chemical state (reduced or oxidized) and potential functional roles of the extracellular cysteines of SR-BI.[16−19] There are six cysteine residues in the

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extracellular loop of SR-BI that are highly conserved in SR-BI homologues, and some of them are found in equivalent positions in CD36 and LIMPII (see Discussion). Two of them (Cys^{251} and Cys^{384}) are in a reduced state with free thiols, and four (Cys^{280}, Cys^{321}, Cys^{323}, and Cys^{334}) participate in two intramolecular disulfide bonds.[16] Several studies have focused their attention on these cysteine residues in SR-BI[16−19] (see Discussion and Supporting Information). For example, we have shown using a $C^{384}S$ mutant that Cys^{384} is apparently important for inhibition of the receptor by the small molecule, thiosemicarbazone inhibitor BLT-1[16,20,21] (apparent IC_{50} of ∼50 nM) because free thiol groups can directly or indirectly interact with thiosemicarbazones and a $C^{384}S$ mutation in mouse SR-BI renders the receptor completely resistant to this inhibitor. Others have systematically mutated each extracellular loop cysteine to either Ser or Gly.[17−19] Nevertheless, there remain many outstanding issues regarding these cysteines, including the precise disulfide bonding pattern and how these conserved extracellular cysteines contribute to normal receptor activities such as SR-BI-mediated HDL binding and lipid uptake.

Here, we have extended the biophysical and functional analysis of the cysteines in mouse SR-BI. We have directly determined that there is a Cys^{321}−Cys^{323} disulfide bond and infer that the second disulfide bond links Cys^{280} to Cys^{334}. We also have used mutagenesis to focus on the roles of Cys^{321}, Cys^{323}, and Cys^{384}. We found that some mutations in the Cys^{321}-Pro^{322}-Cys^{323} (CPC) motif, which includes an intramotif disulfide bond and is highly conserved among SR-BI homologues, can significantly reduce intrinsic receptor activity. The effects on receptor activity of any given mutation in the CPC motif can be context-dependent. For example, full intrinsic receptor activity depends on a proline at position 322 when it is flanked by Cys^{321} and Cys^{323}, but not when both cysteines have been mutated. It is possible that the intervening proline in the CPC motif stabilizes the disulfide bond between Cys^{321} and Cys^{323}. Although the CPC motif in SR-BI is highly conserved, it is not critical for full, intrinsic receptor activity, as a GGG replacement of the triplet exhibits WT receptor activity. In addition, we have shown that substitution of the Cys at position 384 with residues other than serine ($C^{384}X$, where X = T, L, Y, G, or A) prevents BLT-1 inhibition, and that these $C^{384}X$ mutations can either enhance or weaken SR-BI-mediated HDL binding and lipid uptake, depending on the size of the side chain.

EXPERIMENTAL PROCEDURES

Lipoproteins and Inhibitor. Human HDL was isolated from donors by density gradient ultracentrifugation and labeled with ^{125}I ([^{125}I]HDL), or [^{3}H]cholesteryl ester ([^{3}H]CE or [^{3}H]CE-HDL) as described previously.[2,22−25] BLT-1 was obtained as described previously[20,21] and stored in 5 mM stock solutions in DMSO at −20 °C.

Mass Spectrometric Analysis of the Free Thiols in Recombinant SR-BI-t1. We used immunoaffinity chromatography to isolate essentially homogeneously pure mSR-BI-t1 as described previously.[21,26] SR-BI-t1, a C-terminally epitope-tagged mouse SR-BI, was expressed in *N*-acetylglucosaminyltransferase I (GnTI)-deficient HEK293S cells.[21,27] Purified SR-BI-t1 (5 μg) was deglycosylated with 1000 units of PNGase F (Prozyme) in 20 mM MES (pH 6), 15 mM NaCl, and 1.5% octyl glucoside. The surfactant Rapigest [1% (v/v), Waters Corp.] was added to final concentration of 0.2% to facilitate proteolytic digestion. The first step of digestion was initiated by adding sequencing grade trypsin (Promega, 1:10 enzyme:substrate ratio, 37 °C, overnight). The second step of digestion used the protease GluC (Promega) under the same conditions. Rapigest was removed by acidification with 1% trifluoroacetic acid, precipitation, and centrifugation. The samples next were desalted on C18 stagetips,[28] and then the peptides were analyzed by liquid chromatography and tandem mass spectrometry (LC−MS/MS). LC−MS/MS was performed on a Q-Exactive instrument (Thermo, Inc.), employing a "top 12" data-dependent MS/MS analysis over a 120 min gradient. Spectra were searched against the Uniprot mouse proteome using Spectrum Mill. Initial search parameters included a 30% minimal matched peak intensity and ±30 and ±50 ppm tolerances on precursor and product ion masses, respectively, allowing a maximum of five missed enzymatic cleavages. Allowed modifications included oxidized methionine, oxidized linear cysteine (for the disulfide bond mass shift), and deamidated asparagine. All data were analyzed as previously described.[16]

Generation of Mutant cDNAs. Site-directed mutagenesis to generate mutants was performed using the QuikChange II kit (Stratagene) on templates of either WT mouse SR-BI cDNA in the pcDNA3.1 vector or mutant SR-BI cDNAs ($P^{322}G$ and $P^{322}A$) in the same vector. Table S1 (Supporting Information) lists the amino acid substitution mutants and polymerase chain reaction (PCR) primers used to generate the corresponding PCR products. The PCR products were treated with endonuclease DpnI and transformed into XL1-Blue supercompetent *Escherichia coli* cells by heat shock. The plasmid DNAs of selected ampicillin-resistant colonies were isolated and sequenced to confirm the presence of the mutations. The plasmid DNAs were then transformed into *E. coli* and isolated.

COS Cells and Cell Culture. COS cells were maintained and assay incubations performed at 37 °C in a humidified 5% CO_2/95% air incubator as previously described.[16] Cellular protein levels were determined by the method of Lowry et al.[29]

Transient Expression of cDNAs in COS Cells. COS cells were grown in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin (medium A) supplemented with 10% (v/v) fetal bovine serum (FBS) (medium B) and transiently transfected with WT and mutant SR-BI cDNAs embedded in pcDNA3.1-based expression vectors. For transfections, COS cells were seeded on day 0 in the wells of 24-well dishes (50000 cells per well) in medium B without antibiotics and grown at 37 °C overnight. On day 1, the cells were treated with 10 μg per well of the indicated expression vector DNA [WT SR-BI, mutated forms of SR-BI, or the "empty" vector (EV) pcDNA3 without an expression cassette insert as a control] using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. Antibiotics were added to the medium on day 2, and then receptor activity assays were performed on day 3.

