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Tryptophan 415 is Critical for the Cholesterol Transport Functions of Scavenger Receptor BI

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Abstract

High density lipoproteins (HDL) are anti-atherogenic particles, primarily due to their role in the reverse cholesterol transport pathway whereby HDL delivers cholesteryl esters (CE) to the liver for excretion upon interaction with its receptor, scavenger receptor BI (SR-BI). We designed experiments to test the hypothesis that one or more of the eight highly conserved tryptophan (Trp; W) residues in SR-BI are critical for mediating function. We created a series of Trp-to-phenylalanine (Phe, F) mutant receptors, as well as Trp-less SR-BI (W-SR-BI), and assessed their ability to mediate cholesterol transport. Wild-type (WT) or mutant SR-BI receptors were transiently expressed in COS-7 cells, and cell surface expression was confirmed. Next, we showed that Trp-less- and W415F-SR-BI had significantly decreased abilities to bind HDL and promote selective uptake of HDL-CE, albeit with higher selective uptake efficiency as compared to WT-SR-BI. Interestingly, only Trp-less-, but not W415F-SR-BI, showed an impaired ability to mediate efflux of free cholesterol (FC). Furthermore, both W415F- and Trp-less-SR-BI were unable to reorganize plasma membrane pools of FC based on lack of sensitivity to exogenous cholesterol oxidase. Restoration of Trp 415 into the Trp-less-SR-BI background was unable to rescue Trp-less-SR-BI's impaired functions, suggesting that Trp 415 is critical, but not sufficient for full receptor function. Furthermore, with the exception of Trp 262, restoration of individual extracellular Trp residues, in combination with Trp 415, into the Trp-less-SR-BI background partially rescued SR-BI function, indicating that Trp 415 must be present in combination with other Trp residues for proper cholesterol transport functions.

Graphical abstract

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NOTES

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Keywords

HDL-CE; selective uptake; SR-BI; efflux; tryptophan; cholesterol oxidase; LIMP-2

Removal of circulating cholesterol via the reverse cholesterol transport pathway (RCT) is an anti-atherogenic mechanism vital to combating cardiovascular disease (CVD)^{1–3}. In this pathway, high-density lipoprotein (HDL) transports cholesterol from peripheral tissues to the liver for excretion via bile formation. Scavenger receptor class B type I (SR-BI) is the most physiologically relevant HDL receptor that is critical for mediating cholesteryl ester (CE) delivery from HDL into hepatic cells in the final steps of RCT⁴. Overexpression of SR-BI in mice results in decreased HDL-cholesterol (HDL-C) levels, increased cholesterol excretion, and decreased progression of atherosclerosis^{5–7}. On the other hand, SR-BI-null mice display increased progression of atherosclerosis, despite higher HDL-C levels^{8–10}. Recently, mutations in SR-BI have been identified in patients with high HDL-C levels^{11, 12}. Paralleling observations in mice, functional analysis of these mutant receptors also revealed impaired SR-BI-mediated cholesterol transport functions^{12, 13}.

SR-BI is an 82 kDa heavily glycosylated cell surface receptor with no available high-resolution structural information. Hydropathy plot analyses predict that SR-BI consists of a very large extracellular (EC) domain (408 amino acids) anchored by two membrane-spanning domains and N- and C-terminal cytosolic domains¹⁴. SR-BI-mediated delivery of HDL-CE into cells occurs via selective uptake, a two-step process that requires binding of HDL to the EC domain of SR-BI^{15–19}, followed by the selective transfer of only the HDL core lipids into the cell^{17, 18, 20}. The selective uptake of HDL-CE likely requires proper alignment between the ligand and receptor²¹, and may be facilitated by the formation of SR-BI homo-oligomers^{22, 23} that constitute a ‘hydrophobic channel’ through which HDL-CE can be delivered to cells in a concentration-dependent manner²⁴. Structural features of the EC domain may also facilitate selective uptake of HDL-CE, and these include intramolecular disulfide bond formation between extracellular cysteine residues^{25, 26}, as well as a region of hydrophobicity in the N-terminal half of the EC domain²⁷.

In the current study, we determined the importance of eight highly conserved extracellular tryptophan (Trp; W) residues in SR-BI-mediated cholesterol transport functions. Our

findings suggest that mutation of all Trp residues to phenylalanine (Phe; F) in SR-BI impairs all cholesterol transport functions of SR-BI. Specifically, Trp 415 was identified to be critical for mediating the binding of HDL to SR-BI, as well as the selective uptake of HDL-CE into cells. However, restoration of Trp 415 into a Trp-less background did not completely restore receptor function, indicating the requirement for a combination of tryptophan residues for optimal SR-BI function.

