Glycine 420 Near the C-terminal Transmembrane Domain of SR-BI Is Critical for Proper Delivery and Metabolism of High Density Lipoprotein Cholesteryl Ester*

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Scavenger receptor BI, SR-BI, is a physiologically relevant receptor for high density lipoprotein (HDL) that mediates the uptake of cholesteryl esters and delivers them to a metabolically active membrane pool where they are subsequently hydrolyzed. A previously characterized SR-BI mutant, A-VI, with an epitope tag inserted into the extracellular domain near the C-terminal transmembrane segment, revealed a separation-of-function between SR-BI-mediated HDL cholesteryl ester uptake and cholesterol efflux to HDL, on one hand, and cholesterol release to small unilamellar phospholipid vesicle acceptors and an increased cholesterol oxidase-sensitive pool of membrane free cholesterol on the other. To further elucidate amino acid residues responsible for this separation-of-function phenotype, we engineered alanine substitutions and point mutations in and around the site of epitope tag insertion, and tested these for various cholesterol transport functions. We found that changing amino acid 420 from glycine to histidine had a profound effect on SR-BI function. Despite the ability to mediate selective HDL cholesteryl ester uptake, the G420H receptor had a greatly reduced ability to: 1) Enlarge the cholesterol oxidase-sensitive pool of membrane free cholesterol, 2) Mediate cholesterol efflux to HDL, even at low concentrations of HDL acceptor where binding-dependent cholesterol efflux predominates, and 3) Accumulate cholesterol mass within the cell. Most importantly, the G420H mutant was unable to deliver the HDL cholesteryl ester to a metabolically active membrane compartment for efficient hydrolysis. These observations have important implications regarding SR-BI function as related to its structure near the C-terminal transmembrane domain.

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§ The abbreviations used are: SR-BI, scavenger receptor class B type I; HDL, high density lipoprotein; FC, free cholesterol; POPC, palmitoyloleoylphosphatidylcholine; CE, cholesteryl ester/cholesterol oleate; COE, cholesteryl oleyl ether; DLT, dilactitol tyramine; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; ACAT, acyl CoA:cholesterol acyltransferase; apoA-I, apolipoprotein A-I; RCT, reverse cholesterol transport.
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All plasmids were prepared using endotoxin-free Qiagen Maxi-prep kits and sequenced throughout the SR-BI coding region for the correct mutation or epitope tag addition and to ensure that no undesired point mutations had been generated during the amplification process. DNA sequencing was performed by the automated sequencing facility at the State University of New York at Stony Brook. Reactions were prepared using a dye termination cycle sequencing kit and analyzed on Applied Biosystems Model 3100 DNA Sequencer with an Excel upgrade as recommended by the manufacturer (PE Applied Biosystems).

**Transient Transfection of COS-7 Cells**—COS-7 cells were maintained and transfected as previously described (28). The cells were assayed 48 h post-transfection unless otherwise indicated. Cell lysates were prepared as previously described (30, 31), and protein concentrations were determined by the Lowry method (32). Protein lysates were electrophoresed, transferred onto nitrocellulose membranes, and detected using a polyclonal anti-SR-BI C-terminal antibody (Novus Biologicals, Inc.) (1:5,000), a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) (1:10,000), and SuperSignal West Pico reagent (Pierce).

**Preparation of [125I]DLT-[3H]COE-HDL, [3H]CE-HDL, and [125I]HDL**—Human HDL (1.215 g/ml < ρ < 1.210 g/ml), herein referred to as HDL, was isolated by sequential ultracentrifugation (33). The HDL was labeled with either nonhydrolyzable [3H]cholesterol deyl ether ([3H]COE) (Amershams Biosciences) or hydrolyzable [3H]cholesterol acetate ([3H]CA) (Amersham Pharmacia Biotech) using recombinant cholesterol ester transfer protein (Cardiovascular Targets, Inc.) as described (34) with modifications (27). Labeled particles were re-isolated by gel exclusion chromatography and then labeled with [125I]diiodotyramine (DLT) as described previously (28). The average specific activity of the [125I]DLT-[3H]COE-HDL was 700 dpm/ng of protein for [125I]DLT and 6.0 dpm/ng of protein (15.4 dpm/ng of CE) for [3H]. The average specific activity of the [3H]CE-HDL was 7.4 dpm/ng of protein (27.5 dpm/ng of CE) for [3H]. For some experiments, HDL was labeled using the iodine monochloride method (35), and the average specific activity of the [125I]HDL was 478 dpm/ng of protein.