Assays. *[^{125}I]HDL Binding and Uptake of [^{3}H]CE from [^{3}H]CE-HDL.* On day 3, cells plated and transfected as described above were washed twice with prewarmed (37 °C) medium A with 0.5% (w/v) bovine serum albumin (BSA) (medium C). [^{125}I]HDL binding and cellular uptake of [^{3}H]CE from [^{3}H]CE-HDL then were measured in medium C by adding 10 μg of protein/mL of the radiolabeled HDL and incubating the cells at 37 °C for 2 h in the absence (total activity, duplicate determinations) or presence (nonspecific activity, duplicate

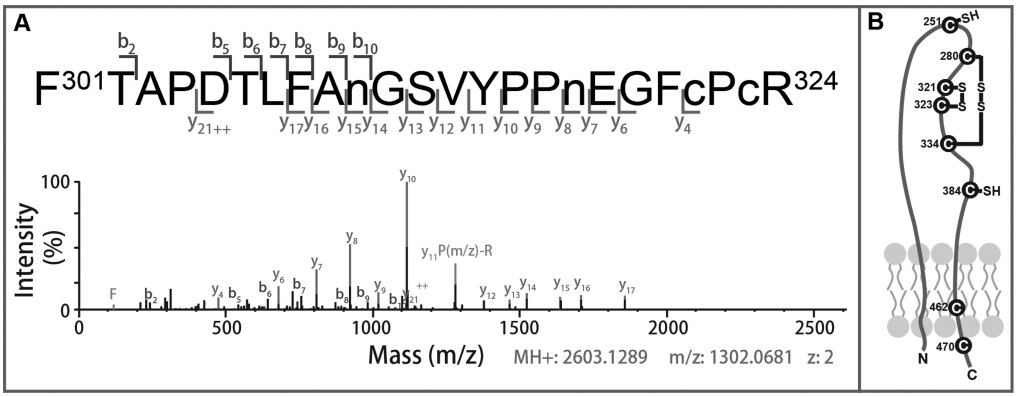

Figure 1. Identification of the disulfide bond pattern in mouse SR-BI by mass spectrometry. (A) Purified SR-BI-t1 was deglycosylated and proteolytically digested (trypsin and GluC), and peptides produced without reduction and alkylation were subjected to LC−MS/MS analysis as described in Experimental Procedures. The MS/MS spectrum of the F^{301}TAPDTLFAnGSVYPPnEGFcPcR^{324} peptide is shown together with the peptide's sequence in which lowercase letters denote amino acids that are modified: deamidated asparagine (n) and oxidized linearized cysteine (c). Both doubly and triply charged forms of the peptide were observed at m/z 1302.0681 and 868.38, respectively, the masses expected for the peptide with a Cys^{321}−Cys^{323} disulfide bond. (A) MS/MS spectrum for the fragmented doubly charged peptide. The amino acid composition of the doubly charged 1302.0681 mass is assigned by the fragmentation pattern in which the y and b ion series represent the different cleavage points in the amino acid sequence from the C- and N-termini, respectively (100% of the intensity = 1.2×10^7). (B) Model of mouse SR-BI illustrating the approximate locations of the cysteines in the extracellular loop. Cysteines 251 and 384 are fully reduced [free thiols (-SH)[16]], and disulfide bonds (S−S) link Cys^{321} to Cys^{323} and Cys^{280} to Cys^{334}.

determinations) of a 40-fold excess of unlabeled HDL.[2,6] The amounts of [^{125}I]HDL or [^{3}H]CE associated with the cells were determined as previously described.[2,6,16,30] At least two and usually four independent experiments were performed for all mutants. The data shown in Figures 2−5 are from representative individual experiments. The data in Figure S3 of the Supporting Information represent averages from three to four independent experiments, as indicated.

Specific [^{125}I]HDL binding and [^{3}H]CE uptake were calculated as the difference between total and nonspecific values. The amounts of cell-associated [^{3}H]CE are expressed as the equivalent amount of [^{3}H]CE-HDL protein (nanograms) to permit comparisons of the relative amounts of [^{125}I]HDL binding and [^{3}H]CE uptake on the same scale.[30] All errors and error bars represent standard errors of the mean. In assays using BLT-1 [37 °C, medium C with 0.5% (v/v) DMSO (medium D)], cells were preincubated with or without 1 μM BLT-1 for 1 h and then incubated with radiolabeled lipoproteins as described above, all in medium D in the presence or absence of 1 μM BLT-1.

Flow Cytometric Analysis of SR-BI Cell Surface Expression. Relative levels of SR-BI expressed on the cell surface were determined using anti-SR-BI antibody KKB-1 and flow cytometry as previously described.[16,31] Briefly, COS cells were incubated with antibody KKB-1 (1/1000 dilution) at 37 °C for 1 h in medium C containing 25 M Hepes (pH 7.4), followed by a second incubation with FITC-conjugated goat anti-rabbit IgG (Amersham Biosciences, 1/1000 dilution) at 37 °C for 1 h in the same medium, and then harvested in phosphate-buffered saline (PBS) containing 2 mM EDTA. The levels of cell surface SR-BI in these unfixed cells were determined by flow cytometry using FITC fluorescence filters.[31] Cell surface receptor expression was used to normalize the results for receptor activity assays ([^{125}I]HDL binding and [^{3}H]CE uptake) and thus permit determination of the intrinsic receptor activities in a manner independent of the surface expression level. Normalization of binding and uptake data based on surface receptor expression assumes that the mutations did not interfere with KKB-1 antibody binding.

Data Analysis. Data were analyzed using Prism 5 (GraphPad Software, Inc., San Diego, CA). Statistical analyses comparing either HDL binding or lipid uptake at a single substrate concentration for WT SR-BI and the individual mutants, as well as the uptake-to-binding ratio, were performed using one-way analysis of variance (ANOVA) with Tukey post-testing.

RESULTS

Mass Spectrometric Determination of the Disulfide Bond Pattern in Mouse SR-BI. Previously, we used mass spectrometry (MS) to show that two of the six extracellular cysteines, Cys^{251} and Cys^{384}, in homogeneously pure, epitope-tagged (t1), and active recombinant mouse SR-BI (SR-BI-t1) are reduced (free thiol side chains) and the other four (Cys^{280}, Cys^{321}, Cys^{323}, and Cys^{334}) are involved in two intramolecular disulfide bonds, but we did not establish the bonding pattern at that time.[16] To identify the precise disulfide bonding pattern, we digested recombinant, purified SR-BI-t1 (see Experimental Procedures) with trypsin and GluC and analyzed the peptide products by LC−MS/MS. Digestion was performed after deglycosylation without prior reduction and alkylation to permit direct identification of disulfide bonds. Analysis of the unreduced receptor yielded peptides covering approximately 78% of the full-length protein sequence (Figure S1 of the Supporting Information). One of the peptides identified and characterized was F^{301}TAPDTLFANGSVYPPNEGFCPCR^{324} that contains two cysteines, Cys^{321} and Cys^{323}. MS analysis of this peptide established that it contains an intrapeptide disulfide bond between its two cysteines (Figure 1A). Thus, there is a disulfide bond linking Cys^{321} and Cys^{323} in mouse SR-BI (Figure 1B). We were unable to identify peptides containing Cys^{280} and Cys^{334} despite many attempts using a variety of digestion conditions, presumably because of incomplete digestion of the unreduced protein. Nevertheless, we infer that the second disulfide bond in SR-BI must link Cys^{280} to Cys^{334} (Figure 1B) because we previously established that these two cysteines participate in intramolecular disulfide bonding.[16] Below we will describe additional studies of the influence of