EXPERIMENTAL PROCEDURES

Materials

The following antibodies were used: polyclonal anti-SR-BI directed against the C-terminal cytoplasmic domain or the extracellular domain (Novus Biologicals, Inc., Littleton, CO); FITC-conjugated goat anti-rabbit IgG secondary antibody (BD Pharmingen, Pittsburgh, PA); horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham- GE Healthcare), anti-mouse IgG (Amersham- GE Healthcare). [³H]Cholesteryl oleoyl ether (COE) was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). [¹²⁵I]Sodium iodide and [³H]cholesterol were purchased from Perkin-Elmer. Cholesterol oxidase from *Streptomyces*, cholesterol, 4-cholesten-3-one, and cholesteryl oleate standards were purchased from Sigma.

Plasmids

Site-directed mutations at W9, W56, W181, W237, W246, W262, W415 and/or W474 were introduced into wild-type (WT) murine SR-BI [pSG5(SR-BI)]¹⁷ (Invitrogen, Grand Island, NY) where the Trp residue was mutated to phenylalanine (Phe; F). Mutagenesis and sequencing of plasmids were performed by Top Gene Technologies (Pointe-Claire, QC). We also generated a Trp-less (W) SR-BI receptor where all eight Trp residues were mutated to Phe. Mutants where Trp was reincorporated into a Trp-less SR-BI receptor were named according to the Trp residue that was restored. For example, the W^{W415} mutant harbors only Trp415 in the Trp-less SR-BI receptor background.

Cell Culture and Transfection

COS-7 cells were cultured in DMEM (Invitrogen) containing 10% calf serum (Invitrogen), 2 mM L-glutamine, 50 units/mL penicillin, 50 µg/mL streptomycin, and 1 mM sodium pyruvate. Cells were seeded in 10-cm dishes with 10 mL fresh media and transfected once they reached 60–70% confluence as described¹⁷. Briefly, prior to transfection, 30 µL FuGENE 6 (Promega, Madison, WI) was incubated with 10 µg of cDNA encoding pSG5 vector, wild-type or mutant SR-BI receptors (ratio of 3:1 FuGENE 6:DNA) for 15 min at room temperature in polystyrene round-bottom tubes. The FuGENE 6:DNA mixture was then added drop-wise to COS-7 cells in 10 mL media. Cellular assays were performed 48 h post-transfection, unless otherwise noted.

Cell lysis

Forty-eight hours post-transfection, COS-7 cells were washed twice with cold PBS (pH 7.4) and lysed with 1% NP-40 cell lysis buffer containing protease inhibitors. Protein concentrations were determined by the Lowry method as previously described²⁸.

Immunoblot analysis

Cell lysates were separated on 8% SDS-PAGE gels, transferred to nitrocellulose membranes and detected using an antibody specific for the C-terminal domain of SR-BI followed by horseradish peroxidase-conjugated anti-rabbit secondary antibody. Antigen-antibody complexes were visualized with SuperSignal West Pico reagent (Thermo Scientific).

Flow cytometry

COS-7 cells transiently expressing wild-type or mutant SR-BI receptors were trypsinized, washed, resuspended in DMEM and assessed for cell surface expression using flow cytometry as previously described²⁶, with minor modifications. Briefly, primary antibody (1:100 dilution) was incubated with cells for 1 h on ice. Following a wash, secondary antibody conjugated to a FITC probe was incubated with cells for 20 minutes on ice. Cytometric analysis was performed using an Accuri 6 cytometer (BD Biosciences; Blood Center of Wisconsin) equipped with a laser emitting at 488 nm. Analysis was performed using CFlow Plus software. Green fluorescence was measured and gates were set to exclude necrotic cells and cellular debris. Fluorescence intensity of events within the gated regions was quantified. Data were collected from 10,000 events for each sample. Determination of the relative fluorescent signal of transfected cells was based on the inclusion of only cells exhibiting high levels of fluorescence and exclusion of cells adjacent to auto-fluorescent, non-transfected or pSG5-transfected cells. Cells chosen for analysis were based off gating according to the FITC fluorescent signal so that empty vector-transfected cells constituted 1–2% of total cells in order to provide a baseline. Following baseline elimination, FITC relative mean fluorescence values were obtained for empty vector and WT or Trp-mutant receptors. Data are presented as “relative mean fluorescence intensity” to reflect the exclusion of non-fluorescent cells from each independent experiment.

Cell surface expression by immunofluorescence

Transiently-transfected COS-7 cells were re-plated 24 h post-transfection onto glass coverslips in 6-well plates. After an additional 24 h, cells were washed with cold PBS and fixed with 1% paraformaldehyde/PBS for 15 min at room temperature. Cells were then stained using a primary antibody directed against the extracellular domain of SR-BI (1:100) and a FITC-conjugated goat anti-rabbit IgG secondary antibody. Nuclei were stained with DAPI (Thermo Fisher). Cells were examined on a Nikon A1 confocal microscope imaging system.

