Effects of amino acid substitutions at glycine 420 on SR-BI cholesterol transport function

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Abstract Scavenger receptor class B type I (SR-BI) facilitates the uptake of HDL cholesteryl esters (CEs) in a two-step process involving binding of HDL to its extracellular domain and transfer of HDL core CEs to a metabolically active membrane pool, where they are subsequently hydrolyzed by a neutral CE hydrolase. Recently, we characterized a mutant, G420H, which replaced glycine 420 in the extracellular domain of SR-BI with a histidine residue and had a profound effect on SR-BI function. The G420H mutant receptor exhibited a reduced ability to mediate selective HDL CE uptake and was unable to deliver HDL CE for hydrolysis, despite the fact that it retained the ability to bind HDL. This did not hold true if glycine 420 was replaced with an alanine residue; G420A maintained wild-type HDL binding and cholesterol transport activity. To further understand the role that glycine 420 plays in SR-BI function and why there was a disparity between replacing glycine 420 with a histidine versus an alanine, we generated a battery of point mutants by substituting glycine 420 with amino acids possessing side chains that were charged, hydrophobic, polar, or bulky and tested the resulting mutants for their ability to support HDL binding, HDL cholesterol transport, and delivery for hydrolysis. The results indicated that substitution with a negatively charged residue or a proline impaired cell surface expression of SR-BI or its interaction with HDL, respectively. Furthermore, substitution of glycine 420 with a positively charged residue reduced HDL CE uptake as well as its subsequent hydrolysis.—Parathath, S., Y. F. Darlington, M. de la Llera Moya, D. Drazul-Schrader, D. L. Williams, M. C. Phillips, G. H. Rothblat, and M. A. Connelly. **Effects of amino acid substitutions at glycine 420 on SR-BI cholesterol transport function. *J. Lipid Res.* 2007.** 48: **1386–1395.**

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Scavenger receptor class B type I (SR-BI) is a physiologically relevant HDL receptor that participates in many aspects of HDL cholesterol metabolism (for review, see Ref. 1–6). The most studied function of SR-BI is the selective uptake of HDL cholesteryl ester (CE), the process whereby HDL core CE is taken into the cell without degradation of the whole particle and its apolipoproteins (7–11). SR-BI-mediated selective uptake is the major route for the delivery of HDL CE to the liver and steroidogenic tissues in rodents (12–16) as well as in human steroidogenic cells (17, 18). Additionally, SR-BI has been shown to facilitate the bidirectional flux of free cholesterol (FC) (19–22). These observations suggest that SR-BI is important at both ends of the reverse cholesterol transport pathway: efflux of FC from cells within the vessel wall and selective uptake of HDL CE and FC into the liver. Although it is well established that SR-BI's role in the reverse cholesterol transport pathway is particularly important for cholesterol metabolism in rodents, it is not clear whether this is true in humans. However, several recent studies have reported genetic polymorphisms that implicate SR-BI as a significant player in human HDL cholesterol metabolism as well (23–30).

SR-BI-mediated HDL CE selective uptake is a two-step process involving *1*) lipoprotein binding to the extracellular domain of SR-BI and *2*) lipid transfer from the HDL particle to the cell (31–33). Recent reports showed conclusively that it is not only the presence but also the proper orientation of apolipoprotein A-I (apoA-I) that is necessary for optimal SR-BI-mediated HDL CE selective uptake (34–37). In addition, BLT-1, a compound reported to increase the affinity of HDL for SR-BI, blocks SR-BI-mediated lipid transport (38). Together, these observations suggest that *1*) binding of HDL to SR-BI is not

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Abbreviations: apoA-I, apolipoprotein A-I; BLT-1, blocks lipid transport-1; CE, cholesteryl ester; COE, cholesteryl oleyl ether; FC, free cholesterol; SR-BI, scavenger receptor class B type I.

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sufficient for lipid transfer and *2*) the HDL particle must be properly positioned for the formation of a "productive complex" to allow efficient lipid uptake to occur.