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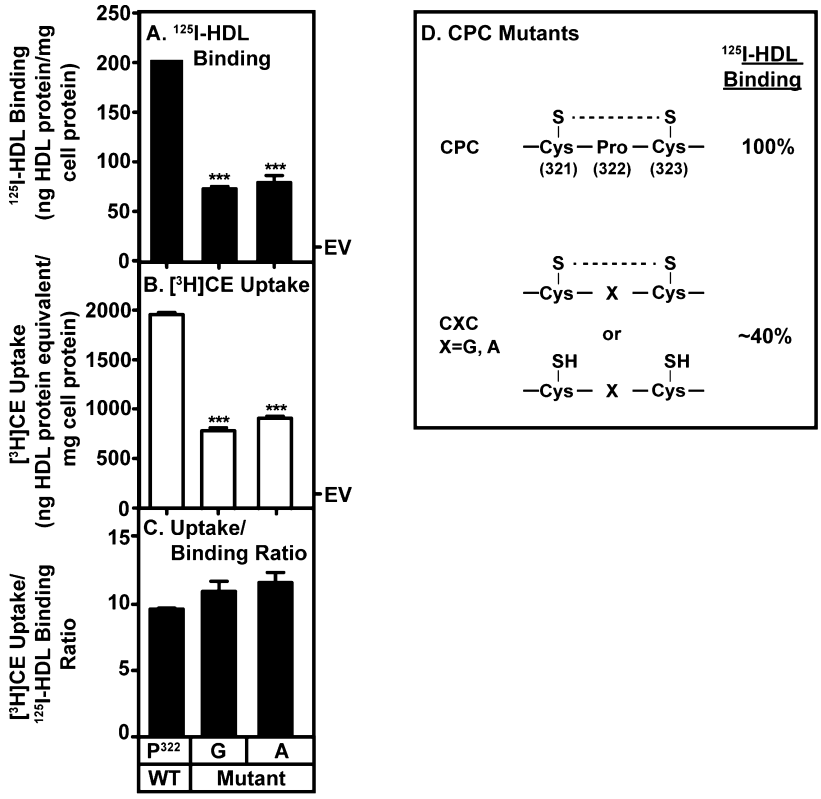

Figure 2. Effects of CXC (Pro^{322}) mutations on [^{125}I]HDL binding (A), uptake of [^{3}H]cholesteryl ester ([^{3}H]CE) from [^{3}H]CE-HDL (B), and the lipid transport efficiency (uptake-to-binding ratio) (C). Receptor-specific values were calculated as the differences between the total binding or uptake values (duplicate determinations) and the corresponding nonspecific values measured in the presence of a 40-fold excess of unlabeled HDL (duplicate determinations). Lipid transport efficiency (C) was determined as the ratio of specific [^{3}H]CE uptake to specific [^{125}I]HDL binding. Binding and uptake values were normalized to correct for differences in receptor surface expression relative to WT SR-BI based on flow cytometry (1.0 for WT SR-BI, 0.7 for P^{322}G, and 0.7 for P^{322}A). EV (right-hand sides of panels A and B) represents the activities of cells transfected with the empty vector (EV) as a negative control. (D) Representations of the P^{322}X mutations introduced into the CPC region of SR-BI (disulfide bonds represented as horizontal dashed lines) and approximate relative [^{125}I]HDL binding values. The P^{322}X mutants are shown either with a CXC intramotif disulfide bond (top) or with side chains of the cysteines indicated by SH (bottom), which could represent either a free thiol or side chain sulfurs in uncharacterized, aberrant disulfide bonds. Statistical analyses comparing WT SR-BI and mutants were performed using one-way ANOVA with Tukey post-testing (***$P < 0.0001$).

both the disulfide bond between Cys^{321} and Cys^{323} and the nature of the side chain at position 384 on SR-BI activity.

Role of Pro^{322} in the CPC Motif in SR-BI-Mediated HDL Binding and Lipid Uptake. Intramolecular disulfide bonds between cysteines separated by a single intervening residue, CXC, are strained and can be thermodynamically unstable.[32] When proline is the intervening residue, the disulfide bond is stabilized relative to other intervening residues.[32,33] Our identification of a disulfide bond in the CPC motif raised the possibility that Pro^{322} might be required to stabilize this disulfide bond. Furthermore, this CPC motif is strongly conserved among members of the SR-BI family of proteins (see Figure S2 of the Supporting Information), suggesting that Pro^{322} may play an important role, in either stabilizing the disulfide bond or otherwise contributing to the protein folding or stability of functional receptors. To investigate the role of Pro^{322}, we generated mutant cDNA expression vectors that encode mouse SR-BI in which the proline at position 322 in the CPC motif was converted to glycine (P^{322}G) or alanine (P^{322}A) (CXC, where X = G or A, as illustrated in Figure 2D). These vectors, as well as vectors encoding WT SR-BI and an empty vector control (EV), were transiently transfected individually into COS cells, and we measured the [^{125}I]HDL binding and [^{3}H]CE uptake activities (37 °C, subsaturating HDL concentration of 10 μg of protein/mL). All results were corrected for the statistically significant differences in relative cell surface expression levels of the mutant receptors relative to WT, which were 1.0 for WT SR-BI, 0.7 for P^{322}G, and 0.7 for P^{322}A.

Mutation of Pro^{322} to either G or A resulted in significant reductions (~60%) in both [^{125}I]HDL binding (Figure 2) and uptake of [^{3}H]CE from [^{3}H]CE-HDL (Figure 2B) compared to those of WT SR-BI. However, the ratio of uptake to binding, the efficiency of lipid transport mediated by these mutants, did not differ from that of WT SR-BI (Figure 2C). These results suggest that the conserved Pro^{322} in the context of the CPC motif significantly contributes to SR-BI-mediated receptor activities, primarily at the level of intrinsic HDL binding.

Role of Cys^{321} and Cys^{323} in the CPC Motif in SR-BI-Mediated HDL Binding and Lipid Uptake Activities. Although the results from the Pro^{322} mutants are consistent with a mechanism by which Pro^{322} influences receptor activity by stabilizing the CPC disulfide bond in mouse SR-BI, a study by Guo et al.[18] suggested this might not be the case. Guo et al. reported that a C^{321}G mutation (GPC) does not alter receptor activity (HDL binding and lipid uptake) in human SR-BI, even though such a mutation would disrupt what we now know to be the intra-CPC motif's disulfide bond. They also reported that a

