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EXTRACELLULAR HYDROPHOBIC REGIONS IN SCAVENGER RECEPTOR BI PLAY A KEY ROLE IN MEDIATING HDL-CHOLESTEROL TRANSPORT

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Abstract

The binding of high density lipoprotein (HDL) to scavenger receptor BI (SR-BI) is responsible for whole-body cholesterol disposal via reverse cholesterol transport. The extracellular domain of SR-BI is required for HDL binding and selective uptake of HDL-cholesterol. We identified six highly hydrophobic regions in this domain that may be important for receptor activity and performed site-directed mutagenesis to investigate the importance of these regions in SR-BI-mediated cholesterol transport. Non-conservative mutation of the regions encompassing V67, L140/L142, V164 or V221 reduced hydrophobicity and impaired the ability of SR-BI to bind HDL, mediate selective uptake of HDL-cholesterol, promote cholesterol efflux, and enlarge the cholesterol oxidase-sensitive pool of membrane free cholesterol. In contrast, conservative mutations at V67, V164 or V221 did not affect the hydrophobicity or these cholesterol transport activities. We conclude that the hydrophobicity of N-terminal extracellular regions of SR-BI is critical for cholesterol transport, possibly by mediating receptor-ligand and/or receptor-membrane interactions.

Supplementary Key Words

SR-BI; selective uptake; efflux; hydrophobicity; extracellular domain; cholesteryl ester

1. INTRODUCTION

The inverse correlation between the risk for developing coronary artery disease and plasma concentrations of high density lipoprotein (HDL)¹ [1,2] has been attributed to the strong athero-protective effects of HDL that include inhibition of low density lipoprotein oxidation [3,4] and

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¹Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; CE, cholesteryl ester; COE, cholesteryl oleoyl ether; DLT, dilactitol tyramine; DMEM, Dulbecco's modified Eagle's medium; FC, free cholesterol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high density lipoprotein; PAGE, polyacrylamide gel electrophoresis; PFO, perfluoro-octanoic acid; SR-BI, scavenger receptor class B type I.

oxidative damage [5], promotion of endothelial nitric oxide production [6,7] and vascular reactivity and integrity [8], inhibition of platelet aggregation and coagulation [9,10] and prevention of thrombosis [11]. However, the primary athero-protective role of HDL stems from its ability to promote the disposal of peripheral cholesterol at the liver via a process termed reverse cholesterol transport [12].

The final step of reverse cholesterol transport involves the movement of cholesterol from HDL to the liver for catabolism. The selective transfer of cholesteryl ester (CE) from HDL to cells is mediated by scavenger receptor class B type I (SR-BI) [13], an 82-kDa glycosylated cell surface receptor [14] highly expressed in the liver and steroidogenic tissues [15–17]. SR-BI (509 amino acids) consists of a large extracellular domain (403 amino acids) anchored by two transmembrane domains and two short cytoplasmic tails [18]. Transgenic overexpression [19–21] or hepatic adenoviral infection [22,23] of SR-BI decreased HDL plasma cholesterol levels and increased cholesterol catabolism and excretion. On the other hand, a 50% reduction in SR-BI expression [17] or full disruption of the SR-BI gene in mice increased plasma HDL-cholesterol levels and reduced neutral lipid stores in the adrenal gland and ovary [24,25]. Thus, SR-BI is the most physiologically relevant HDL receptor.

SR-BI-mediated selective uptake of HDL-CE is a two-step process: (i) HDL must bind to the extracellular domain of SR-BI and (ii) CE is transferred from HDL to the plasma membrane by a non-endocytic mechanism, without holoparticle uptake or degradation of apolipoproteins [26–28]. The critical nature of the extracellular domain of SR-BI in CE transfer has been demonstrated through the use of chimeric receptors [29–31] and insertion of epitope tags into various regions of the extracellular domain of SR-BI [32]. Moreover, antibodies to the extracellular domain blocked HDL-CE-selective uptake and the delivery of HDL-CE to the steroidogenic pathway in cultured adrenocortical cells [33]. In fact, a set of distinct SR-BI-mediated activities appears to be inherent to the extracellular domain, including free cholesterol (FC) efflux and influx, as well as the ability to increase cellular FC mass and enhance sensitivity of membrane FC to exogenous cholesterol oxidase [34].