**HDL Cell Association, Selective COE Uptake, and Apolipoprotein Deposition**—Transiently transfected COS-7 cells (35-mm wells) were washed once with serum-free DMEM, 0.5% BSA, and [125I]DLT-[3H]COE-HDL particles were added at a concentration of 10 μg of protein/ml (unless otherwise indicated) in serum-free DMEM, 0.5% BSA. After incubation for 1.5 h at 37 °C, the medium was removed and the cells were washed three times with PBS, 0.1% BSA (pH 7.4) and one time with PBS (pH 7.4). The cells were lysed with 1 ml of 0.1 N NaOH, and the lysate was processed to determine trichloroacetic acid-insoluble and insoluble 125I radioactivity and organic solvent-extractable 3H radioactivity. The values for cell-associated HDL apolipoprotein, total cell-associated HDL COE, and the selective uptake of HDL COE, were obtained as previously described (28).

**Cholesterol Oxidase-sensitive FC, EF Flaxx, and Cholesterol Mass Assays**—Assays were performed as previously described (27). Statistical comparisons were made by unpaired Student’s t-test (GraphPad Prism version 4.0, GraphPad Software).

**Cholesterol Ester Hydrolysis Assay**—Transiently transfected COS-7 cells (35-mm wells) were preincubated for 2 h at 37 °C in the presence of an acyl CoA:cholesterol acyltransferase (ACAT) inhibitor, CP113,818 (gift from Pfizer), at a final concentration of 2 μg/ml in DMEM containing 0.5% BSA. [3H]COE-HDL particles were added at a concentration of 10 μg of protein/ml in DMEM containing 0.5% BSA and 2.5 μg/ml ACAT inhibitor. After incubation at 37 °C for 2 h (unless otherwise indicated), the medium was removed and the monolayers were washed three times with PBS, 0.1% BSA (pH 7.4). The lipids were extracted using a chloroform/methanol (1:1, v/v) mixture (36), and the aliquot was derivatized with silylating reagent as described (28). The extracts were then quantitated with the aid of a Varian 3600 gas chromatograph (Varian, Inc.) equipped with a flame ionization detector and a nonpolar capillary column, and the levels of cholesterol, cholesterol ester, and free cholesterol were determined. The results were compared with standards run on the same column.

**Preparation of Lipid Extracts**—Lipid extracts were prepared from cells grown at a density of 1 × 10⁶ cells/well in a 12-well plate. The plates were washed three times with PBS, 0.1% BSA, and the monolayers were washed three times with PBS, 0.1% BSA. One-tenth of the extracted lipids were spotted on a TLC plate (Solvus) with FC and CE as unlabeled standards. The samples were then eluted with chloroform/methanol (1:1, v/v) mixture (36), and the aliquot was derivatized with silylating reagent as described (28). The extracts were then quantitated with the aid of a Varian 3600 gas chromatograph (Varian, Inc.) equipped with a flame ionization detector and a nonpolar capillary column, and the levels of cholesterol, cholesterol ester, and free cholesterol were determined. The results were compared with standards run on the same column.