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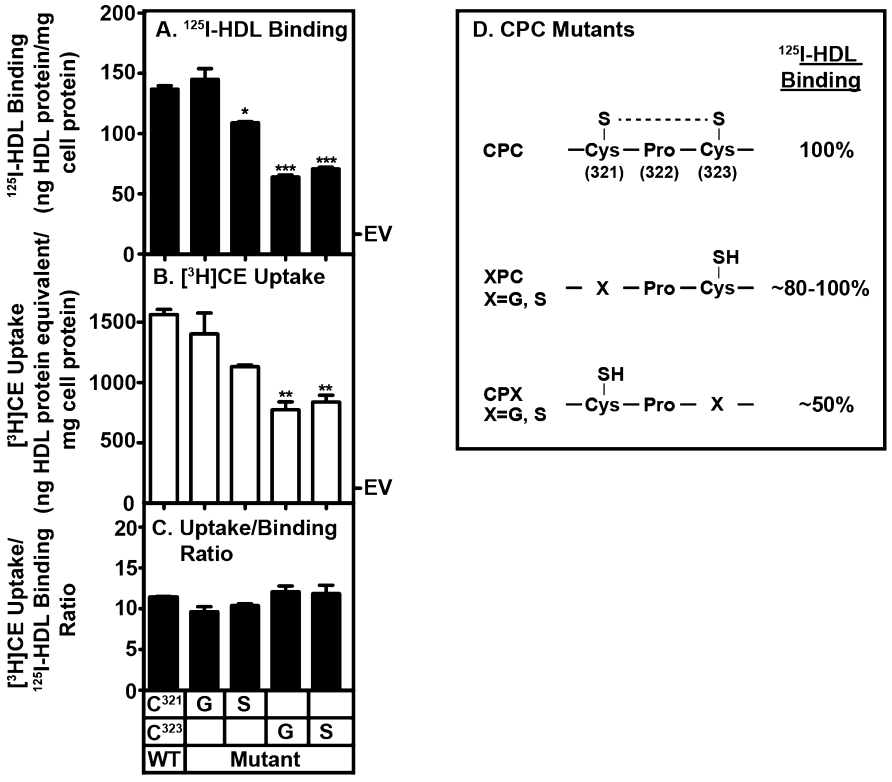

Figure 3. Effects of XPC (Cys^{321}) or CPX (Cys^{323}) mutations on [^{125}I]HDL binding (A), uptake of [^{3}H]cholesteryl ester ([^{3}H]CE) from [^{3}H]CE-HDL (B), and the lipid transport efficiency (uptake-to-binding ratio) (C). Receptor-specific [^{125}I]HDL binding (A) and uptake of [^{3}H]CE from [^{3}H]CE-HDL (B) mediated by WT SR-BI, the indicated C^{321}X or C^{323}X mutant (X = G or S), or the empty vector control (EV) in transfected COS cells were measured, and results were normalized for relative cell surface expression (1.0 for WT, 0.8 for C^{321}G, 0.7 for C^{321}S, 0.7 for C^{323}G, and 0.6 for C^{323}S) as described in the legend of Figure 2. The residues at positions 321 and 323 are indicated (single-letter code) in the grid at the bottom of the figure (an empty grid cell represents the WT amino acid at that position). (D) Representations of the C^{321}X and C^{323}X mutations introduced into the CPC region of SR-BI as described in the legend of Figure 2 and approximate relative [^{125}I]HDL binding values. Statistical analyses comparing WT SR-BI and mutants were performed using one-way ANOVA with Tukey post-testing (*P < 0.01; **P < 0.001; ***P < 0.0001).

C^{323}G mutation (CPG) dramatically reduces both HDL binding and lipid uptake.[18] Others have also reported analyses of C^{321}S and C^{323}S mutants in mouse and rat SR-BI[17,19] (see Discussion and Supporting Information). Using mouse SR-BI, we repeated and extended this work by substituting either Gly or Ser for Cys at these two positions in the CPC motif (illustrated in Figure 3D, XPC or CPX, where X = G or S) and measuring the receptors' activities in transiently transfected COS cells (37 °C, 10 μg of HDL protein/mL). All results were corrected for the statistically significant differences in relative cell surface expression levels of the mutant receptors relative to WT, which were 1.0 for WT SR-BI, 0.8 for C^{321}G, 0.7 for C^{321}S, 0.7 for C^{323}G, and 0.6 for C^{323}S.

Single-amino acid substitutions at Cys^{323} (CPX, where X = G or S) resulted in significant (~50%) reductions in both [^{125}I]HDL binding (Figure 3A) and [^{3}H]CE uptake (Figure 3B) yet did not alter the overall efficiency of lipid transport [uptake-to-binding ratio (Figure 3C)]. Others have also reported decreased activity in Cys^{323} (CPX, where X = G or S) mutants, although the extent of the decrease varied somewhat in different studies.[17−19] In addition, we found that mutations at Cys^{321} (XPC) had no significant effect on HDL binding or lipid uptake (X = G) or resulted in an only slight, although statistically significant, decrease (~20%) in HDL binding, but not in lipid uptake [X = S (Figure 3A,B)]. The XPC mutations did not alter the overall efficiency of lipid transport (Figure 3C). The essentially (80−100%) WT levels of activity of the C^{321}X mutants (XPC) suggest that our results with mouse SR-BI are consistent with those reported by Guo et al.[18] for human SR-BI and confirm that the disulfide bond in the CPC motif is not necessary for essentially normal receptor-mediated HDL binding and lipid uptake activities. However, others have reported different results for the effects of the C^{321}S mutation on SR-BI from mouse[17] or rat[19] (see Discussion and Supporting Information for details).

Consequences of Disrupting Native Disulfide Formation at Cys^{321} on SR-BI-Mediated HDL Binding and Lipid Uptake. We were intrigued by the similarities in the extent (40−50%) to which HDL binding activities were reduced in the Pro^{322} (CXC, where X = A or G) and Cys^{323} (CPX, where X = G or S) mutants. In both sets of mutants, the side chain of Cys^{321} either could not form (CPX, where X ≠ C) or, because of steric and thermodynamic considerations (CXC, where X ≠ P), was not expected to form a stable disulfide bond with the side chain at position 323. In these mutants, the sulfhydryl group of Cys^{321} might either be a free thiol or form a non-native (inappropriate) disulfide bond with a different cysteine either in the same SR-BI molecule (e.g., the free sulfhydryls at Cys^{251} or Cys^{384}) or in some other protein. This raised the possibility that the inability of Cys^{321}'s thiol group to form a disulfide bond with Cys^{323} was responsible for the similarity in the reduction of HDL binding activities for these mutants. To test this possibility, we generated an expression vector encoding a Gly^{321}-Pro^{322}-Gly^{323} (GPG) double mutant