Our detailed analyses also reveal the presence of evolutionarily conserved sequences with high hydrophobicity within the extracellular domain of SR-BI. We hypothesized that these hydrophobic regions may play a role in mediating the cholesterol transport functions of SR-BI. To test this hypothesis, we used site-directed mutagenesis to generate point mutations that would reduce overall hydrophobicity of the particular regions: V67N, L140Q/L142Q, V164N, V221N, L359Q, and L411Q. We then correlated the changes in hydrophobicity to the effects on HDL binding, selective uptake of HDL-CE and other functions of SR-BI. In addition, we created a second set of point mutations that maintained the overall hydrophobicity of the selected regions (V67L, L140V/L142V, V164L, V221L, L359V, and L411V) to test whether the changes in SR-BI function were due to changes in hydrophobicity or changes in amino acid identity.

2. MATERIALS AND METHODS

2.1 Materials

The following antibodies were used: polyclonal anti-SR-BI specific for the C-terminal or the extracellular domain (Novus Biologicals, Inc., Littleton, CO); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Millipore, Billerica, MA); peroxidase-conjugated goat anti-rabbit secondary IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Human HDL (1.063–1.21 g/mL) was purchased from Biomedical Technologies, Inc. [¹²⁵I]Iodine was from Perkin-Elmer, while [³H]cholesterol and [³H]cholesteryl oleoyl ether (COE) were from GE Healthcare (Piscataway, NJ). Cholesterol oxidase (*Nocardia erythropolis*) was from MP Biomedicals, LLC. Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor (Sandoz 58-035),

perfluoro-octanoic acid (PFO) cholesterol, 4-cholesten-3-one and cholesteryl oleate standards were purchased from Sigma. All other reagents were of analytical grade.

2.2 Plasmids and sequencing

Site-directed mutations of V67, V164, V221 and L359 were introduced into wild-type murine SR-BI (pSG5(SR-BI)) [29] using the QuikChange Site-Directed Mutagenesis kit (Stratagene) according to manufacturer's protocols. Oligonucleotide primers were purchased from Integrated DNA Technologies. The following primers were used for mutagenesis: V67N, 5'-CTA CTT GTC TAA CTA CTT CTT CG -3' and 5' – CGA AGA AGT AGT TAG ACA AGT AG -3'; V164N, 5'-CCT TGG CGC TGA ACA CCA TGG GCC -3' and 5'-GGC CCA TGG TGT TCA GCG CCA AGG -3'; V221N, 5'-GGG GTC TTC ACT AAC TTC ACG GGC GTC -3' and 5'-GAC GCC CGT GAA GTT AGT GAA GAC CCC -3'; L359Q, 5'-CAG AAG CTG TTC AAG GTC TGA ACC C -3' and 5'-GGG TTC AGA CCT TGA ACA GCT TCT G -3'. All plasmids were purified using endotoxin-free Qiagen Maxi-Prep kits and sequenced through the coding region to ensure the presence of the correct mutation and the absence of undesired mutations generated during the amplification steps. DNA sequencing was performed on an ABI 3100 at the Protein and Nucleic Acid Facility at the Medical College of Wisconsin. The L140Q/L142Q-, L411Q-, V67L-, L140V/L142V-, V164L-, V221L-, L359V- and L411V-SR-BI mutant receptors were made and sequenced by TOP Gene Technologies (Pointe-Claire, Quebec, Canada).

2.3 Cell culture and transfection

COS-7 cells were maintained in DMEM (Invitrogen), 10% calf serum (Invitrogen), 2 mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin and 1 mM sodium pyruvate, and transfected as previously described [29]. The following day, fresh medium was added and the cells were assayed 48 h post-transfection, with the exception of efflux assays where cells were assayed 72 h post-transfection.

2.4 Cell lysis

Transiently-transfected cells expressing murine wild-type or mutant SR-BI were washed twice in PBS, pH 7.4, and lysed with 300 µl of 1% NP-40 cell lysis buffer [29] containing protease inhibitors (1 µg/ml pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, and 10 µg/ml aprotinin). Protein concentrations were determined by the Lowry method [35].