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FIG. 1. Cell-associated HDL, selective HDL COE uptake, and cholesterol oxidase sensitivity of cells expressing wild type SR-BI and mutant SR-BI receptors with alanine substitutions near the C-terminal transmembrane domain. A, the sequence of mouse SR-BI from amino acids 412–462 is delineated, and the predicted C-terminal transmembrane domain is underlined. The sites of two previously published mutants, A-VI and H-VI, are indicated by an asterisk at the A-VI insertion site and by the six-histidine replacement for H-VI (27). Below the wild type SR-BI sequence are a list of mutants and the positions of the amino acids that were substituted with alanines. COS-7 cells transiently expressing wild type SR-BI or the alanine substitution mutants were incubated at 37 °C for 1.5 h with [125I]DLT-[3H]COE-labeled HDL (10 μg of HDL protein/ml), after which cells were processed to determine cell-associated HDL COE (B) and selective HDL COE uptake (C). Immunoblot analysis of protein lysates, from parallel wells of cells, detected with antibody directed against the C-terminal cytoplasmic domain of SR-BI shows expression levels of each receptor compared with wild type SR-BI (D). Parallel wells of cells expressing SR-BI or the alanine substitution mutants were incubated for 24 h with 5 μCi/ml [3H]cholesterol in serum-containing medium as described under “Experimental Procedures.” After washing, cells were incubated with exogenous cholesterol oxidase for 4 h, and the percentage of cellular [3H]cholesterol oxidized ([3H]cholestenone) was determined (E). Values represent the mean ± S.D. of three replicates and are representative of three separate experiments.
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**Fig. 2.** Comparison of cells expressing mutant receptors to cells expressing wild type SR-BI. (A) Oxidase-sensitive Pool of Membrane FC (Fig. 1B) and oxidase-sensitive Pool of Membrane COE (Fig. 1C). W415A–G420A expressing COS-7 cells exhibited wild type levels of selective HDL COE uptake (Fig. 1C). W415A-G424H expressing COS-7 cells exhibited wild type levels of selective HDL COE uptake (Fig. 1C). Interestingly, these cells also exhibited a reduced oxidase-sensitive pool of membrane FC compared with wild type SR-BI-expressing cells (Fig. 1C). **Point Mutations in SR-BI Reveal a Glycine to Histidine Mutation That Reduces the Ability of the Receptor to Mediate Selective HDL COE Uptake and Increase the Size of the Oxidase-sensitive Pool of Membrane FC**—The ability of the G420A–G424A mutant receptor to mediate efficient selective uptake of HDL COE, but to produce only a minimal increase in the size of the oxidase-sensitive pool of membrane FC, is similar to what we reported previously for the A-VI epitope tag insertion mutant (27). Therefore, we mutated the G420A–G424A residues individually, to determine which were most important for the separation-of-function phenotype. Analysis of the protein sequences from seven different mammals (mouse, rat, human, hamster, rabbit, pig, and cow) showed the residues in the region of SR-BI near the C-terminal transmembrane segment (amino acids 412–462) that are conserved in evolution (Fig. 2). Due to their high degree of cross-species homology, residues Gly-420, Met-422, and Gly-424 were chosen for further mutation (see residues highlighted in boldface, Fig. 2). We modified the SR-BI cDNA to encode alanines (G420A, M422A, and G424A) or histidines (G420H and G424H) at these positions (Fig. 3A). In addition, double alanine and double histidine mutants were generated for Gly-420 and Gly-424 (G420A/G424A and G420H/G424H) at these sites (Fig. 3A). We then expressed the mutant receptors in COS-7 cells and assessed the ability of these point mutants to mediate lipid transport.

**RESULTS**

**Alanine Substitutions Reveal a Span of Five Amino Acids That, When Mutated, Allows SR-BI to Mediate Selective HDL COE Uptake, but Inhibits Its Ability to Increase the Size of the Oxidase-sensitive Pool of Membrane Cholesterol**—We recently reported an SR-BI mutant, A-VI, that contains an epitope tag, from the adenosine virus E4/5 protein, inserted in the extracellular region of SR-BI near its C-terminal transmembrane domain (27). To further define the residues responsible for the A-VI separation-of-function phenotype, we substituted blocks of five amino acids, near the epitope tag insertion site (indicated in Fig. 1A) by alanines (Fig. 1A). When expressed in COS-7 cells, all of the alanine substitution mutants exhibited at or near wild type SR-BI levels of expression as observed by immunoblot (Fig. 1D). Therefore, we proceeded to assess the ability of these alanine substitution mutants to bind HDL and mediate lipid transport.