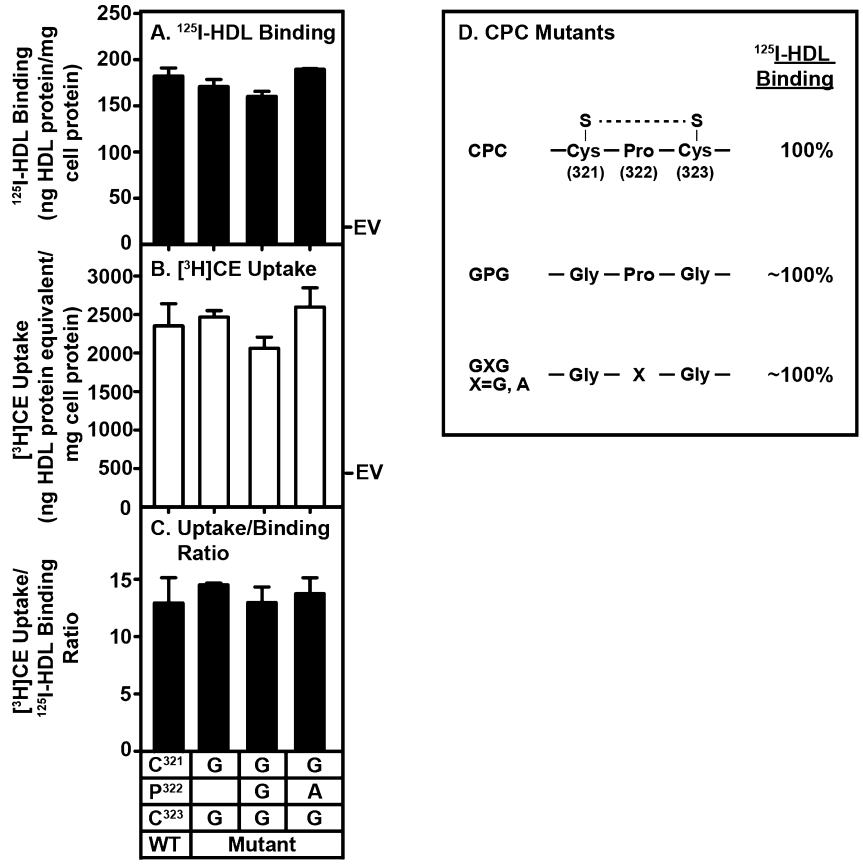

Figure 4. Effects of GPG or GXG mutations on [^{125}I]HDL binding (A), uptake of [^{3}H]cholesteryl ester ([^{3}H]CE) from [^{3}H]CE-HDL (B), and the lipid transport efficiency (uptake-to-binding ratio) (C). Receptor-specific [^{125}I]HDL binding (A) and uptake of [^{3}H]CE from [^{3}H]CE-HDL (B) mediated by WT SR-BI, the indicated GPG or GXG (X = G or A) double or triple mutant at positions 321−323, or the empty vector control (EV) in transfected COS cells were measured, and results were normalized for relative cell surface expression (1.0 for WT, 0.9 for GPG, 1.0 for GGG, and 1.0 for GAG) as described in the legend of Figure 2. The residues at positions 321−323 are indicated (single-letter code) in the grid at the bottom of the figure (an empty grid cell represents the WT amino acid at that position). (D) Representations of the GPG or GXG (X = G or A) double or triple mutations introduced into the CPC region of SR-BI as described in the legend of Figure 2 and approximate relative [^{125}I]HDL binding values. Statistical analyses comparing WT SR-BI and mutants were performed using one-way ANOVA with Tukey post-testing and showed no statistically significant differences.

(illustrated in Figure 4D) that could not form the disulfide bond or have a free thiol or inappropriate disulfide bond at position 321. We expressed the mutant in transiently transfected COS cells and determined its HDL binding and lipid uptake activities as described above. All results were corrected for differences in relative cell surface expression levels of the receptors, which were not statistically significant: 1.0 for WT SR-BI and 0.9 for GPG. Panels A−C of Figure 4 show that the GPG mutant (second bars from the left) exhibited normal HDL binding and lipid uptake activities (compare to those of WT, left-most bars). Thus, Cys is not required at position 321 or 323 for WT levels of receptor-mediated HDL binding and lipid uptake. The reduced HDL binding activities in CXC and CPX mutants were likely a consequence of either a free thiol or inappropriate disulfide bonding at Cys^{321}.

The essentially normal activity of the GPG double mutant permitted us to further assess the role of the conserved Pro^{322} in a manner independent of any effects that the adjacent Cys residues or the Cys^{321}−Cys^{323} disulfide bond might have on it. We generated and analyzed in transfected COS cells the activities of GXG (X = G or A) triple mutants (illustrated in Figure 4D). All results were corrected for relative cell surface expression levels of the receptors, which were not statistically significant: 1.0 for WT SR-BI and 1.0 for GXG (X = G or A). Panels A−C of Figure 4 show that both GXG triple mutants exhibited HDL binding and lipid uptake activities that were not significantly different than those of WT SR-BI or the GPG double mutant. Thus, essentially normal receptor activity does not require a Pro at position 322 when both cysteines are replaced with glycines at positions 321 and 323 (no disulfide bond). These results suggest that the reduction in HDL binding activity in CXC mutants when X ≠ P may be due to the destabilization of the disulfide bond and consequent inappropriate interactions of the side chains of Cys^{321} and/or Cys^{323} (free thiols and/or non-native disulfide bonding). There appears to be no intrinsic dependence on the proline at position 322 for the receptor's functional folding, surface expression, or activity.

To complete the mutational analysis of the CPC motif, we generated and analyzed the activities of two additional mutants that are single-residue modifications of the fully active G^{321}-G^{322}-G^{323} mutant: GGC and CGG (Figure S3 of the Supporting Information). The binding and lipid uptake activities of the GGC mutant (Figure S3A−C of the Supporting Information) were not significantly different from those of the WT receptor, whereas those for the CGG mutant were significantly lower (~20%) than those of the WT receptor. The reduction in uptake activity by the CGG mutant was