HDL labeling, cell association of [¹²⁵I] HDL and uptake of [³H] HDL-COE

Human HDL (Alfa Aesar, Ward Hill, MA) was double-labeled with non-hydrolyzable [³H]COE and [¹²⁵I]dilactitol tyramine as previously described¹⁷. The various preparations of radiolabeled HDL had average specific activities of 341.61 ± 81.94 dpm/ng protein for [³H] and 163.26 ± 71.68 dpm/ng protein for [¹²⁵I]. COS-7 cells transiently transfected with empty pSG5 vector, wild-type SR-BI, or mutant SR-BI receptors were assayed for cell association of [¹²⁵I]-HDL and selective uptake of non-hydrolyzable [³H]-COE as described¹⁹. Data were calculated as ng HDL/mg cell protein or ng HDL-COE/mg cell protein for binding and selective uptake experiments, respectively. Vector (pSG5) values

were subtracted from all wild-type and mutant data. Results are 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.

Measurement of free cholesterol efflux to HDL

COS-7 cells transiently expressing empty vector, wild-type SR-BI, or mutant SR-BI receptors were pre-labeled with [³H]cholesterol 24 h post-transfection as described¹⁹. Seventy-two hours post-transfection, cells were incubated with 50 µg/mL HDL in 0.5% BSA/DMEM for 4 h. Free cholesterol release from cells to HDL was assessed by counting radioactivity associated with media and cells. Statistical comparisons were calculated by one-way ANOVA with Bonferroni post-tests for all groups.

Measurement of cholestenone production

COS-7 cells transiently expressing empty vector, wild-type SR-BI, or mutant SR-BI receptors were pre-labeled with [³H] cholesterol 24 h post-transfection as described¹⁹. Forty-eight hours post-transfection, cells were incubated with 0.5 U/mL cholesterol oxidase for 4 h at 37°C as previously described¹⁹. Statistical comparisons were determined using one-way ANOVA with Bonferroni post-test for all groups.

PFO-PAGE analysis of SR-BI oligomers

COS-7 cells expressing empty vector, wild-type, or mutant-SR-BI receptors were lysed with PBS containing protease inhibitors and sonicated as described²⁷. PFO-PAGE was performed as described²⁷ using 6% polyacrylamide gel and SR-BI detected by immunoblot analysis.

RESULTS

SR-BI mutant receptors are expressed at the cell surface

SR-BI has eight evolutionarily-conserved tryptophan (Trp; W) residues, six of which are located in the critical EC domain. To determine the importance of Trp residues in mediating the various cholesterol transport functions of SR-BI, we used site-directed mutagenesis to create a panel of single point mutations where each Trp residue was mutated to phenylalanine (Phe; F) to generate the following mutant SR-BI receptors: W9F-, W56F-, W181F-, W237F-, W246F-, W262F-, W415F-, and W474F-SR-BI. We also created an SR-BI receptor void of all Trp residues (Trp-less-SR-BI; W). Immunoblot analysis of cell lysates harvested from COS-7 cells transiently expressing wild-type (WT) or mutant SR-BI receptors indicated relatively similar levels of expression of all mutant receptors as compared to WT SR-BI (Figure 1A). Next, we evaluated the ability of the mutant receptors to express specifically at the cell surface by flow cytometry. Using an antibody directed against the EC domain of SR-BI, we demonstrated that WT SR-BI and all mutant SR-BI receptors expressed at comparable levels at the cell surface (Figure 1B). Cell surface expression was further confirmed by confocal microscopy, as well as in cell extensions as previously shown²⁹ (Figure 1C). Together these data demonstrate that mutating one, or all, of the tryptophan residues in SR-BI to phenylalanine did not affect the ability of the receptors to traffic to the cell surface.

Trp-less- and W415F-SR-BI receptors have an impaired ability to bind HDL and mediate selective uptake of HDL-COE, despite higher selective uptake efficiency

SR-BI plays a critical role in the last steps of RCT by mediating the selective delivery of HDL-CE into cells⁴. In order to determine the role of Trp residues in the ability of SR-BI to bind HDL and mediate the selective uptake of HDL-COE, COS-7 cells transiently expressing empty vector, WT or mutant SR-BI receptors were incubated with [¹²⁵I]/[³H]-HDL-COE and SR-BI function was assessed as previously described¹⁷. Our data revealed that mutation of all Trp residues resulted in an 89% decrease in the ability of SR-BI to bind HDL as compared to WT SR-BI (Figure 2A). This was accompanied by a 74% decrease in the ability of W-SR-BI to mediate selective uptake of HDL-COE (Figure 2B). We predict this effect was due to loss of Trp 415, as only W415F-SR-BI, but not other single Trp-to-Phe mutant SR-BI receptors, displayed an impaired ability to mediate HDL binding (46% of WT-SR-BI levels) and delivery of HDL-COE (50% of WT levels). However, both W415F- and W-SR-BI had higher calculated selective uptake efficiencies of HDL-COE (i.e. the amount of HDL-COE uptake based on levels of HDL binding) as compared to WT-SR-BI (Figure 2C). In order to determine if the presence of Trp 415 was sufficient for SR-BI to bind HDL and mediate proper selective uptake of HDL-CE, we restored Trp 415 into the Trp-less receptor background (referred to as W^{W415}) and its cell surface expression was confirmed (Figure S1). Restoration of Trp 415 was not able to rescue the receptor's ability to fully bind HDL and mediate selective uptake of HDL-COE (Figures 2A and B, right of vertical dashed line). However, restoration of Trp 415 did improve the selective uptake efficiency of HDL-COE to levels that are statistically higher than those of WT-SR-BI (Figure 2C, right of vertical dashed line). Similar trends were observed when the values for HDL binding and selective uptake of HDL-COE were normalized to cell surface expression of the respective mutant SR-BI receptors (Figures S2A and B). Together, these data suggest that Trp 415 is required, but not sufficient for full activity of WT SR-BI to bind HDL and mediate CE uptake from HDL.