**Comparison of cells expressing mutant receptors to cells expressing wild type SR-BI revealed that the alanine substitution mutants displayed four different phenotypes (Fig. 1). K425A–T429A expressing COS-7 cells exhibited wild type levels of HDL binding (Fig. 1B), selective HDL COE uptake (Fig. 1C) and cholesterol oxidase-sensitive FC (Fig. 1E). W415A–S419A-expressing cells, on the other hand, did not bind HDL (Fig. 1B), did not mediate selective HDL COE uptake (Fig. 1C), and did not show an increase in the size of the oxidase-sensitive pool of membrane FC (Fig. 1E). This was expected, because this mutation overlaps the glutamine residue (Gln-418) that was previously reported to be involved in HDL binding (36). F430A–L434A- and V435A–E462 expressing cells exhibited reduced HDL binding (Fig. 1B), reduced selective HDL COE uptake (Fig. 1C), and a reduced ability to produce an increase in the oxidase-sensitive pool of membrane FC (Fig. 1E) when compared with wild type SR-BI-expressing cells. However, we did not determine whether the decrease in HDL binding for these two mutants was due to decreased cell surface receptor expression or due to a decreased ability of the mutant to bind HDL. Despite a reduced level of cell surface HDL binding, G420A–G424A-expressing COS-7 cells exhibited wild type levels of selective HDL COE uptake (Fig. 1C). Interestingly, these cells also exhibited a reduced oxidase-sensitive pool of membrane FC compared with wild type SR-BI-expressing cells (Fig. 1E).
COE uptake, cells expressing G420H and the double-histidine mutant G420H/G424H exhibited levels of HDL COE-selective uptake that were 50–60% wild type levels (Fig. 3C). Furthermore, G420H- and G420H/G424H-expressing cells showed no increase in the size of the oxidase-sensitive pool of membrane FC when compared with wild type SR-BI cells (Fig. 3E).

Histidine Replacement of Glycine 420, G420H, Greatly Reduces the Ability of SR-BI to Efflux Cholesterol to HDL—In addition to mediating HDL CE-selective uptake, SR-BI accelerates the release of FC from cells (15, 16, 25). To determine whether the G420H mutation had an effect on the ability of the receptor to mediate cholesterol efflux, COS-7 cells expressing the point mutant receptors were assayed for efflux of cholesterol to HDL (200 μg/ml) (Fig. 4A). The cells expressing G420A, M22A, G424A, G420A/G424A, and G424H were able to efflux cholesterol to HDL in 4 h with the same efficiency as wild type SR-BI-expressing cells (Fig. 4A). G420H- and G420H/G424H-expressing cells, however, showed a reduced ability to release cholesterol to HDL (Fig. 4A). In addition, we tested the ability of G420H to efflux cholesterol to increasing concentrations of HDL. The G420H mutant-expressing cells showed a greatly reduced ability to release cholesterol, even to lower HDL ac-
cells were harvested to determine the amount of [3H]cholesterol released from the cells. Values represent the mean ± S.D. of three replicates and are representative of three separate experiments.

![Cholesterol Efflux](image)

**Fig. 4.** Effects of expression of wild type SR-BI and mutant SR-BI receptors with point mutations on FC efflux to HDL. **A,** COS-7 cells transiently expressing SR-BI or point mutant receptors were prelabeled with [3H]cholesterol and incubated with 200 µg/ml HDL for 4 h to measure the efflux of [3H]cholesterol. After incubation, cells were harvested to determine the amount of [3H]cholesterol released from the cells. **B,** COS-7 cells transiently expressing SR-BI or G420H point mutant receptor were prelabeled with [3H]cholesterol and incubated with increasing concentrations of HDL for 4 h to measure the efflux of [3H]cholesterol. After incubation, cells were harvested to determine the amount of [3H]cholesterol released from the cells. Values represent the mean ± S.D. of three replicates and are representative of three separate experiments.