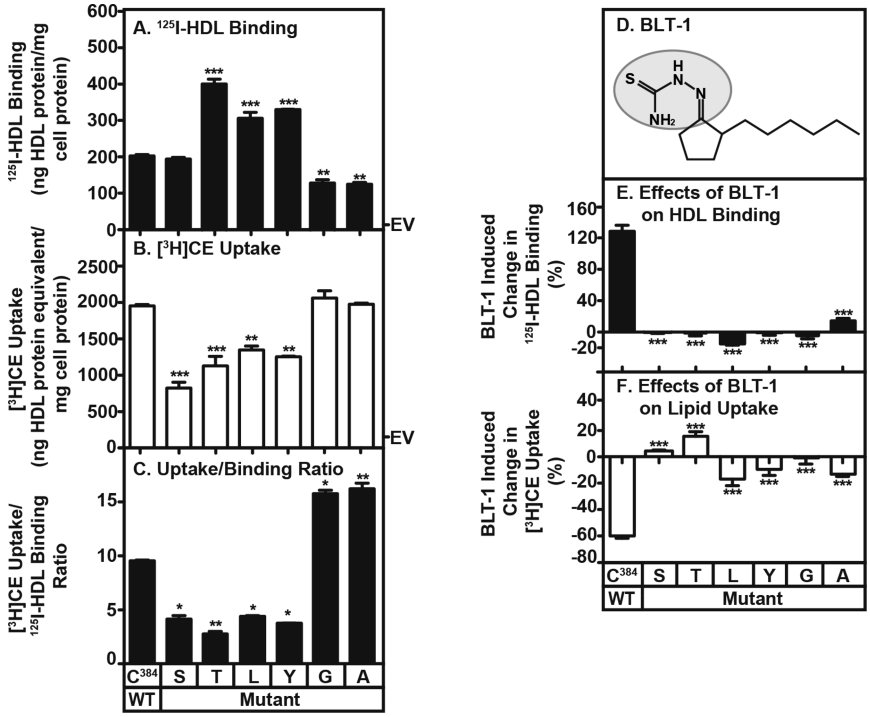

Figure 5. Effects of mutations of Cys^{384} on [^{125}I]HDL binding (A), uptake of [^{3}H]cholesteryl ester ([^{3}H]CE) from [^{3}H]CE-HDL (B), lipid transport efficiency (uptake-to-binding ratio) (C), and sensitivity to the small molecule inhibitor BLT-1 (D–F). Receptor-specific [^{125}I]HDL binding (A) and uptake of [^{3}H]CE from [^{3}H]CE-HDL (B) mediated by WT SR-BI or the indicated C^{384}X mutants (X = S, T, L, Y, G, or A; one-letter amino acid code) in transiently transfected COS cells were measured at 37 °C with a subsaturating concentration of HDL (10 μg of protein/mL) as described in Experimental Procedures. Receptor-specific [^{125}I]HDL binding (A) and uptake of [^{3}H]CE from [^{3}H]CE-HDL (B) mediated by WT SR-BI, the indicated P^{322}X mutants (X = G or A), or the empty vector control (EV) in transfected COS cells were measured, and results were normalized for relative cell surface expression (1.0 for WT SR-BI, 0.9 for C^{384}S, 1.0 for C^{384}T, 1.0 for C^{384}L, 1.0 for C^{384}Y, 0.9 for C^{384}G, and 0.9 for C^{384}A) as described in Figure 2. (D) Structure of BLT-1 with its thiosemicarbazone moiety shaded. [^{125}I]HDL binding (E) and uptake of [^{3}H]CE from [^{3}H]CE-HDL (F) of WT SR-BI and C^{384}X mutants were determined in the presence or absence of an excess (1 μM) of BLT-1. Values are expressed as the percentage change in receptor activity induced by BLT-1 and are calculated as the difference between activities in the presence and absence of BLT-1, divided by the activity in the absence of BLT-1. The activities without BLT-1 in the same experiment are shown in panels A (binding) and B (uptake). Negative values in panels E and F represent the loss of activity in the presence of BLT-1. Statistical analyses comparing WT SR-BI and mutants were performed using one-way ANOVA with Tukey post-testing (*P < 0.01; **P < 0.001; ***P < 0.0001).

significantly smaller than that by the CPG mutant [~50% (Figure 3B and Figure S3B of the Supporting Information)]. Perhaps steric or entropic constraints imposed by Pro^{322}, but not Gly^{322}, in CXG mutants force the side chain of Cys^{321}, and thus the receptor's extracellular domain, into a less productive conformation when a Cys^{321}–Cys^{323} disulfide cannot form. These findings confirm that when Cys^{321} cannot form a disulfide bond with Cys^{323}, the Cys^{321} reduces the intrinsic receptor activity.

Effect of the Identity of the Amino Acid at Position 384 on SR-BI-Mediated HDL Binding and Lipid Uptake Activities and Sensitivity to BLT-1 Inhibition. We previously reported that a C^{384}S mutation significantly altered the lipid uptake activity of mouse SR-BI.[16] To determine if variation in the size of the amino acid side chain at position 384 affects SR-BI-mediated HDL binding and lipid uptake activities, we generated multiple mutant cDNA expression vectors that encode mouse SR-BI in which Cys^{384} was substituted with amino acids with larger side chains [threonine (C^{384}T), leucine (C^{384}L), and tyrosine (C^{384}Y)], smaller side chains [glycine (C^{384}G) and alanine (C^{384}A)], or a similarly sized side chain [serine (C^{384}S)]. These vectors, as well as vectors encoding WT SR-BI and an empty vector control (EV), were transiently transfected individually into COS cells. Receptor activities, [^{125}I]HDL binding and uptake of [^{3}H]CE from [^{3}H]CE-HDL, of each mutant in these cells were measured at 37 °C at a subsaturating concentration of HDL (10 μg of protein/mL). All results were corrected for differences in relative cell surface expression levels of the receptors [determined by flow cytometry (see Experimental Procedures)]. The relative surface expression levels, which were not statistically significant from the WT receptor, were as follows: 1.0 for WT SR-BI, 0.9 for C^{384}S, 1.0 for C^{384}T, 1.0 for C^{384}L, 1.0 for C^{384}Y, 0.9 for C^{384}G, and 0.9 for C^{384}A.

Figure 5A shows that compared to WT SR-BI, the C^{384}T, C^{384}L, and C^{384}Y variants with the larger side chains exhibited significant, ~150–200% increases in HDL binding activity, while the level of binding to the C^{384}G and C^{384}A variants with the smaller side chains was reduced by ~35% (one-way ANOVA analysis with Tukey post-testing). As we reported previously,[16] binding of [^{125}I]HDL to C^{384}S did not differ from that to WT SR-BI. There also were significant reductions relative to the WT control in the uptake of [^{3}H]CE from [^{3}H]CE-HDL mediated by the C^{384}T, C^{384}L, or C^{384}Y variant (larger side chains), but not the C^{384}G or C^{384}A mutants with smaller side chains (Figure 5B). To compare the lipid transport efficiencies of WT SR-BI and its variants (amount of lipid transport normalized for surface [^{125}I]HDL binding), we calculated the ratio of [^{3}H]CE uptake to [^{125}I]HDL binding (Figure 5C). The efficiency of lipid transport mediated by

mutants in which Cys[384] was replaced with residues with larger side chains (C[384]T, C[384]L, and C[384]Y) was significantly reduced, whereas it was significantly increased for those (C[384]G or C[384]A) whose side chains are substantially smaller. As previously reported,[16] the lipid transport efficiency of C[384]S was significantly lower than that of WT SR-BI but exhibited no alteration in HDL binding. The relationship of these findings to those reported by others[17−19] for mutations at Cys[384] will be considered in Discussion and Supporting Information.

The apparent increase in HDL binding and reduction in lipid uptake in mutants at position 384 whose side chains are larger than that of cysteine (T, L, and Y) closely resemble the effects of the small molecule inhibitor BLT-1 [apparent IC_{50} of ∼50 nM (Figure 5D)] on WT SR-BI: increased binding at subsaturating HDL concentrations accompanied by decreased lipid uptake.[20] On the basis of structure−activity relationship analysis, which showed the thiosemicarbazone moiety in BLT-1 was important for its activity,[21] as well as loss of BLT-1 sensitivity of the C[384]S mutant, we suggested that a free sulfhydryl side chain at position 384 is required for BLT-1 sensitivity.[16] Panels E and F of Figure 5 show that all of the other C[384]X mutants (X = T, L, Y, G, or A) expressed transiently in COS cells also are resistant to BLT-1 (1 μM) with no BLT-1-induced significant change in HDL binding (panel E) or lipid uptake (panel F). These results support the proposal that in WT SR-BI the free sulfhydryl at position 384 may directly or indirectly interact with BLT-1, and they raise the possibility that larger side chains at position 384 might mimic the effects of BLT-1 by occupying some of the space into which BLT-1 binds, although other explanations are possible.