Although there is some controversy over how SR-BI facilitates the transfer of HDL lipids to the cell, which may be attributable in part to cell type-specific differences in cholesterol metabolism, there is less disagreement over the fate of HDL CE after uptake has occurred. In fact, SR-BI has been shown to deliver HDL CE into a metabolically active membrane pool, where they are hydrolyzed by cell type-specific neutral CE hydrolases (39). In conjunction with these activities, SR-BI increases cellular cholesterol mass and alters cholesterol distribution in plasma membrane domains as judged by the enhanced sensitivity of membrane cholesterol to extracellular cholesterol oxidase (40, 41). These data support the idea that SR-BI delivers HDL CE and FC into a metabolically active membrane pool where they are efficiently metabolized.

In a recent publication, we reported that changing a glycine residue in the extracellular region of SR-BI near the C-terminal transmembrane domain to histidine had a profound effect on the ability of the receptor to deliver HDL CE in a way that allowed its efficient metabolism (42). Despite the fact that the mutant receptor, G420H, retained the ability to bind HDL particles, it exhibited a reduced ability to mediate selective HDL CE uptake and deliver HDL CE to a membrane pool, where it could be acted upon by a neutral CE hydrolase (42). This was evident by a decrease in the percentage of HDL CE that was hydrolyzed as well as by a lack of augmentation in the pool of membrane cholesterol that was sensitive to cholesterol oxidase. Interestingly, replacement of glycine 420 with an alanine, G420A, had no affect on receptor function (42). In this study, we examined the consequences of further amino acid substitution at glycine 420 to understand the disparity between the functional consequences of these two mutant receptors, G420H and G420A. The results revealed that substitution of glycine 420 with amino acids containing positively charged side chains disrupted SR-BI cholesterol transport function, whereas those containing large bulky side chains did not. In addition, substitution of glycine 420 with amino acids containing either a negatively charged side chain or a proline residue reduced receptor cell surface expression or disrupted the formation of a productive complex between SR-BI and HDL, respectively. These results, coupled with the fact that glycine 420 and the amino acid residues surrounding it are evolutionarily conserved, lend support to the supposition that this region of the receptor is critical for SR-BI-mediated CE and FC delivery and its subsequent metabolism.

METHODS

Plasmids and sequencing

PCR amplifications were performed using a Perkin-Elmer Cetus DNA Thermal Cycler 9700 (Perkin-Elmer, Inc.). Oligonucleotides were purchased from Integrated DNA Technologies. The cloning procedure for pSG5(SR-BI), which contains the mouse SR-BI coding region, was described previously (43). The following primers were used to amplify pSG5(SR-BI) and introduce mutations into mouse SR-BI. Cloning of G420A and G420H was described previously (42). G420K, 5′-AGCCAGCTCTTCAAGCAAGGCAATGGGTGGCAAGCCCCTGAGCACG-3′ and 5′-AGCCAGCTCTTCAGCTCTGTTCGAACCACAGCAACGGCAGAAC-3′; G420E, 5′-AGCCAGCTCTTCAAGCGAAGCAATGGGTGGCAAGCCCCTGAGCACG-3′ and 5′-AGCCAGCTCTTCAGCTCTGTTCGAACCACAGCAACGGCAGAAC-3′; G420L, 5′-AGCCAGCTCTTCAAGCCTAGCAATGGGTGGCAAGCCCCTGAGCACG-3′ and 5′-AGCCAGCTCTTCAGCTCTGTTCGAACCACAGCAACGGCAGA-AC-3′; G420I, 5′-AGCCAGCTCTTCAAGCATAGCAATGGGTGGCAAGCCCCTGAGCACG-3′ and 5′-AGCCAGCTCTTCAGCTCTGTTCGAACCACAGCAACGGCAGAAC-3′; G420P, 5′-AGCCAGCTCTTCAAGCCCAGCAATGGGTGGCAAGCCCCTGAGCACG-3′ and 5′-AGCCAGCTCTTCAGCTCTGTTCGAACCACAGCAACGGCAGAAC-3′; G420Q, 5′-AGCCAGCTCTTCAAGCCAAGCAATGGGTGGCAAGCCCCTGAGCACG-3′ and 5′-AGCCAGCTCTTCAGCTCTGTTCGAACCACAGCAACGGCAGAAC-3′; G420F, 5′-AGCCAGCTCTTCAAGCTTCGCAATGGGTGGCAAGCCCCTGAGCACG-3′ and 5′-AGCCAGCTCTTCAGCTCTGTTCGAACCACAGCAACGGCAGAAC-3′. The resulting PCR products were digested with Sap I (New England Biolabs, Inc.) and recircularized. All plasmids were prepared using Endotoxin-free Qiagen Maxi-prep kits and sequenced throughout the SR-BI coding region to confirm the correct point mutation and to ensure that no undesired mutations had been generated during the amplification process. DNA sequencing was performed by the automated sequencing facility at Stony Brook University. Reactions were prepared using a dye termination cycle sequencing kit and analyzed on an 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 described previously (43). The cells were assayed at 48 h after transfection unless indicated otherwise. Cell lysates were made (44, 45), and protein concentrations were determined by the method of Lowry et al. (46). 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, Inc.).