G420H Reduces the Ability of SR-BI to Deliver HDL CE, and the CE That It Does Deliver Is Not Efficiently Hydrolyzed—In earlier studies it was shown that SR-BI delivers HDL CE to a metabolically active membrane compartment, where the CE is efficiently hydrolyzed by a neutral CE hydrolase (24). To determine whether the point mutations affected the ability of SR-BI to direct HDL CE to a neutral CE hydrolytic pathway, we expressed the mutant receptors in COS-7 cells and assayed the cells for the amount of HDL CE incorporated and the fraction of this CE that was hydrolyzed. Although cells expressing G420A, M422A, G424A, G420A/G424A, and G424H exhibited wild type levels of HDL CE uptake, G420H and the double-histidine mutant G420H/G424H-expressing cells exhibited levels of HDL CE uptake that were about 25–30% of wild type levels (Fig. 5A). In addition, the HDL CE taken into the cells by cells expressing G420A, M422A, G424A, G420A/G424A, and G424H was hydrolyzed as efficiently as that delivered by wild type SR-BI (Fig. 5B). On the contrary, much of the HDL CE delivered to the cells by G420H and G420H/G424H remained esterified (Fig. 5B). To determine if the decreased hydrolysis in the G420H- and G420H/G424H-expressing cells was due to less uptake of HDL CE or to a change in the way the HDL CE is delivered to the cell, we compared the HDL CE taken into the cell to the amount of FC generated for cells expressing each of the mutants discussed so far. We found a very good correlation ($r^2$ of 0.9991) between the amount of HDL CE uptake and the amount of FC generated by CE hydrolysis (Fig. 5C). In fact, all of the mutants, including the G420A–G424A mutant, fall on the line (indicating ~77% hydrolysis), except for the two mutants with the G420H mutation. These two mutants, G420H and G420H/G424H, clearly fall below the line (indicating ~38% hydrolysis). This indicates that these two mutants generated less FC than would be expected for the amount of CE that they delivered to the cells.

Relative to SR-BI-expressing cells, G420H-expressing cells took up considerably less HDL CE (30% compared with SR-BI) than they did HDL COE (60% compared with SR-BI). To test whether the reduced uptake of HDL CE in the G420H-expressing cells was due to a direct inability of this mutant receptor to mediate uptake or due to a saturation of the membrane with unhydrolyzed HDL CE, we performed a time course of HDL CE uptake and hydrolysis. At the earliest time points, 15 and 30 min, the G420H mutant-expressing cells incorporated 85 and 58%, respectively, the amount of HDL CE incorporated by wild type SR-BI-expressing cells (Fig. 6A). After 1 h, however, G420H-expressing COS-7 cells showed about 32% of the HDL CE uptake seen with wild type SR-BI-expressing cells (Fig. 6A). In addition, when the amount of HDL CE accumulation was plotted over time, it became clear that HDL CE accumulated to the same extent in both the SR-BI- and G420H-expressing cells (Fig. 6B). The amount of FC, on the other hand, increased with time in the SR-BI-expressing cells due to hydrolysis of the CE delivered to the cell from the HDL particles by SR-BI. In contrast, the amount of FC in the G420H-expressing cells remained the same as those observed with cells transfected with vector alone (Fig. 6C). Clearly, the amount of HDL CE uptake in the G420H-expressing cells slowed down greatly with time, presumably due to the failure to hydrolyze the HDL CE.