Mutation of all Trp residues impairs the ability of SR-BI to efflux free cholesterol from cells to HDL

SR-BI also mediates the efflux of free cholesterol (FC) from cells to HDL acceptor particles in circulation^{30–32}. In order to determine the role of Trp residues in mediating SR-BI's ability to promote efflux of FC to HDL, COS-7 cells transiently expressing empty vector, WT or mutant SR-BI receptors were preloaded with [³H]cholesterol, and efflux of FC to HDL was measured. Only W-SR-BI displayed an impaired ability to promote efflux of FC to HDL acceptors (Figure 3), suggesting that loss of all Trp residues completely impairs the receptor's ability to release FC from cells to HDL. Interestingly, no single Trp residue could be attributed to mediating optimal efflux function. While loss of Trp 415 alone did not impair SR-BI's cholesterol efflux function, the restoration of W415 into W-SR-BI significantly increased the ability of W-SR-BI to efflux FC to HDL, demonstrating that while not required, Trp 415 likely contributes to SR-BI's cholesterol efflux function. Normalization of efflux values to cell surface expression showed similar trends (Figure S2C).

To determine whether a defect in the ability of W415F-SR-BI to mediate the efflux of FC to HDL occurred at earlier time points, we repeated the FC efflux assay, as well as assays to measure HDL binding and selective uptake of HDL-COE, over a course of time. As shown in Figure 4 (Panels A and B), differences in the abilities of WT- and W415F-SR-BI to bind HDL and mediate selective uptake of HDL-COE were evident at time points as early as 15 min of incubation with HDL. WT-SR-BI had a Bmax of 19.2 ± 1.8 ng HDL/mg cell protein, while W415F-SR-BI had a Bmax of 10.2 ± 1.0 ng HDL/mg cell protein. However the rate of efflux of FC to HDL was similar between WT- and W415F-SR-BI at all the time points tested (Figure 4C).

Trp-less- and W415F-SR-BI are unable to reorganize pools of plasma membrane FC

The ability of SR-BI to increase the plasma membrane pool of FC available for oxidation by exogenous cholesterol oxidase (as judged by a higher membrane content of cholestenone) is a unique function of this receptor and does not require the presence of HDL³³. To determine whether loss of one or all Trp residues affected the re-distribution of plasma membrane cholesterol by SR-BI, COS-7 cells transiently expressing empty vector, WT or mutant SR-BI receptors were incubated with exogenous cholesterol oxidase 48 h post-transfection and cellular levels of cholestenone were assessed by thin layer chromatography. Our data revealed that decreased cholestenone production was only observed upon expression of either Trp-less- or W415F-SR-BI (Figure 5). Normalization of cholestenone production values to cell surface expression of the mutant receptors showed similar patterns (Figure S2D). These results parallel the trends observed for HDL binding and selective uptake of HDL-CE with these same mutant receptors. Therefore, these data suggest that Trp 415 is required for a variety of SR-BI's functions that are both dependent and independent of the presence of HDL.

Restoration of individual extracellular Trp residues, but not Trp 262, in combination with Trp 415, partially rescues SR-BI's cholesterol transport functions

As restoration of Trp 415 alone could not rescue all of SR-BI's cholesterol transport functions, we hypothesized that receptor function might be fully restored if Trp 415 was present in combination with another extracellular Trp residue. To test this hypothesis, mutant SR-BI receptors were designed such that Trp 415 and one of each of the other extracellular Trp residues were reincorporated into the Trp-less SR-BI background receptor (e.g.

W^{W415,W56} is an SR-BI receptor that harbors only Trp 415 and Trp 56). These mutant receptors were transiently expressed in COS-7 cells and their cell surface expression was confirmed (Figure S3). Our data indicate that all extracellular Trp residues, with the exception of Trp 262 (i.e. W^{W415,W262}), were able to partially or completely rescue

W^{W415} SR-BI's ability to bind HDL (Figure 6A) or mediate the selective uptake of HDL-COE (Figure 6B). Similar trends were observed when assessing cholestenone production, where with the exception of Trp 262, restoration of any Trp residue into the W^{W415} SR-BI background also partially rescued W^{W415} SR-BI's ability to promote sensitivity of membrane FC to exogenous cholesterol oxidase (Figure 6C).