Preparation of ^{125}I-dilactitol tyramine-[^{3}H]cholesteryl oleyl ether-HDL, [^{3}H]CE-HDL, and ^{125}I-HDL

Human HDL_3 ($1.125 < d < 1.210$ g/ml), herein referred to as HDL, was isolated by sequential ultracentrifugation (47). The HDL was labeled with either nonhydrolyzable [^{3}H]cholesteryl oleyl ether ([^{3}H]COE) (Amersham Life Sciences) or hydrolyzable [^{3}H]cholesteryl oleate/[^{3}H]CE (Amersham Life Sciences) using recombinant CE transfer protein (Cardiovascular Targets, Inc.) as described (48) with modifications (49). Labeled particles were reisolated by gel exclusion chromatography and then labeled with ^{125}I-dilactitol tyramine as described previously (43). The average specific activity of the ^{125}I-dilactitol tyramine-[^{3}H]COE-HDL was 330 dpm/ng protein for ^{125}I and 4.0 dpm/ng protein (16.7 dpm/ng CE) for ^{3}H. The average specific activity of the [^{3}H]CE-HDL was 13.5 dpm/ng protein (52.1 dpm/ng CE) for ^{3}H.

HDL cell association, selective COE uptake, and apolipoprotein degradation

Transiently transfected COS-7 cells (in 35 mm wells) were washed once with serum-free DMEM and 0.5% BSA, and ^{125}I-

dilactitol tyramine-[^{3}H]COE-HDL particles were added at a concentration of 10 μg protein/ml (unless indicated otherwise) in serum-free DMEM and 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 and 0.1% BSA (pH 7.4) and one time with PBS (pH 7.4). The cells were lysed with 1.1 ml of 0.1 N NaOH, and the lysate was processed to determine trichloroacetic acid-soluble and -insoluble ^{125}I radioactivity and organic solvent-extractable ^{3}H radioactivity. The values for cell-associated HDL apolipoprotein, total cell-associated HDL COE, and the selective uptake of HDL COE were obtained as described previously (43). The efficiency of HDL CE selective uptake was determined by subtracting the values for vector-transfected cells and normalizing the amount of HDL CE selective uptake to the amount of cell-associated HDL particles. Statistical comparisons were made by one-way ANOVA with Bonferroni posttest for all groups (* $P < 0.05$, ** $P < 0.001$) compared with wild-type SR-BI (GraphPad Prism version 4.0 software).

Cell surface biotinylation experiments

Transiently transfected COS-7 cells (in 35 mm wells) were washed with cold PBS (with calcium and magnesium) and incubated for 1 h at 4°C in the presence of biotin (1 mg/ml). Cells were washed with PBS containing 100 mM glycine and lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing Halt Protease Inhibitor Cocktail (Pierce, Inc.). After centrifugation, a portion of the clarified lysate was incubated with Neutravidin beads (Pierce, Inc.) for 1 h at room temperature. The beads were pelleted, washed four times with 1 ml of RIPA buffer per wash, and resuspended in 50–200 μl of gel loading buffer. Aliquots of total lysate and surface proteins, equivalent to the original amount of total lysate that was precipitated by the Neutravidin beads, were electrophoresed on 10% polyacrylamide gels, transferred onto nitrocellulose membranes, and detected using a polyclonal anti-SR-BI C-terminal antibody as described above.