DISCUSSION

Relatively little is known about the detailed structure of SR-BI or how its structure contributes to its functions. For example, only relatively recently has there been analysis of the chemical state (reduced or oxidized) and potential functional roles of the extracellular cysteines of SR-BI.[16−19] In this study, we have determined the disulfide bonding pattern in recombinant mouse (m)SR-BI purified from mammalian cells overexpressing the protein using mass spectrometry. We previously showed that purified mSR-BI's six extracellular cysteines form two disulfide bonds involving Cys[280], Cys[321], Cys[322], and Cys[334] and that the side chains of Cys[251] and Cys[384] are in the reduced, thiol state.[16] Independently, Papale et al.[17] suggested on the basis of mutagenesis studies that there might be two disulfide bonds linking cysteines at positions 280, 321, 323, and 334. Subsequently, Hu et al.[19] also suggested that cysteines 280, 321, 323, and 334 might be involved in disulfide bond formation based on the reduced cell surface levels of expression of rat (r)SR-BI with serine mutations at these positions. We have now established directly that there is a disulfide bond linking Cys[321] and Cys[323] and therefore that the second disulfide bond links Cys[280] and Cys[334] (Figures 1B and 6). Papale et al.[17] and Metcalfe et al.[34] have reported that the side chain of SR-BI's Cys[384] in intact cells is resistant to modification by sulfhydryl reagents. This resistance might have been due to its participation in a disulfide bond.[17,34] Alternatively, direct MS analysis suggests that the side chain of Cys[384] is a free sulfhydryl group[16] that may be buried (see below) and thus inaccessible to these reagents unless the native structure is perturbed.[34] It is noteworthy that SR-BI's disulfide pattern (Figure 6, left panel) differs considerably from that reported by Rasmussen et al.[35] for SR-BI's homologue, bovine CD36 (Figure 6, middle panel),

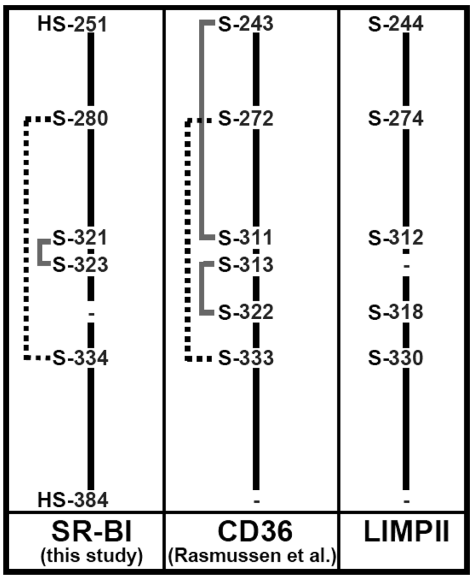

Figure 6. Alignment of extracellular cysteine residues in members of the mammalian CD36 superfamily: SR-BI, CD36, and LIMPII. The approximate relative locations of the six extracellular cysteines in both SR-BI and CD36 and five extracellular cysteines in LIMPII are illustrated (positions in the amino acid sequences are shown). Dashes indicate the absence of cysteines when compared to the presence of cysteines at equivalent locations in other family members (e.g., there are no cysteines in CD36 or LIMPII equivalent to Cys[384] in SR-BI). The disulfide bonds reported for mouse SR-BI (this study) and bovine CD36[35] are shown as lines connecting the sulfur atoms in the side chains of the cysteines. The dotted lines represent disulfide bonds that are in equivalent positions in the SR-BI and CD36 sequences. The disulfide bonding pattern of LIMPII has not yet been reported.

the first and only other report of the disulfide pattern in a member of the CD36 superfamily of proteins. In SR-BI and CD36, five of the six extracellular loop cysteines are in precisely or nearly equivalent positions in the aligned (BLAST[36]) primary sequences (Figure 6): 251/243 (SR-BI/CD36), 280/272, 321/311, 323/313, and 334/333. There is no cysteine in CD36's sequence that is near Cys[384] of SR-BI. Because of the relatively low level of sequence similarity in the region containing cysteines 321, 323, and 334 in SR-BI and cysteines 311, 313, 322, and 333 in CD36, as well as a gap in that region in CD36 relative to SR-BI, the relative alignment of cysteines in this region is ambiguous and that shown in Figure 6 is slightly modified relative to that produced by BLAST to align the two CXC motifs in SR-BI and CD36.

The six extracellular loop cysteines in bovine CD36 are reported to form three disulfide bonds (Figure 6): Cys[243]−Cys[311], Cys[272]−Cys[333], and Cys[313]−Cys[322].[35] The disulfide of CD36 linking Cys[272] to Cys[333] and that of SR-BI linking Cys[280] to Cys[334] are in equivalent positions in the sequences based on BLAST and COBALT sequence alignments[36,37] and thus are presumably in structurally equivalent positions (denoted by dotted lines in Figure 6). The other mammalian CD36 family member, LIMPII, has only five extracellular loop cysteines (Figure 6, right), three of which have clear equivalents in SR-BI: 251/224 (SR-BI/LIMPII), 280/274, and 334/330. The other two cysteines in LIMPII, at positions 312 and 318, are close to but because of sequence gaps difficult to match unequivocally with Cys[321], Cys[323], and Cys[334] of SR-BI. At least one of the cysteine side chains in LIMPII must either be a free thiol or participate in intermolecular disulfide bonding. It seems

possible that there may be a disulfide bond in LIMPII linking Cys^{274} to Cys^{330} that would be equivalent to the apparently common disulfide bond in SR-BI (Cys^{280}–Cys^{334}) and CD36 (Cys^{272}–Cys^{333}). There is no CXC motif in LIMPII, but there is one in CD36, C^{311}-F^{312}-C^{313}, that seems likely to be in a position analogous to that of the C^{321}-P^{322}-C^{323} motif in SR-BI. However, as might be expected because of the intervening phenylalanine, these two cysteines in CD36 (C^{311}-F^{312}-C^{313}) apparently are not disulfide-bonded to each other as they are in SR-BI (ref 35 and see below).

We[16] and others[17−19] previously have studied the influence of cysteines on SR-BI's activities by mutating its cysteines individually or in groups to either Ser or Gly and then measuring the cell surface expression and receptor activities of the mutants expressed from transfected cDNAs in cultured cells. Four different groups have reported results using either mouse (m), rat (r), or human (h) SR-BI and a variety of expression vectors and assays.[16−19] Papale et al.,[17] Guo et al.,[18] and Hu et al.[19] independently have systematically mutated each of the six extracellular loop cysteines and studied their effects on HDL binding and lipid uptake. All of the published reports considered cell surface expression as well as absolute levels of receptor activity (binding and lipid uptake). While some of the results for some of the mutations in these independent reports and this study are similar (e.g., no effect of a $C^{321}G$ mutation on receptor activity reported by Guo et al.[18] or in this study), no two of these reports are in full accord. Some examples of the similarities and differences are described in the Supplemental Discussion of the Supporting Information. Here we have repeated and in some cases extended previously reported analyses of mutations of cysteines at positions 384, 321, and 323, exclusively using mSR-BI, a common expression vector, and the same methods for transiently transfecting expression vectors into COS cells and measuring receptor activities, with explicit normalization for cell surface expression differences.