G420H Mutation Reduces the Ability of SR-BI to Accumulate Cellular CE and FC—A striking characteristic of SR-BI is that it alters cholesterol content and distribution in cells (26). To test whether or not this mutation had an effect on the mass distribution of cholesterol, we expressed G420H, G420H/G424H, and G420A–G424A mutants in COS-7 cells and measured total and free cholesterol by gas chromatography. As previously observed, when compared with vector-transfected cells, SR-BI-expressing cells had an increased amount of total cholesterol, which included a statistically significant increase in free and esterified cholesterol (Table I). Like SR-BI, G420A–G424A-expressing cells also exhibited an increase in the levels of total, free, and esterified cholesterol, which was consistent with its ability to mediate wild type levels of selective HDL CE uptake (Fig. 1C) and to deliver the HDL CE for hydrolysis (Fig. 5C). G420H- and G420H/G424H-expressing cells, on the other hand, had a greatly reduced pool of esterified cholesterol compared with wild type SR-BI-expressing cells. This observation is consistent with the ability of these mutant receptors to deliver some HDL CE to the cell, but not to a membrane pool of CE that would allow its efficient hydrolysis. Notably, the reduced pool of CE seen in the G420H- and G420H/G424H-expressing cells was still statistically higher than that observed with cells transfected with vector alone, which is also consistent with the observation that these receptors do allow some uptake of HDL CE to occur. Although there is a reduction in the cellular accumulation of CE in G420H- and G420H/G424H-expressing cells, the values for cellular FC mass are intermediate to cells expressing wild type SR-BI. Although we have not tested this directly, G420H- and G420H/G424H-ex-
pressing cells probably retained the ability to mediate influx of HDL FC, which could account for the increase in cellular FC and not CE. We repeated this experiment in the presence of acyl CoA:cholesterol acyltransferase (ACAT) inhibitor and found that in cells that deliver HDL CE for hydrolysis, such as SR-BI- and G420A–G424A-expressing cells, there is a decrease in the amount of CE that accumulated in the cells. This was presumably because the cells that were not treated with ACAT inhibitor were able to hydrolyze and re-esterify a fraction of the HDL CE that was incorporated, and the cells treated with the ACAT inhibitor could not re-esterify the FC generated by hydrolysis. The G420H- and G420H/G424H-expressing cells, on the other hand, exhibit the same CE accumulation in the presence or absence of ACAT inhibitor, which indicates that the HDL CE came directly from the HDL particle and was not due to hydrolysis and re-esterification. This further supports the observation that, unlike wild type SR-BI, the G420H mutation does not permit the receptor to deliver the HDL CE for efficient CE hydrolysis.

**G420H Mutation Has No Effect on the Ability of the Receptor to Homo-oligomerize**—Interestingly, the region of SR-BI near and including part of the C-terminal transmembrane domain contains a leucine zipper motif (37). In addition, the critical glycine residue, Gly-420, is in register with the six highly conserved leucines that form this motif (see arrowheads, Fig. 2). Because leucine zipper regions are protein dimerization motifs and recent evidence shows that SR-BI is able to form homo-multimeric complexes (38), we decided to test whether the G420A–G424A or G420H mutations had an effect on homo-

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When both myc-SR-BI and FLAG-SR-BI were co-transfected, transiently expressing SR-BI or G420H were incubated at 37 °C for different time points with 10 μg/ml [3H]CE-labeled HDL in the presence of an ACAT inhibitor at a final concentration of 2 μg/ml. After incubation, the cells were processed to determine the total cell associated counts in dpm/mg cell protein (A), the amount of CE in dpm/mg of cell protein (B) and the amount of FC in dpm/mg of cell protein (C). Values represent the mean ± S.D. of three replicates and are representative of two separate experiments.