2.5 HDL labeling

HDL was labeled with dilactitol tyramine and non-hydrolyzable [³H]COE as described [29]. Average specific activities were 8.3 dpm/ng protein for ³H and 330.4 dpm/ng protein for ¹²⁵I.

2.6 Cell association of [¹²⁵I]HDL and selective uptake of [³H]HDL-COE

COS-7 cells were transiently transfected with pSG5 or vectors encoding wild-type and mutant SR-BI cDNAs. Cell association of [¹²⁵I]HDL and selective uptake assays of non-hydrolyzable [³H]COE were performed as previously described [29]. Data represent the average of four separate experiments performed in triplicate. Data were calculated as ng HDL/mg cell protein or ng HDL-COE/mg cell protein for binding and selective uptake experiments, respectively. Empty vector values were subtracted from all wild-type and mutant values, and data were normalized to wild-type SR-BI. Statistical comparisons were calculated by one-way ANOVA with Bonferroni post-tests for all groups.

2.7 Free cholesterol efflux and cholesterol oxidase sensitivity assays

Assays were performed as previously described [32], unless otherwise specified. Data represent the average of four separate experiments, each performed in quadruplicate. Statistical comparisons were calculated by one-way ANOVA with Bonferroni post-tests for all groups.

2.8 Immunofluorescence

Transiently-transfected COS-7 cells were re-plated 24 hours post-transfection onto glass coverslips in 12-well plates. After 24 hours, the media were removed, cells were washed with PBS and fixed with 4% paraformaldehyde/PBS for 10 minutes. Cells were then stained as described by Peng *et al.* [36] using a primary antibody directed against the extracellular domain of SR-BI (1:200) and an Alexa 488-conjugated goat anti-rabbit IgG secondary antibody (Molecular Probes, Inc., 1:1000). Cells were examined on a Leica TCS SP2 Laser Scanning confocal microscope imaging system.

2.9 Immunoblot analysis

Transiently-transfected cells expressing wild-type or mutant SR-BI receptors were washed twice with PBS, and lysed with 1% NP-40 cell lysis buffer containing the above-mentioned protease inhibitors. Equal amounts of total cellular proteins were separated on 8% SDS-PAGE gels, transferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ) 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 (Pierce).

2.10 Cell surface biotinylation

Transiently-transfected COS-7 cells expressing wild-type or mutant SR-BI receptors were washed twice with PBS and incubated with 1 mg/mL EZ-Link Sulfo-NHS-LC-biotin (Pierce) in PBS for 30 min at 4 °C in the dark. Cells were lysed with 1% NP-40 cell lysis buffer containing the above-mentioned protease inhibitors. A portion of the cell lysate was incubated with streptavidin agarose (Pierce) for 1 hour at room temperature. The beads were pelleted, washed 4 times in cell lysis buffer and resuspended in sample treatment buffer. Biotinylated SR-BI was detected by immunoblot analysis as described above.

2.11 PFO-PAGE analysis

PFO-PAGE was performed as described [37], but with the following modifications. Forty-eight hours post-transfection, COS7 cells expressing wild-type or mutant SR-BI receptors were washed twice with PBS and lysed in PBS containing the aforementioned protease inhibitors. Prior to electrophoresis, lysates were sonicated using a Fisher Scientific 550 Sonic Dismembrator on ice five times at 30% power (power level 3) for 3 s each, with a 1-min cooling period between sonications. Equal amounts of total cellular proteins were solubilized in sample treatment buffer (5% PFO, 100 mM Tris, 20% glycerol, 0.005% bromophenol blue). Samples were rotated for 30 min at room temperature and separated on 5% polyacrylamide gels (lacking SDS) in running buffer (25 mM Tris, 192 mM glycine, 0.5% PFO, final pH 8.5). Proteins were transferred to nitrocellulose membranes and detected by immunoblot analysis as described above.

3. RESULTS

3.1 Rationale for mutagenesis

The traditional depiction of SR-BI with a “horseshoe-like” topology is based upon hydropathy analysis [38] of the predicted amino acid sequence and reveals two membrane-spanning regions. These N- and C-terminal domains display a hydrophobicity index much greater than

the typical threshold value and are long enough to span the membrane bilayer [39,40]. However, the hydropathy plot (Figure 1) also revealed that the extracellular domain of SR-BI contains other evolutionarily conserved sequences with a high hydrophobicity index (Figure 2).