Additional assays for cholesterol transport and metabolism

Assays for cholesterol influx (4 h of incubation with 10 μg/ml HDL_3 as the donor particles) (49), cholesterol efflux (4 h of incubation with 50 μg/ml HDL_3 as the acceptor particles), and CE hydrolysis (2 h of incubation with 10 μg/ml HDL_3) (42) were performed as described previously. Statistical comparisons were made by one-way ANOVA with Bonferroni posttest for all groups (* $P < 0.05$, ** $P < 0.001$) compared with wild-type SR-BI (GraphPad Prism version 4.0 software).

RESULTS

Glycine 420 is highly conserved in evolution and resides on an uncharged face of a large α-helical domain

To understand the role that glycine 420 plays in SR-BI function and to investigate the functional differences when replacing glycine 420 with a histidine versus an alanine, we examined the amino acid sequence surrounding this residue. Analysis of the protein sequence near SR-BI's C-terminal transmembrane segment (amino acids 412–439) from eight different mammals (mouse, rat, human, hamster, rabbit, pig, cow, and dog), chicken, and freshwater pufferfish revealed a remarkable degree of evolutionary conservation (**Fig. 1A**). In fact, glycine 420 was conserved in all 12 of the sequences we found in the Protein DataBank, including tree shrew and zebrafish SR-BI (data not shown). As noted previously, secondary structure prediction programs such as PredictProtein (available at www.embl-heidelberg.de/predictprotein or www.predictprotein.org) predict that the region surrounding glycine 420 is part of a large α-helical domain that extends into SR-BI's C-terminal transmembrane segment (49). This prediction especially holds true when comparing several species of SR-BI simultaneously in this program, even though the primary amino acid sequences of individual species of SR-BI (e.g., mouse, hamster, and dog) contain an extra proline (P426) in this region, which would be predicted to cause a break or kink in the α-helix.

Because glycine 420 is highly conserved in evolution and appears on the uncharged/less polar side of this long α-helix, we hypothesized that glycine 420 might play a dual role in the structure of SR-BI: *1*) it is uncharged, so it would maintain the hydrophobic nature of the uncharged side of the α-helix; *2*) it is small and flexible, which may allow the helix to mediate conformational changes that may be necessary for HDL binding and membrane channel formation to occur, the latter being true for glycine residues that are found in GXXXG motifs within transmembrane domains (50, 51). To test these hypotheses, we substituted glycine 420 with amino acids possessing side chains that were charged, hydrophobic, polar, or bulky and tested the resulting mutants for their ability to support HDL binding, HDL cholesterol transport, and delivery of HDL CE for metabolism.

Effects of replacing glycine 420 on HDL binding and HDL COE selective uptake

To test its role in the function of SR-BI, we substituted glycine 420 with alanine (G420A), leucine (G420L), isoleucine (G420I), phenylalanine (G420F), cysteine (G420C), histidine (G420H), glutamine (G420Q), lysine (G420K), glutamate (G420E), and proline (G420P). COS-7 cells transiently expressing most of the point mutant receptors exhibited wild-type or nearly wild-type levels of HDL binding (**Fig. 2A**). G420E- and G420P-expressing cells, however, exhibited significantly reduced HDL binding compared with wild-type SR-BI-expressing cells. G420K-expressing cells appeared to have reduced HDL binding, but the decrease in binding with these cells did not reach statistical significance ($P < 0.07$). In addition to wild-type levels of HDL binding, cells expressing G420A, G420L, G420I, G420F, and G420C exhibited wild-type or greater levels of selective HDL COE uptake (Fig. 2B). In contrast, cells expressing G420H, G420Q, G420K, G420E, and G420P exhibited significantly reduced HDL COE selective uptake (Fig. 2B). After calculating the selective uptake efficiency, it was clear that the G420E-expressing cells exhibited wild-type selective uptake efficiency despite the reduction in HDL binding by these cells (Fig. 2C). This also held true for cells expressing G420P; however, it was omitted from Fig. 2C because the selective uptake efficiency was artificially high as a result of the low amount of HDL