Loss of function is independent of SR-BI oligomerization

SR-BI has been shown to form dimers and higher-order oligomers^{22, 23, 34}, and it is hypothesized that oligomerization of SR-BI is important for mediating HDL-CE delivery into cells²⁴. In order to determine if the impaired cholesterol transport functions observed for selected mutant SR-BI receptors were due to changes in receptor oligomerization, we analyzed cell lysates expressing WT or mutant SR-BI receptors by PFO-PAGE, a technique suitable for assessing the oligomeric status of membrane-bound proteins³⁵. Our data revealed that all mutant receptors maintained their ability to form dimers and higher order oligomers, similar to WT-SR-BI (Figure 7). These data suggest that receptor oligomerization is independent of the Trp mutations.

DISCUSSION

Whole body cholesterol regulation is dependent on the ability of SR-BI to promote the bidirectional flux of cholesterol to and from HDL, and this function is key to the early and late stages of RCT, respectively³⁶. However, a mechanistic understanding of how cholesterol transport is regulated by HDL/SR-BI interactions remains hampered by the lack of a high-resolution structure of the full-length SR-BI receptor, and in particular, of the extracellular domain that is critical for SR-BI function^{15–19, 37}. In this study, we used a mutagenesis approach to understand the role of eight highly-conserved tryptophan residues in mediating SR-BI's cholesterol transport functions. Our data revealed that Trp-less-SR-BI (i.e. mutation of all Trp residues to Phe) was unable to bind HDL, mediate the selective uptake of HDL-COE, and increase the sensitivity of membrane FC to exogenous cholesterol oxidase, most likely due, in part, to the specific loss of Trp 415. However, as restoration of Trp 415 into the Trp-less-SR-BI background was not able to rescue Trp-less-SR-BI's impaired functions, it appears that Trp 415 is critical but not sufficient for proper receptor function. Furthermore, with the exception of Trp 262, restoration of individual extracellular Trp residues, in combination with Trp 415, into the Trp-less-SR-BI background partially rescued SR-BI functions, thus indicating that Trp 415 must be present in combination with other Trp residues in order to mediate proper cholesterol transport functions.

One interesting observation from our studies was that the decrease in HDL-COE uptake by W415F- and W-SR-BI did not parallel the decrease in HDL binding to the same extent (Figure 2A and B), thus resulting in an increased selective uptake efficiency of HDL-COE for these two mutant receptors as compared to WT-SR-BI (Figure 2C). It is possible that the levels of selective uptake efficiency were artificially high as a result of the low amount of HDL binding. This trend has been observed in a previous study³⁸. It is also possible that mutation of Trp 415 to Phe results in a conformational change within the receptor that makes it less favorable for HDL binding. However, the HDL particles that do bind are better positioned on the extracellular domain of SR-BI for more efficient transfer of HDL-COE to cells, thus reinforcing the idea of “productive complex” formation between SR-BI and HDL to facilitate HDL-CE uptake by SR-BI²¹. *In vivo* studies of W415F- and W-SR-BI should clarify the impact of these mutations on reverse cholesterol transport.

The selective uptake of HDL-CE by SR-BI likely requires the formation of a hydrophobic channel²⁴ via SR-BI oligomerization^{22–24, 39}. Our lab has used FRET strategies to

demonstrate that dimerization occurs via regions in and/or around the C-terminal transmembrane domain of SR-BI²³, and is consistent with the presence of two putative dimerization domains within this region: a GxxxG motif (residues 420-424), as well a leucine zipper motif (between residues 413 and 455)^{40, 41}. Tryptophan 415 of SR-BI lies within the leucine zipper region, just upstream of the GxxxG motif, yet all mutant SR-BI receptors used in these studies displayed oligomerization patterns similar to WT-SR-BI (Figure 7). These findings suggest that formation of oligomers is not dependent on any of the Trp mutations. Further, any functional changes caused by these mutations are likely the result of conformational changes that do not affect oligomer formation. Impaired receptor function was also not due to changes in receptor trafficking, as all mutant SR-BI receptors were expressed at similar levels at the cell surface (Figure 1, Figures S1 and S3).

In addition to mediating the delivery of CE from HDL into cells, SR-BI can also stimulate the flux of FC out of cells to HDL and other acceptor particles³⁰⁻³². Loss of all Trp residues in SR-BI resulted in a receptor that had an impaired ability to efflux free cholesterol to HDL. However, unlike what we observed for selective uptake of HDL-CE, the impaired efflux function could not be attributed to a particular Trp residue, as all single Trp mutant receptors displayed similar levels of FC efflux to HDL as compared to WT-SR-BI. We were not surprised that efflux function remained normal despite mutation of Trp 415, as our findings are consistent with previous observations that residues in the C-terminal half of the EC domain of SR-BI may not be important for mediating the receptor's efflux functions. Furthermore, our data corroborate previous findings and provide additional support for the notion that the efflux function of SR-BI is not necessarily dependent on binding of HDL to the receptor^{19, 26, 37}. While mutation of Trp 415 alone did not impair SR-BI's efflux function, restoration of Trp 415 into the Trp-less receptor resulted in a significant increase in Trp-less-SR-BI's ability to efflux free cholesterol to HDL. These data suggest that, while not required for this function, the presence of Trp 415 helps support SR-BI's efflux functions, further emphasizing its importance.