In order to examine the functional role of these hydrophobic regions, we created a panel of strategic point mutations that greatly reduced the overall hydrophobicity of the given region [38]. In this panel, leucine (Leu, L) or valine (Val, V) residues were mutated to either glutamine (Gln, Q) or asparagine (Asn, N), respectively, to generate non-conservative substitutions. We also developed a second group of point mutations designed to maintain the overall hydrophobicity of the given region. In this group, conservative substitutions were created by mutating Leu residues to Val residues, and Val residues to Leu residues. Table I outlines the amino acids chosen for mutagenesis and the effect of these mutations on the overall hydrophobicity of that region. We chose to design the L140Q/L142Q double point mutation since mutation of both Leu residues resulted in a greater decrease in hydropathy index than mutating either one individually; therefore, our second group of point mutations also contained a double point mutation (L140V/L142V). Further, although residues at positions 164 and 359 are not identical among species, the hydrophobicity of the region remains high among species (hydrophobicity indices range from 1.69–2.17 or 1.77–2.17 for windows containing residue 164 or 359, respectively).

3.2 Wild-type and mutant SR-BI proteins express at the cell surface

Plasmids encoding wild-type and mutant SR-BI were transiently-transfected into COS-7 cells and examined for expression by fluorescent microscopy. Mutant receptors containing nonconservative and conservative substitutions exhibited similar staining patterns as wild-type SR-BI in fixed COS-7 cells [36] as judged by bright fluorescence at the plasma membrane and cell surface extensions (Figure 3). No staining was observed with non-transfected COS-7 cells (data not shown). Biotinylation experiments also confirmed cell surface expression of all mutant SR-BI proteins (Figure 4A). Immunoblot analysis of SR-BI expression in total cell lysates is also shown (Figure 4B). The lower expression of the V221N mutant receptor in both the total cell lysate and at the cell surface is due to variations in transient expression of the mutant receptor since we have previously observed wild-type or slightly below wild-type levels of total cell lysate expression and cell surface biotinylation in separate experiments (data not shown).

3.3 Specific mutant receptors are unable to bind HDL and mediate selective uptake of HDL-COE

In order to determine the functional consequences of our point mutations in the putative extracellular hydrophobic regions, COS-7 cells were transiently transfected with vectors encoding wild-type or mutant SR-BI. Forty-eight hours post-transfection, cells were assessed for HDL binding and selective uptake of HDL-COE. Analysis of the non-conservative set of mutations revealed four mutant receptors—V67N-, L140Q/L142Q-, V164N- and V221N-SR-BI—with significantly reduced abilities to bind HDL (29%, 40%, 18%, and 12% of wild-type SR-BI binding, respectively; Figure 5A)). A similar pattern was observed for the selective uptake of HDL-COE, with the V67N, L140Q/L142Q, V164N, and V221N mutant receptors being virtually unable to mediate this transport function (only 14%, 4%, 5%, and 6% of wild-type HDL-COE uptake, respectively), despite measurable levels of HDL binding (Figure 5B). Analysis of the conservative set of mutations revealed that the V67L, V164L and V221L mutant receptors regained their ability to mediate wild-type levels of HDL binding and HDL-COE selective uptake once their hydrophobicity was restored. While conservative mutations at positions L140/L142 were unable to improve HDL binding, the L140V/L142V mutant receptor displayed an enhanced ability to mediate selective uptake of HDL-COE (60% greater levels

than the L140Q/L142Q receptor, Figure 5B). Receptors with mutations at L359 or L411 displayed wild-type levels of HDL binding and selective uptake of HDL-COE.