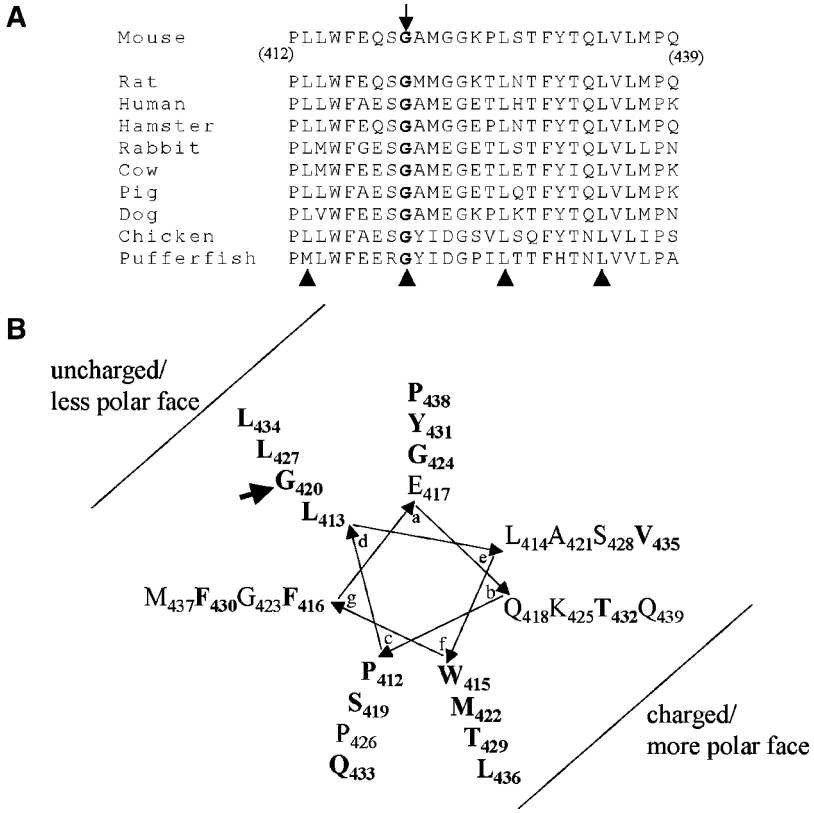

Fig. 1. Cross-species comparison and helical wheel representation of scavenger receptor class B type I (SR-BI) amino acid residues 412–439. A: The sequences of amino acids 412–439 from mouse, rat, human, hamster, rabbit, cow, pig, dog, chicken, and freshwater pufferfish SR-BI are shown [GenBank accession numbers NM_016741 (11), U76205 (70), NM_005505 (71), A53920 (72), AY283277 (73), ALL75567, O18824 (74), XP_543366, XP_415106, and CAG05393, respectively]. Four of the seven leucine residues in the putative seven heptad repeat-leucine zipper are indicated by closed arrowheads. Glycine 420 is indicated by an arrow. B: Helical wheel representation of amino acids 412–439 indicating the uncharged/less polar and charged/more polar sides of the α-helix through this region of SR-BI. The residues in boldface are highly conserved in evolution.

binding. On the other hand, G420H-, G420Q-, and G420K-expressing cells exhibited a significant reduction in selective uptake efficiency (Fig. 2C), as observed previously for COS-7 cells expressing the G420H mutant (42). Therefore, substitution of glycine 420 with an amino acid containing a long or bulky hydrophobic side chain (G420L, G420I, G420F) or a short and polar side chain (G420A, G420C) had no effect on receptor function with respect to HDL binding and uptake. Substitution with a negatively charged (G420E) or with a helix breaking/kinking (G420P) amino acid affected HDL binding but not the efficiency of HDL COE selective uptake. Moreover, substitution of glycine 420 with a positively charged (G420K) or with a long or bulky polar (G420Q, G420H) amino acid had little effect on HDL binding but had a significant effect on the efficiency of HDL COE selective uptake.