Cys^{321}-Pro^{322}-Cys^{323}. Sequence analysis (BLAST and COBALT) of members of the CD36 superfamily suggests that SR-BI homologues are the only members of the superfamily to have a Cys^{321}-Pro^{322}-Cys^{323} (CPC) triplet motif (unpublished and see sequences of SR-BI homologues in Figure S2 of the Supporting Information), which we have shown contains an intramotif disulfide bond in mSR-BI. Those SR-BI homologues identified using BLAST [includes mammals (primates, rodents, and a marsupial), birds, an amphibian, and a reptile] all have a Cys at position 384, except fish (five of five have V^{384}, four of five have Cys^{251}, and all have Cys^{280}, Cys^{321}, Cys^{323}, and Cys^{334}). Although invertebrates have many CD36 superfamily members, we have not identified equivalent CPC-containing SR-BI homologues in invertebrates. CX_nC ($n \geq 1$) motifs can mediate oxidation sensitive metal binding,[38−40] and CPC and CXXC motifs can mimic the activity of thioredoxin and protein disulfide isomerase (PDI) (thiol−disulfide exchange).[33,41,42] Thiol−disulfide exchange has been proposed to be a key trigger of envelope protein-induced fusion of some viral envelopes and their target cell plasma membranes, and the fusion proteins of some viruses contain the CXXC motif.[43−45] One proposed mechanism by which SR-BI may mediate selective lipid uptake is hemifusion[31,46,47] of the phospholipid/protein monolayer shell of HDL with the plasma membrane, a process that would be somewhat similar to at least the first steps of viral membrane fusion. An additional potential role of Cys^{323}, first described by Li and colleagues,[18,48,49] is that Cys^{323} in the CPC motif is also part of another short, conserved sequence in SR-BI, $^{323}CXXS^{326}$, that belongs to the CXXC/S redox family motif. Guo et al. have suggested that the site of binding of HDL to SR-BI may lie close to the CXXS (and thus the CPC) motif.[18]

We examined the potential contributions of each of the residues in the CPC motif to SR-BI activity by generating single-substitution mutations at each position followed by testing the HDL binding and lipid uptake activities of the variants. Intramolecular disulfide bonds between cysteines separated by a single intervening residue (CXC) can be thermodynamically unstable because of entropic considerations.[32] When proline is the intervening residue, such as the CPC in SR-BI, the disulfide bond is stabilized relative to other intervening residues.[32,33] The full WT activity of SR-BI required Pro^{322} and Cys^{323} only when Cys^{321} was present; there were reduced intrinsic activities of CXC and CPX mutants, but essentially wild-type activities of XXC, XPX, and XXX mutants (X ≠ WT residue, e.g., GGG mutant). Indeed, in all independent reports to date (refs 17−19 and this study), CPX mutants ($X^{323} \neq C$) exhibited significantly reduced receptor activities (e.g., ≤50% of WT). WT levels of intrinsic receptor HDL binding and lipid uptake activities do not require absolutely (1) a disulfide bond linking positions 321 and 323, (2) cysteines at either of these two positions, or (3) a proline at position 322. Nevertheless, a free thiol side chain at position 321 that cannot form a disulfide bond with Cys^{323} is deleterious, perhaps because of aberrant intra- or intermolecular disulfide bond formation with other cysteines (e.g., the free sufhydryls at Cys^{251} or Cys^{384} in SR-BI). Others[17,19] have also noted that mutations disrupting potential disulfide bonds in SR-BI could result in the formation of aberrant disulfide bonds that could interfere with surface expression, HDL binding, and/or lipid uptake. It appears likely that the conserved Pro^{322} stabilizes an otherwise strained CPC disulfide bond, thus supporting full SR-BI HDL binding and lipid uptake activities. Why is the CPC motif with an intramotif disulfide bond so highly conserved among apparent SR-BI homologues (Figure S2 of the Supporting Information), but not other members of the CD36 superfamily, yet it can be replaced by a GGG triplet without altering HDL binding and lipid uptake? Perhaps the $^{321}CPC^{323}$ and/or $^{323}CXXS^{326}$ motifs play critical roles in SR-BI activities (e.g., see refs 48 and 49) that are independent of HDL binding and lipid uptake activities examined in this study. In this regard, it is noteworthy that in addition to mediating the transport of the lipid between cells and lipoproteins, SR-BI can serve as a signaling receptor to activate intracellular signaling cascades[50−52] and as a receptor that mediates the entry of hepatitis C virus into hepatocytes.[53−61]

Cys^{384}. We previously reported that for mSR-BI a $C^{384}S$ mutation resulted in a receptor that was completely resistant to the inhibitor BLT-1.[16] BLT-1's inhibitory activity depends on its thiosemicarbazone group,[21] which we suggested is likely to interact directly or indirectly with Cys^{384}.[16] Treatment of WT SR-BI with BLT-1 irreversibly inhibits lipid uptake while increasing HDL binding affinity.[20] Thus, the efficiency of lipid uptake in WT SR-BI (uptake-to-binding ratio) is reduced by BLT-1. We also observed in the mSR-BI $C^{384}S$ mutant a significant (60%) decrease in lipid uptake activity without an alteration in HDL binding.[16] However, others have reported contemporaneously or soon thereafter that a $C^{384}S$ substitution in mSR-BI[17] or rSR-BI[19] or a $C^{384}G$ substitution in hSR-BI[18] had little or no significant effect on HDL binding or lipid uptake activity. We revisited the role of Cys^{384} by analyzing

HDL binding and lipid uptake as well as the inhibitory activity of BLT-1 on a series of $C^{384}X$ mutants, where the side chains of the replacement residues were either larger than (X = T, L, or Y), smaller than (X = G or A), or approximatety the same size as (X = S) cysteine.

We found that all of the substitutions at Cys^{384} resulted in complete resistance to inhibition by BLT-1, confirming our earlier results with $C^{384}S$.[16] We also found that the influence of these Cys^{384} substitutions on receptor activity varied depending on the replacement's side chain. The efficiency of lipid transport was significantly reduced when Cys^{384} was replaced with residues with larger side chains because of increased binding and decreased uptake, a result similar to that seen in WT SR-BI treated with BLT-1.[20] In contrast, the efficiency of lipid transport was significantly increased for those residues with substantially smaller side chains because of reduced binding but essentially normal uptake. One potential explanation of these findings is that in mutants with larger side chains at position 384, those side chains might occupy the same binding site that BLT-1 does or otherwise distort the native conformation of the receptor in a fashion similar to that of BLT-1 and thus mimic the effect of BLT-1. It is possible that the free sulfhydryl at Cys^{384} in WT SR-BI directly or indirectly (e.g., via ligating to an intermediary metal ion) interacts with BLT-1,[16] with the bulk of the bound BLT-1 molecule occupying a cavity immediately adjacent to Cys^{384}'s side chain. In contrast, the smaller side chains in the $Cys^{384}X$ (X = G or A) mutants apparently have an opposite influence (increased efficiency of lipid transport).

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Discussion, amino acid substitution mutants and PCR primers used to generate the corresponding mutant cDNA expression vectors (Table S1), amino acid sequence of the recombinant protein mSR-BI-t1 that was used for mass spectrometry analysis and those peptides in the unreduced protein detected by mass spectrometry (Figure S1), alignment of portions of the sequences of 52 SR-BI homologues (Figure S2), and effects of GGC or CGG mutations on [^{125}I]HDL binding, uptake of [^{3}H]cholesteryl ester ([^{3}H]CE) from [^{3}H]CE-HDL, and lipid transport efficiency (uptake-to-binding ratio) (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

HDL, high-density lipoprotein; SR-BI, scavenger receptor class B, type I; CE, cholesteryl ester; Cys or C, cysteine; Gly or G, glycine; Pro or P, proline; Ser or S, serine; LC−MS/MS, liquid chromatography and tandem mass spectrometry; WT, wild-type.

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