3.4 Changes in hydrophobicity also affect cholesterol efflux and oxidase-sensitivity of membrane free cholesterol

In addition to its role in HDL-CE uptake, SR-BI also has the ability to stimulate the release of free cholesterol from cells [41–43]. In order to determine whether our point mutations altered SR-BI-mediated cholesterol efflux, COS-7 cells expressing the mutant receptors were assayed for their ability to promote efflux of cholesterol to HDL. Analysis of the first set of mutations revealed that the V67N, V164N, and V221N mutant receptors displayed significantly reduced levels of efflux to HDL (64%, 28%, and 36%, respectively, of wild-type levels). Restoration of hydrophobicity at V67, V164 and V221 allowed the mutant receptors to regain wild-type levels of efflux (Figure 6). Interestingly, despite an inability to bind HDL, the L140Q/L142Q mutant receptor was able to efflux FC to HDL similar to wild-type SR-BI.

SR-BI can also increase the pool of plasma membrane FC available for oxidation by cholesterol oxidase, as judged by a higher membrane content of cholestenone [34]. To determine whether our point mutations affected the re-distribution of plasma membrane cholesterol, COS-7 cells expressing wild-type and mutant SR-BI were treated with exogenous cholesterol oxidase 48 hours post-transfection. With our non-conservative set of mutations, we observed a pattern that paralleled the HDL binding and HDL-CE selective uptake trends where expression of V67N-, L140Q/L142Q-, V164N-, and V221N-SR-BI significantly decreased the size of the oxidase-sensitive pool of membrane FC (35%, 18%, 54%, and 36% of wild-type levels, respectively). From the conservative set of mutations, the V67L and L140V/L142V mutant receptors displayed a trend for increased cholestenone production, yet the values remained significantly lower than wild-type SR-BI based on our statistical analyses. While the V164L and V221L mutant receptors appeared to regain their ability to increase the oxidase-sensitive pool of FC due to their statistically similar levels of cholestenone production as wild-type SR-BI, they were unable to mediate significant increases in cholestenone in comparison to the V164N and V221N receptors, respectively (Figure 7).

3.5 Loss of cholesterol transport function is independent of SR-BI oligomerization

We [44,45] and others [16,46–48] have demonstrated the presence of SR-BI oligomers in steroidogenic tissues, as well as in a diverse collection of cell lines. It has been hypothesized that SR-BI oligomerization facilitates cholesterol transport between HDL and the plasma membrane via formation of a “hydrophobic channel” [27]. In order to determine whether the loss of cholesterol transport functions reflected a change in the oligomeric status of SR-BI, we performed native polyacrylamide gel electrophoresis (PAGE) of mutant receptors with nonconservative substitutions in the presence of PFO. PFO-PAGE is used to evaluate the molecular mass of homo-multimeric membrane protein complexes [37,49–51]. We found that all mutant receptors existed as dimers and higher order oligomers similar to wild-type SR-BI (data not shown).

4. DISCUSSION

Since the identification of SR-BI as the HDL receptor [15,52], numerous studies have examined the mechanism of the HDL-CE selective uptake process via SR-BI. However, few of these reports have commented on how the topology and/or structural organization of SR-BI at the plasma membrane may influence this crucial step in reverse cholesterol transport. We investigated the role of several putative hydrophobic regions of the extracellular domain of SR-BI by creating amino acid substitutions that modified the overall hydrophobicity of a given region in a predictable manner. In general, non-conservative substitutions in the C-terminal

portion of the extracellular domain (L359Q and L411Q) did not have major effects on the various SR-BI-mediated cholesterol functions. However, our functional data revealed that loss of hydrophobicity within the N-terminal half of the extracellular domain (V67N, L140Q/L142Q, V164N or V221N mutations) resulted in an inability of SR-BI to properly: (i) bind HDL and mediate selective uptake of HDL-CE, (ii) promote the release of FC to HDL (with the exception of L140Q/L142Q) and (iii) re-organize plasma membrane cholesterol pools to render them sensitive to exogenous cholesterol oxidase. In contrast, most mutant receptors that retained their hydrophobicity (V67L, V164L and V221L) displayed wild-type levels of SR-BI function in all our assays tested. Taken together, these data suggest that the hydrophobic portion (s) of the N-terminal half of the extracellular domain of SR-BI, namely the region encompassing residues 67 to 221, is critical for facilitating the cholesterol transport functions of SR-BI.