To clarify whether the decrease in HDL binding for mutants G420E and G420P was attributable to a reduction in cell surface expression of SR-BI or to the disruption of HDL binding to the receptor, we performed cell surface biotinylation experiments at 4°C (**Fig. 3**). The results revealed that all but one of the mutant receptors, G420E, expressed the majority of the receptor at the cell surface. With G420E, however, there was reproducible reduction in its cell surface expression, even when expressed at equivalent levels with wild-type SR-BI in the transient transfections (data not shown).

Effects of replacing glycine 420 on HDL CE hydrolysis

To determine whether additional amino acid substitutions at residue 420 affect 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 as well as the amount hydrolyzed. We found that COS-7 cells expressing G420A, G420L, G420I, G420F, and G420C exhibited wild-type levels of HDL CE uptake (**Fig. 4A**). However, cells expressing G420H, G420Q, G420K, G420E, and G420P exhibited significantly reduced levels of HDL CE uptake (Fig. 4A). Furthermore, cells expressing G420A, G420L, G420I, G420F, and G420C exhibited wild-type levels of HDL CE hydrolysis, as noted by the ability of these cells to decrease the amount of CE remaining in the cells (Fig. 4B). Despite the significant reduction in the ability to

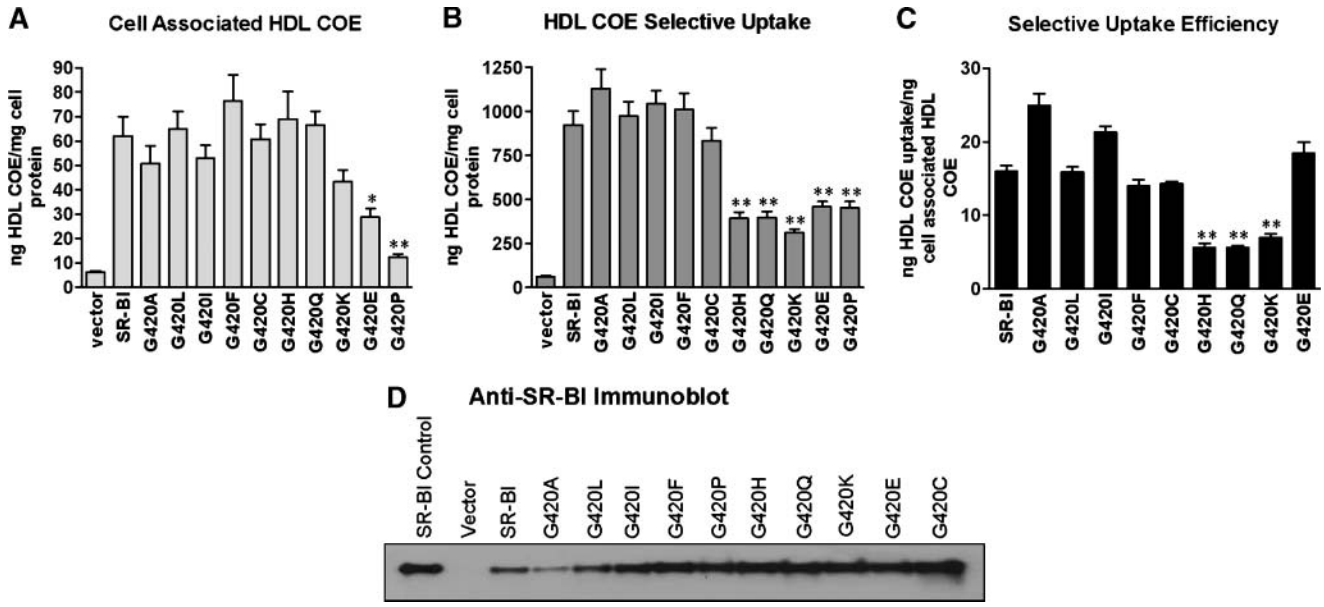

Fig. 2. Cell-associated HDL, selective HDL cholesteryl oleyl ether (COE) uptake, and selective uptake efficiency of cells expressing wild-type and glycine 420 mutant SR-BI receptors. A, B: COS-7 cells transiently expressing wild-type SR-BI or the glycine 420 amino acid substitution mutants were incubated at 37°C for 1.5 h with ^{125}I-dilactitol tyramine-[^{3}H]COE-labeled HDL (10 μg HDL protein/ml), after which cells were processed to determine cell-associated HDL COE (A) and selective HDL COE uptake (B). C: The efficiency of HDL COE selective uptake was determined by subtracting the values from vector-transfected cells and normalizing the amount of HDL COE selective uptake to the amount of cell-associated HDL particles. D: Immunoblot analysis of protein lysates, from parallel wells of cells, detected with antibody directed against SR-BI's C-terminal cytoplasmic domain illustrates typical expression levels of each receptor compared with wild-type SR-BI. Values represent means ± SD of six replicates from two separate experiments and are representative of five experiments overall. Statistical significance was determined by one-way ANOVA with Bonferroni posttest for all groups (* $P < 0.05$, ** $P < 0.001$) compared with wild-type SR-BI.