The key finding in this study is the identification of glycine 420 as a critical residue for the function of SR-BI. Substitution of histidine for glycine in G420H did not disrupt HDL binding and only modestly reduced the uptake of cholesteryl ether from HDL. In contrast, the uptake of HDL cholesteryl ester was much more compromised. The uptake of HDL cholesteryl ester via G420H was only modestly reduced at early times but progressively decreased with incubation time. This decrease was not due to a change in HDL CE accumulation in the cell as compared with SR-BI but to the failure to hydrolyze the CE (see Fig. 6B). Thus, in the absence of SR-BI-directed CE hydrolysis, membrane CE content appeared to saturate. This is the expected result, because the capacity of the plasma membrane to accommodate CE is limited to 2–3 mol% with respect to membrane phospholipid. Thus, in the absence of ongoing CE hydrolysis, the rate of net CE transfer from HDL declines. The G420H receptor provides support for the importance of the coupling between selective CE uptake and an efficient CE hydrolysis process that is necessary for selective uptake to continue. Additionally, results with the G420H receptor indicate that SR-BI is directly responsible for the efficient delivery of HDL CE to the CE hydrolysis process. The mechanism of this delivery is unclear at present. It could reflect the ability of SR-BI to recruit a neutral CE hydrolase to the plasma membrane, although the ability of SR-BI to facilitate CE hydrolysis by apparently different CE hydrolases in diverse cell types (24) argues against a direct protein-protein interaction in such a recruitment process. Alternatively, the failure of the G420H receptor to facilitate HDL CE hydrolysis might reflect the ability of SR-BI to organize membrane lipids in a manner that permits access of a cytoplasmic CE hydrolase to the CE within the membrane bilayer.

In addition to the loss of SR-BI-mediated HDL CE hydrolysis, the G420H receptor shows a complete loss of the cholesterol oxidase-sensitive pool of membrane FC, suggesting an inability of G420H to alter membrane lipid organization. One possibility is that the inability of G420H to facilitate HDL CE hydrolysis is responsible for the loss of the cholesterol oxidase-sensitive oligomerization. To demonstrate oligomer formation of wild type SR-BI, COS-7 cells were transfected with either of two SR-BI cDNA constructs containing C-terminal additions of either the c-myc (myc-SR-BI) or FLAG (FLAG-SR-BI) epitope tags (29). Cell lysates from COS-7 cells expressing myc-SR-BI or FLAG-SR-BI were immunoprecipitated with anti-myc or anti-FLAG antibodies, respectively, and immunoblots, probed with the appropriate antibodies, showed high levels of expression of both epitope-tagged receptors (Fig. 7, top panels, lanes 1 and 4). Cells transfected with myc-SR-BI and immunoprecipitated with anti-FLAG antibody or cells transfected with FLAG-SR-BI and immunoprecipitated with anti-myc antibody showed no detectable protein on the immunoblot, indicating that there was no nonspecific immunoprecipitation (lanes 2 and 3, Fig. 7). When both myc-SR-BI and FLAG-SR-BI were co-transfected, both epitope-tagged receptors could be co-immunoprecipitated by their opposing antibody, indicating that they were complexed with each other (lanes 5 and 6, Fig. 7). As a control for nonspecific complex formation, or aggregation, lysates of either myc-SR-BI- or FLAG-SR-BI-transfected cells were mixed and then immunoprecipitated. In this case, neither of the epitope-tagged receptors could be immunoprecipitated by their opposing antibody (lanes 7 and 8, Fig. 7), indicating that there was no co-immunoprecipitation of the epitope-tagged receptors if they were not expressed at the same time in the COS-7 cells. These results support the previous conclusion that wild type SR-BI is able to homo-oligomerize. In a similar fashion, the same series of co-transfections with myc-tagged and FLAG-tagged G420A-G424A or myc-tagged and FLAG-tagged G420H (Fig. 7, bottom panels) demonstrated that these receptors also homo-oligomerize.
COS-7 cells transiently expressing SR-BI, SR-BI mutants G420H, G420H/G424H, or G420A-G424A were incubated 72 h in DMEM containing 10% calf serum. Lipids were extracted, and cholesterol mass was measured by gas chromatography. Values are the mean (±S.D.) of three replicate determinations and are representative of two experiments.