While the majority of our data support a role for N-terminal extracellular hydrophobic regions in regulating SR-BI-mediated cholesterol flux, the L140V/L142V mutant receptor stands alone in that these conservative amino acid substitutions also reduce HDL binding, HDL-CE selective uptake or plasma membrane cholesterol levels. One obvious difference is that the L140V/L142V receptor contains two amino acid substitutions while V67L-, V164L- and V221L-SR-BI contain only single substitutions. This is not a likely source of the discrepancy as individual substitutions (L140V or L142V) do not significantly disrupt the predicted hydrophobicity of the region. Rather, it is probable that one or both of these Leu residues make direct contact with another residue in SR-BI to maintain the receptor in the required conformation. In other words, these residues are not likely to interact with HDL since substituting these residues affects SR-BI activities that are both dependent upon (CE selective uptake) and independent of (oxidase sensitivity [53], FC efflux [41]) HDL engagement. A thorough mutagenic analysis of these two amino acid positions is required to fully appreciate the structure/function relationships of L140 and L142.

As described above, we have characterized four novel amino acid positions in the extracellular domain of SR-BI that are critical for HDL binding: V67, L140/L142, V164 and V221. Despite the low levels of HDL binding exhibited by some of these mutant receptors, other functions remained mostly intact. For example, the V164N and V221N mutant receptors maintained accessibility to exogenous cholesterol oxidase, consistent with previous observations that HDL binding is not required for this function of SR-BI [53]. Similarly, the V67N and L140Q/L142Q mutants displayed modest to normal levels of FC efflux to HDL, compared to wild-type SR-BI, thus supporting the notion that SR-BI-mediated FC efflux is mostly independent of direct SR-BI-acceptor interactions [41]. Our data is thus consistent with numerous other studies that describe discrete functional subdomains present in the extracellular domain of SR-BI that mediate the various lipid transport activities [27,33,54].

Understanding the conformation and/or topology of the extracellular domain of SR-BI, as well as the changes that might occur upon ligand engagement, is critical to understanding the role this region plays in mediating the various cholesterol transport functions. The mere binding of HDL to SR-BI is not sufficient for lipid transfer to occur. Rather, efficient selective uptake of HDL-CE requires the formation of a “productive complex” [32,55] where the lipoprotein and receptor must be precisely aligned and/or have the capacity to undergo conformational changes in order to support lipid transfer. How then might these mutations alter “productive complex” formation? It seems likely that the N-terminal half of the extracellular domain of SR-BI physically interacts with the HDL ligand. In addition, we hypothesize that this region may interact with or “dip” into the plasma membrane to facilitate lipid transfer, as has been described for CD36, a member of the class B sub-family of scavenger receptors that shares 70% sequence similarity with SR-BI [29]. The “dipping” of an extracellular domain into a lipid bilayer has also been reported for several other integral membrane proteins including rhomboid protease

GlpG [57] and the Ste2p G-protein-coupled receptor [58]. Therefore, we propose that loss of hydrophobicity in specific extracellular regions of SR-BI alters receptor topology such that proper “productive complex” formation between SR-BI and HDL is prevented.

In conclusion, the N-terminal region of the extracellular domain of SR-BI contains hydrophobic regions critical for receptor-mediated cholesterol flux. We hypothesize that these regions interact with HDL and/or the plasma membrane to facilitate lipid transfer.

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Fig. 1.

Kyte-Doolittle hydropathy analysis of SR-BI. Kyte-Doolittle hydropathy (<http://gcat.davidson.edu/rakarnik/KD.html>) analysis of the murine SR-BI amino acid sequence reveals the presence of N- and C-terminal transmembrane domains (N-TMD and C-TMD, respectively). Six putative regions of hydrophobicity within the extracellular domain are also indicated (A–F). A window size of 9 was chosen for our analysis and refers to the number of amino acids examined at a time to determine the hydrophobic character of that region. The solid line (hydropathy index = 1.8) indicates the theoretical threshold for hydrophobicity.