bind HDL and deliver HDL CE to the cells, G420E and G420P mutant-expressing cells maintained the ability to deliver HDL CE for efficient hydrolysis (Fig. 4B). Most interestingly, much of the HDL CE delivered to the cells by G420H, G420Q, and G420K remained esterified (Fig. 4B).

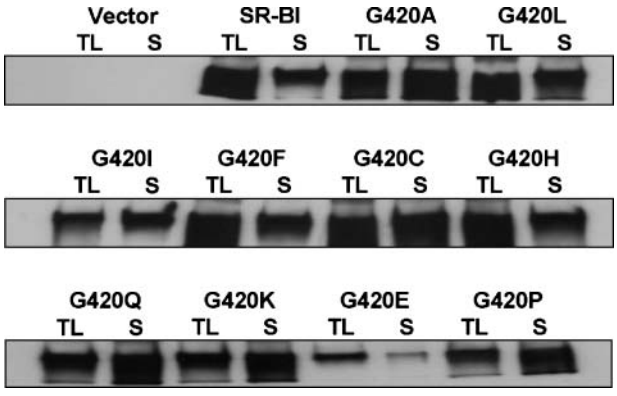

Fig. 3. Cell surface biotinylation of COS-7 cells expressing wild-type and glycine 420 mutant SR-BI receptors. COS-7 cells transiently expressing SR-BI or point mutant receptors were incubated for 1 h at 4°C in the presence of biotin reagent. The cells were lysed, and the resulting cell lysis product was incubated with Neutravidin beads to bind the biotinylated cell surface proteins. Immunoblotting was performed comparing total cell lysates (TL) with cell surface proteins (S) at equivalent concentrations. Results are representative of two separate experiments.

Therefore, the mutant receptors containing a positively charged or a long, bulky polar amino acid side chain at residue 420 (G420H, G420Q, G420K) were unable to deliver the HDL CE to a metabolically active pool of cholesterol, where they could be efficiently hydrolyzed.

Effects of replacing glycine 420 on HDL FC influx

Because our earlier studies revealed that G420H-expressing COS-7 cells showed no increase in the size of the cholesterol oxidase-sensitive pool of membrane FC compared with wild-type SR-BI cells, we wished to determine whether amino acid substitutions at this residue had an effect on the ability of SR-BI to facilitate the influx of HDL FC. To address this question, we transiently expressed the amino acid substitution mutants in COS-7 cells and tested for the ability of these cells to facilitate HDL FC influx. Cells expressing G420A, G420L, G420I, G420F, and G420C exhibited wild-type levels of HDL FC uptake, whereas cells expressing G420H, G420Q, G420K, G420E, and G420P exhibited reduced HDL FC influx (**Fig. 5**). Therefore, we could not separate the HDL CE and FC uptake functions of the receptor with the expression of mutants with amino acid substitutions at glycine 420.