Previously, our group^{24, 25, 36} and others^{19, 38, 42} have identified residues in SR-BI that, when mutated, impair HDL-CE selective uptake, yet do not disrupt SR-BI's ability to efflux free cholesterol to HDL, suggesting that the cholesterol transport functions of SR-BI are separable. Indeed, time course studies (Figure 4) demonstrated that both WT- and W415F-SR-BI promote similar rates of efflux of free cholesterol to HDL, statistically above background levels at incubation times ranging from 15 to 240 minutes, whereas differences in the ability of WT- and W415F-SR-BI to bind HDL and mediate selective uptake of HDL-COE were evident as early as 15 minutes in presence of HDL. Together these data provide additional support for the idea that these two functions of SR-BI are mechanistically distinct.

In the presence of SR-BI, an increased pool of plasma membrane FC is available for oxidation to cholestenone by exogenous cholesterol oxidase, a function that is independent of the presence of HDL³³. When all Trp residues in SR-BI are mutated to Phe, this function is impaired, again, likely due to the contribution of Trp 415 (Figure 5). The inability of W415F-SR-BI to rearrange pools of FC in the membrane supports our hypothesis that Trp 415 may be important for stabilizing a functional conformation of SR-BI at the membrane. These studies and others^{33, 43} also suggest that this region of the receptor may be required

for SR-BI's role in regulating cellular cholesterol levels. Interestingly, the dependency of the efflux function of SR-BI on the ability of SR-BI to redistribute membrane pools of FC remains unresolved. Indeed, certain mutations of SR-BI impair the abilities of the receptor to mediate efflux FC to HDL, as well as redistribute pools of membrane FC^{13, 43}. Yet, in the current study, these two functions of SR-BI appear to be independent of one another upon mutation of Trp 415. Similar findings were observed upon mutation of specific cysteine residues in SR-BI²⁶. Furthermore, mutation of Q445 in SR-BI impaired the ability of the receptor to interact with plasma membrane cholesterol, while efflux of FC to HDL was unaffected by this mutation⁴⁴. More detailed investigations into the role of SR-BI in modulating plasma membrane cholesterol levels are warranted.

SR-BI is a member of the superfamily of class B scavenger receptors that includes the oxidized LDL receptor, cluster of differentiation 36 (CD36)⁴⁵, as well as lysosome integral membrane protein 2 (LIMP2), a receptor involved in the transfer of beta-glucocerebrosidase into the lysosome^{46, 47}. Recently, the X-ray crystal structure of the extracellular domain of human LIMP-2 was solved by Neculai *et al*⁴⁸. LIMP-2 shares 34% sequence identity and 56% sequence homology with human SR-BI⁴⁸. Using this structure as a guide, we generated a homology model of the extracellular domain of murine SR-BI as a tool to elucidate the structural importance of the tryptophan residues investigated in these studies. As shown in Figure 8A, this homology model includes only the six extracellular Trp residues. Not included in this model are Trp 9 and Trp 474, each located at the interface of the N-terminal and C-terminal cytoplasmic domains, respectively. These two residues are the least conserved between CD36 and LIMP2, and their contribution to SR-BI is negligible as loss of these residues, either by mutagenesis (data presented herein) or by deletion of the cytoplasmic domains^{15, 18} does not impair SR-BI function.

According to our model, Trp 415 of SR-BI could potentially be involved in stabilization of the beta sheet through base stacking interactions with Tyr 68. Tryptophan and tyrosine residues have been known to stabilize β strands, particularly in β -hairpins⁴⁹. Neighboring Trp 415 and Tyr 68 are Pro 91 and Pro 345, which may also contribute to ring stability (Figure 8B). Our homology model also suggests that Trp 415 could potentially form a hydrogen bond with the nearby residue Glu 88 (Figure 8C). These molecular interactions may help to explain the importance of Trp 415 in SR-BI's functions. The importance of Trp 415 in mediating proper SR-BI function is also consistent with findings by Parathath *et al.* who used alanine scanning mutagenesis to demonstrate that mutation of residues 415 to 419 resulted in an impaired ability to mediate HDL binding and selective uptake of HDL-CE⁴³. Tryptophan 415 is highly conserved between species of SR-BI, however Trp 415 of human SR-BI aligns with Tyr 410 of human LIMP2, suggesting this residue may be important for the functional differences between the two receptors. SR-BI contains 11 N-linked glycosylation sites at residues 102, 108, 116, 173, 212, 227, 255, 288, 310, 330, and 383⁵⁰, however, none of the Trp mutations disrupts any of these N-linked glycosylation sequences (NxS/T). SR-BI is also predicted to contain several intramolecular disulfide bonds^{25, 26}. Our homology model suggests that Trp 415 is not located near these potential bonds, suggesting this mutation likely does not impair disulfide bond formation. Additional mutagenic analyses to test some of these hypotheses and assess changes in receptor function are required.