MOUSE	58	EIPVPFYL	SVVFF	EVVNPN	76	131	SDYIVLPN	ILW	GG	SILMESK	151
Rat	58	EIPVPFYL	SVVFF	EVVNPN	76	131	SDYIVLPN	ILW	GG	AVVMEDK	151
Human	58	EIPFPFYL	SVVFF	VDVNP	76	131	SDYIVMPN	ILW	GA	AVMENK	151
Hamster	58	EIPVPFYL	SVVFF	EVVNPN	76	131	SDYIILPN	ILW	GG	AVVMESK	151
Pig	58	EIPVPFYL	SVVFF	VDVNP	76	131	SDYIVIPN	ILW	GA	SVMEDR	151
Cow	58	EIPVPFYL	SVVFF	IVNPN	76	131	SDYIVMPN	ILW	GA	SVMMENR	151
Dog	118	EIPVPFYL	SVVFF	VDVNP	136	191	SDYIVMPN	ILW	GA	AVMMENR	201
Rabbit	58	EIPAPFYL	SVVFF	VDVNP	76	131	SDYIVIPN	ILW	GA	SVMEHR	151
Tree shrew	58	EIPVPFYL	SVVFF	VDVNP	76	131	SDYIVMPN	ILW	GA	AVMMENR	151
		***	*****	:::*.:			*****	:::*.:			
MOUSE	155	LKLMMTLAL	VTMG	QRAFMN	173	212	NNSNSGVFT	VFTG	VQNFSR	230	
Rat	155	LKLMTLGLV	TMG	QRAFMN	173	212	NDSSSGVFT	VFTG	VQNFSK	230	
Human	155	LKLIMTLAFT	TLGER	AFMN	173	212	NNSDSGLFT	VFTG	VQNFSR	230	
Hamster	155	LKLMMTLGLA	VTMG	QRAFMN	173	212	NNSDSGLFT	VFTG	VQNFSK	230	
Pig	155	LKLIMTFAPS	ALGER	AFMN	173	212	NNSDSGLFT	VFTG	VKDFSR	230	
Cow	155	LKLMMTLAFS	TLGER	AFMN	173	212	NNSDSGLFT	VFTG	VKNFSR	230	
Dog	215	LKLIMTLAFS	TLGER	AFMN	233	272	NNSDSGIFT	VFTG	VKDFSR	290	
Rabbit	155	LKLIMTLAFS	ALGER	AFMN	173	212	NDSSSGVFT	VFTG	VKDFSR	230	
Tree shrew	155	LKLMMTLAFS	TLGER	AFMN	173	212	NNSNSGLFT	VFTG	VKDFQR	230	
		:::*.:	:::				*:::***	:::***			
MOUSE	350	ADPVLSEAVL	GLNPN	PKHE	368	402	QTGKIEPVV	PLW	FEQSG	420	
Rat	350	ADPVLSEAVL	GLNPD	PKHE	368	402	QTGKIEPVV	PLW	FEQSG	420	
Human	350	ADPVLAEAVT	GLHPN	QEAH	368	402	QTGKIEPVV	PLW	FAESG	420	
Hamster	350	ADPVLSEAVL	GLNPD	PREH	368	402	QTGKIEPVV	PLW	FEQSG	420	
Pig	350	ADPVLAEAVS	GLHPN	TEEH	368	402	QTGKIEPVV	PLW	FAESG	420	
Cow	350	ADPVLAEAVS	GLHPN	PKHE	368	402	QTGNIQPVV	PLM	WFEEG	420	
Dog	410	ADPMLAEAVL	GLHPN	QEEH	428	462	QTGKIEPVV	PLW	FEESG	480	
Rabbit	350	ADPVLAEAVL	GLHPN	EEH	368	402	QTGKIEPVV	PLW	FEESG	420	
Tree shrew	350	ADPVLAEAVL	GLHPN	QEEH	368	402	QTGKIEPVV	PLW	FEESG	420	
		:::	GLH:::*				***:::***	GLH:::*			

Fig. 2.

Comparison of extracellular hydrophobic regions of SR-BI across species. Sequence fragments from mouse, rat, human, hamster, pig, cow, dog, rabbit, and Northern tree shrew SR-BI are shown (predicted protein sequences were obtained from GenBankTM accession number NM_016741, NP_113729.1, U76205, NM_005505, A53920, ALL75567, O18824, XM_543366, AY283277 and EU379936 respectively.) Residues chosen for mutation in the mouse sequence and their conservation among species are highlighted in black. “*”, identical residues; “:”, conserved residues; “.”, semi-conserved residues.

Fig. 3.