| Receptor           | Total cholesterol (ACAT inhibitor) | Free cholesterol (ACAT inhibitor) | Cholesteryl ester (ACAT inhibitor) |
|--------------------|-----------------------------------|----------------------------------|-------------------------------------|
|                    | µg/mg of cell protein             |                                  |                                     |
| Vector             | 23.9 ± 2.1                        | 18.9 ± 1.3                       | 5.0 ± 0.8                           |
| SR-BI              | 48.2 ± 4.0                        | 29.3 ± 3.1                       | 18.9 ± 1.0                          |
| G420H              | 33.3 ± 0.7**                      | 26.2 ± 1.4                       | 7.1 ± 0.7**                         |
| G420H/G424H        | 29.8 ± 0.5                        | 23.2 ± 0.4                       | 6.6 ± 0.4                           |
| G420A-G424A        | 36.9 ± 0.4                        | 25.2 ± 0.5                       | 11.6 ± 0.5                          |

*p < 0.005 compared to vector.
*p < 0.05 compared to wild type SR-BI.

*Fig. 7. Formation of multimers of wild type SR-BI as well as G420H mutant receptors. Multimerization of immunoprecipitated myc-SR-BI and FLAG-SR-BI, myc-G420A–G424A and FLAG-G420A–G424A, and myc-G420H and FLAG-G420H. COS-7 cells transiently transfected with myc-SR-BI, FLAG-SR-BI, myc-G420A–G424A, FLAG-G420A–G424A, myc-G420H, FLAG-G420H, myc-SR-BI/FLAG-SR-BI, myc-G420A–G424A/FLAG-G420A–G424A, or myc-G420H/FLAG-G420H were lysed in Nonidet P-40 cell lysis buffer and immunoprecipitated with myc or FLAG antibody as described under “Experimental Procedures”. Immunoprecipitated SR-BI proteins were electrophoresed by 8% SDS-PAGE and analyzed by immunoblot using anti-myc or anti-FLAG antibodies.

As judged by fluorescence microscopy (data not shown) and the oligomerization assay (Fig. 7), disruption of SR-BI function in the G420H receptor is not due to an altered cell surface distribution of SR-BI or to the inability of the receptor to form homo-oligomers. This result was surprising considering the mutated glycine is in register with seven leucines that form a heptad repeat reminiscent of a leucine zipper motif (37, 40). The context of the Gly-420 residue (GAMGG) also closely resembles a GXXG motif, which is another motif responsible for dimerization of membrane channel/transport proteins (41–45). Therefore, we initially hypothesized that the alpha helical region, which includes the C-terminal transmembrane segment and the extracellular domain to residue 413 (see Fig. 2), might assist the receptor in homo-oligomerization as has been found for many other leucine-zipper containing proteins. Although this region, and Gly-420, may participate in such interactions, it does not appear necessary for homo-oligomerization.

Intriguingly, the extracellular part of this predicted alpha helix, which lies just outside of the C-terminal transmembrane segment of SR-BI, is amphipathic. As was expected, the side of the alpha helix that contains the leucine residues, as well as glycine 420, is hydrophobic, whereas the opposite side of the alpha helix has polar and charged residues. One possibility is that one side of the amphipathic helix interacts with apoA-I on HDL, and the opposite side interacts with the plasma membrane to draw the HDL close enough to properly deliver the HDL CE. Previous studies showed that the class A amphipathic alpha helix of apoA-I binds directly to SR-BI (46, 47). The apoA-I helix could interact with the polar face of the predicted SR-BI amphipathic helix in the Leu-413 through Gln-439 region. Histidine at residue 420 may disrupt the orientation of this alpha helix due to its bulk or a potential charge on the hydrophobic face. In contrast, glycine or alanine at this position is well tolerated. Further mutational analysis should be informative as to how residue 420 of SR-BI influences the interaction with HDL and the formation of the hydrophobic channel that transports the HDL CE to its site of metabolism.

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