Tryptophan residues are known to bridge the membrane-water interface of cells^{51–53}. According to our homology model, tryptophan residues 181, 237 (not shown), and 415 are all located in the mid-region of the beta-barrel-like EC domain and are solvent-exposed (Figure 7A, right panel). It has been suggested that binding of HDL induces a conformational change within SR-BI²³. As such, it is certainly possible that upon binding of HDL to SR-BI, conformational changes within the EC domain of SR-BI may bring of Trp 415 closer to the membrane-water interface and facilitate CE delivery into cells from HDL. Indeed, this hypothesis warrants further investigation. Whether Trp 181 and 237 are also more proximal to the membrane upon HDL binding to be determined, but based on the findings from our mutagenesis studies, their role in bridging the membrane-water interface may not be as critical to receptor function.

Understanding the molecular architecture of the extracellular domain of SR-BI is important for elucidating the mechanisms by which this receptor facilitates net cholesterol excretion and protects against cardiovascular disease. While our studies demonstrate the importance of Trp 415 in mediating the cholesterol transport functions of SR-BI, further investigations will reveal whether this residue also plays a role in other important function of SR-BI such as mediating stress response⁵⁴ and macrophage migration⁵⁵. These studies are currently underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CD36	cluster of differentiation 36
CE	cholesteryl ester
COE	cholesteryl oleyl ether
CVD	cardiovascular disease
DMEM	Dulbecco's modified Eagle's medium
EC	extracellular
FC	free cholesterol
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol

LDL	low-density lipoprotein
RCT	reverse cholesterol transport
SR-BI	scavenger receptor BI
WT	wild-type
tryptophan	Trp, W
phenylalanine	Phe, F

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Figure 1. Mutant SR-BI receptors express in cell lysates and at the cell surface

(A) COS-7 cells transiently expressing empty vector, WT or mutant SR-BI receptors were lysed 48 h post-transfection and assessed for SR-BI expression by immunoblot analysis following separation of total cell lysates by 8% SDS-PAGE. Surface expression of WT or SR-BI mutant receptors was assessed by (B) flow cytometry using an antibody against the extracellular domain of SR-BI or by (C) immunofluorescence, where cells were fixed and stained with an antibody against the extracellular domain of SR-BI followed by FITC-conjugated secondary antibody (green). Nuclei are stained with DAPI (blue).

Figure 2. Trp-less- and W415F-SR-BI receptors have decreased abilities to bind HDL and promote selective uptake of HDL-COE

COS-7 cells transiently expressing empty vector, WT or mutant SR-BI receptors were incubated with [125 I]/[3 H]-COE-labeled HDL (10 μ g protein/mL) for 1.5 h at 37°C and assessed for (A) HDL binding, (B) selective uptake of HDL-COE and (C) selective uptake efficiency are shown. Values represent the mean $\pm$ SEM of six independent experiments, each performed in triplicate. Bars to the right of the dashed line represent data from restoration of Trp 415 into the Trp-less SR-BI background (five independent experiments, each performed in triplicate). All data sets are presented following subtraction of empty vector values and were normalized to respective WT SR-BI (normalized value = 100%). The mean experimental values in Panel A for HDL binding (before subtraction of the value for vector-transfected cells) for vector and WT SR-BI were 17.2 and 77.0 ng HDL/mg cell protein. For selective uptake of HDL-COE, the mean experimental values for Panel B (before subtraction of the value for vector-transfected cells) were 878.1 and 1479.2 ng HDL-COE/mg cell protein for vector and WT SR-BI, respectively. Statistical analyses were determined by one-way ANOVA comparing each mutant to WT SR-BI from respective experiments, unless otherwise noted by a connecting line. * p <0.05, ** p <0.01 and *** p <0.001.

Figure 3. Trp-less-SR-BI has a decreased capacity to promote efflux of free cholesterol (FC) to HDL

COS-7 cells transiently expressing empty vector, WT SR-BI or Trp-mutant receptors were pre-labeled with [3 H]cholesterol, and then incubated with 50 μ g/mL HDL for 4 h. The radioactivity released to the media and remaining in the cells was used to calculate a percent efflux of free cholesterol to HDL. Values represent the mean $\pm$ SEM of five independent experiments, each performed in quadruplicate. All data sets are presented following subtraction of empty vector values. Bars to the right of the dashed line represent data from restoration of Trp 415 into the Trp-less SR-BI background (three independent experiments, each performed in quadruplicate). The mean experimental values (before subtraction of the value for vector-transfected cells) for vector and WT SR-BI were 5.1% and 10.3%, respectively. Statistical analyses were determined by one-way ANOVA to respective WT SR-BI (shown above each data set) or to Trp-less SR-BI (indicated by line connecting data sets), ** $p < 0.01$ *** $p < 0.001$.