Cell surface staining patterns of wild-type SR-BI and mutant SR-BI receptors. COS-7 cells expressing wild-type SR-BI or mutant SR-BI receptors were plated onto glass coverslips 24-h post-transfection. Non-permeabilized cells were fixed and stained with antibody directed against the extracellular domain of SR-BI and visualized with Alexa 488-conjugated secondary antibody. Scale bar, 15.75 μm .

Fig. 4.

Cell surface expression of mutant SR-BI receptors. COS-7 cells expressing wild-type or mutant SR-BI receptors were incubated with Sulfo-NHS-LC-Biotin and biotinylated proteins were immunoprecipitated as described in Experimental Procedures. Immunoblot analysis of SR-BI expression at (A) the cell surface by biotinylation (from ~200 μ g of total lysate) and (B) in 20 μ g of total cell lysate is shown. Bands were detected with antibody directed against the C-terminal cytoplasmic domain of SR-BI. (C) GAPDH was detected as a loading control.

Fig. 5.

HDL binding and selective uptake of HDL-COE in cells expressing wild-type SR-BI and mutant SR-BI receptors. COS-7 cells transiently expressing wild-type SR-BI or mutant SR-BI receptors were incubated at 37 °C for 1.5 h with [125 I]DLT/[3 H]COE-labeled HDL (10 μ g of HDL protein/ml), after which cells were processed as previously described [29]. Cell-associated HDL (**panel A**) and selective uptake of HDL-COE (**panel B**) are shown. For both panels, data sets were normalized to wild-type SR-BI (normalized value = 1.0) following subtraction of empty vector values. Data represent the mean $\pm$ SD of four independent experiments, each performed in triplicate. * p <0.05, ** p <0.01, *** p <0.001, as determined by one-way ANOVA.

Fig. 6.

Efflux of [^3H]cholesterol to HDL acceptors by wild-type SR-BI and mutant SR-BI receptors. COS-7 cells transiently expressing wild-type SR-BI or mutant SR-BI receptors were pre-labeled with [^3H]cholesterol and incubated with 50 $\mu\text{g}/\text{mL}$ HDL for 4 hours to measure the efflux of [^3H]cholesterol to the HDL acceptor. Values represent the mean $\pm$ SD of four independent experiments, each performed in quadruplicate. For both panels, all data sets were normalized to wild-type SR-BI (normalized value = 100%) following subtraction of empty vector values. * $p < 0.05$, *** $p < 0.001$ as determined by one-way ANOVA.

Fig. 7.

Sensitivity of cells expressing wild-type SR-BI and mutant SR-BI receptors to cholesterol oxidase. COS-7 cells transiently expressing wild-type SR-BI or point mutants of SR-BI were pre-labeled with [3 H]cholesterol and incubated with exogenous cholesterol oxidase (0.5 U/mL) for 4 hours to measure the conversion of free cholesterol to cholestenone. Values represent the mean $\pm$ SD of three independent experiments, each performed in quadruplicate. ** $p < 0.01$, *** $p < 0.001$ as determined by one-way ANOVA.

Table 1

Hydropathy indices of evolutionarily conserved extracellular sequences before and after mutation. A moving average of hydropathy scores was calculated using a window size of 9. The amino acid(s) that offered the greatest contribution to the hydrophobicity of the indicated peak (i.e. region of 9 amino acids) was/were chosen for mutation.

Hydrophobic Region		Mutated Residue(s)	Hydropathy Index ^b		
Region ^a	Sequence		Original	Non-conservative (V to N, L to Q)	Conservative (V to L, L to V)
A	LSVYFFE V	V67	1.82	0.97	1.78
B	IVLPN IL VL	L140/L142	2.64	1.01	2.72
C	LMMTLAL VT	V164	2.20	1.34	2.16
D	GVFTVFTGV	V221	1.78	0.92	1.74
E	VLSEAV L GL	L359	1.88	1.07	1.93
F	IIEPVV L PLL	L411	1.96	1.14	2.00

^aRegions of hydrophobicity are indicated in Figure 1. Murine SR-BI amino acid sequences of each hydrophobic region are indicated. The residue with the greatest contribution to hydrophobicity in that region is indicated in bold.

^bCalculated using a window size of 9.