Figure 4. Mutation of Trp 415 impairs the ability of SR-BI to bind HDL and mediate selective uptake of HDL-COE in a time-dependent manner, but not FC efflux to HDL

COS-7 cells transiently expressing empty vector (triangles), WT SR-BI (circles) or W415F-SR-BI (squares) were assayed at various time points for (A) HDL-binding, (B) selective uptake of HDL-COE, or (C) efflux of FC to HDL as previously described. Bmax values for HDL-binding were determined by GraphPad PRISM software using a nonlinear, one site, specific-binding analysis. Statistical analyses were determined using a two-tailed paired t-test. For panels A and B, $p < 0.01$ for vector vs. WT-SR-BI, vector vs. W415F-SR-BI, and WT-SR-BI vs. W415F-SR-BI, unless otherwise noted where $*p < 0.05$ and $***p < 0.001$. For panel C, $p < 0.01$ for vector vs. WT-SR-BI and vector vs. W415F-SR-BI, unless otherwise noted where $***p < 0.001$ for vector vs. WT-SR-BI, $*p < 0.05$ vector vs. W415F-SR-BI. There was no statistical significance between WT- and W415F-SR-BI at all the time points tested.

Figure 5. Expression of Trp-less- and W415F-SR-BI receptors make membrane FC less sensitive to exogenous cholesterol oxidase

COS-7 cells transiently expressing empty vector, WT, or mutant SR-BI receptors were preloaded with [3 H]-cholesterol and then incubated with cholesterol oxidase (0.5 U/mL) for 4 h. Lipids were extracted and separated by thin layer chromatography. The radioactivity associated with cholestenone, FC and CE was measured and percent cholestenone reported. Values represent the mean $\pm$ SEM of three independent experiments, each performed in quadruplicate. Bars to the right of the dashed line represent data from restoration of Trp 415 into the Trp-less SR-BI background (two independent experiments, each performed in quadruplicate). The mean experimental values (before subtraction of the value for vector-transfected cells) for vector and WT SR-BI were 11.9% and 41.0% for cholestenone production, respectively. Statistical analyses were determined by one-way ANOVA to WT SR-BI, * p <0.01, *** p <0.001.

Figure 6. Restoration of individual extracellular Trp residues to W^{W415}-SR-BI (besides Trp262) partially rescues HDL-binding, HDL-COE uptake, and sensitivity of membrane FC to exogenous cholesterol oxidase

COS-7 cells transiently expressing empty vector, WT or mutant SR-BI receptors were assessed for (A) HDL-binding, (B) selective uptake of HDL-COE and (C) sensitivity of membrane FC to exogenous cholesterol oxidase as previously described. Values represent the mean $\pm$ SEM of four independent experiments, each performed in triplicate (A and B) or two experiments performed in quadruplicate (C), with the exception of W415F-, and W^{W415}-SR-BI mutants, which represent a single experiment and were included for reference. Following subtraction of empty vector values, data sets were normalized to WT SR-BI (normalized value = 100%). The mean experimental values set for HDL binding (before subtraction of the value for vector-transfected cells) for vector and WT SR-BI were 10.2 and 49.4 ng HDL/mg cell protein, respectively. For the selective uptake of HDL-COE, the mean experimental values (before subtraction of the value for vector-transfected cells) were 377.8 and 622.2 ng HDL-COE/mg cell protein for vector and WT SR-BI, respectively. The mean experimental values for cholestenone production (before subtraction of the value for vector-transfected cells) for vector and WT SR-BI were 10.7% and 30.0%, respectively.

Statistical analyses were determined by one-way ANOVA to WT-SR-BI, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

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Figure 7. WT and mutant SR-BI receptors display similar patterns of oligomerization
COS-7 cells transiently expressing WT or mutant SR-BI receptors were separated by PFO-PAGE using 6% polyacrylamide gels. SR-BI was detected by immunoblot analysis. Blots are representative of four independent experiments.

Figure 8. Homology model of the extracellular domain of SR-BI

(A). A ribbon representation and homology model of the extracellular domain of murine SR-BI was generated by MODELLER (reviewed in ⁵⁶) and based on the structure of the extracellular domain of LIMP-2 [pdb file of LIMP-2 (4Q4F)]. Extracellular tryptophan residues in the EC domain of SR-BI are shown in pink. The homology model is shown in ribbon (left) and a space-filling (right) form. Solvent-exposed Trp residues (model on right) are shown in pink. (B). Enlarged image demonstrating potential base stacking between Trp 415 (pink) and Tyr 68, Pro 91, and Pro 345 (green). (C). Trp 415 (pink) potentially forms a hydrogen bond (shown in blue and circled with dashed line) with Glu 88 (light blue).