Effects of replacing glycine 420 on HDL FC efflux

Expression of SR-BI leads to increased cholesterol efflux (19–21), and our earlier studies demonstrated that

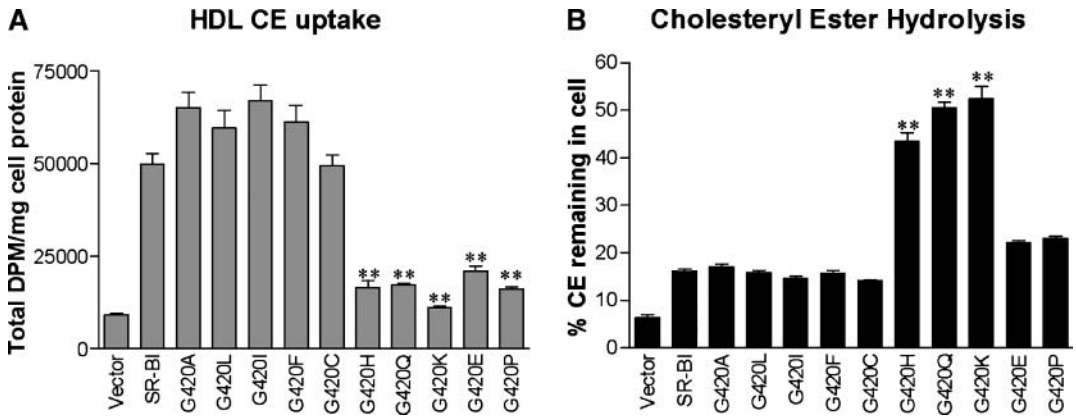

Fig. 4. Uptake and hydrolysis of HDL cholesteryl ester (CE) by cells expressing wild-type and glycine 420 mutant SR-BI receptors. COS-7 cells transiently expressing SR-BI or point mutant receptors were incubated at 37°C for 2 h with 10 μg/ml [^{3}H]CE-labeled HDL in the presence of an ACAT inhibitor at a final concentration of 2 μg/ml. After incubation, the cells were processed to determine the total cell-associated dpm/mg cell protein (A) and the percentage of the total number of counts that remained as [^{3}H]CE within the cells (B). Cells from the same transient transfection were used for HDL binding and selective HDL COE uptake, represented in Fig. 2, as well as for HDL CE uptake and hydrolysis, represented here, to have the same protein expression analysis for both experiments. Values represent means ± SD of six replicates from two separate experiments and are representative of five experiments overall. Statistical significance was determined by one-way ANOVA with Bonferroni posttest for all groups (** $P < 0.001$) compared with wild-type SR-BI.

G420H-expressing COS-7 cells showed a reduction in the amount of FC efflux compared with cells expressing wild-type SR-BI (42). To determine how different amino acid substitutions would alter FC efflux to HDL as an acceptor, we transiently expressed the glycine 420 mutants in COS-7 cells and tested for the ability of these cells to facilitate HDL FC efflux. Cells expressing G420A, G420L, G420I, G420F, and G420C exhibited wild-type levels of HDL FC efflux, whereas cells expressing G420H, G420Q, G420K, G420E, and G420P exhibited reduced HDL FC efflux (**Fig. 6**). Of these mutants, cells expressing G420H and G420K exhibited the lowest degree of efflux compared with cells expressing wild-type SR-BI. Interestingly, cells expressing G420Q, G420P, and G420E exhibited reduced FC efflux compared with SR-BI; however, the values were statistically higher than those observed with G420H- and G420K-expressing cells. Therefore, charged amino acids at glycine 420 resulted in a reduction in FC efflux, but positively charged amino acids resulted in an even larger reduction in FC efflux compared with wild-type SR-BI.

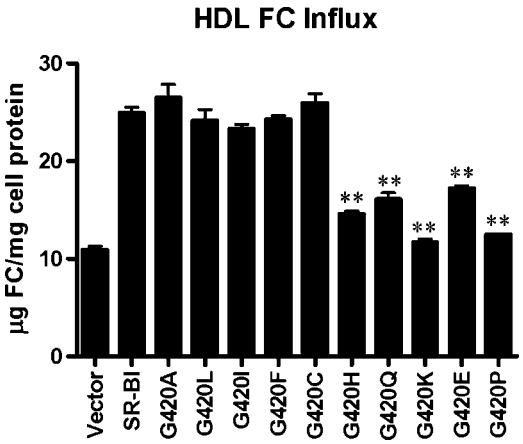

Fig. 5. Effects of the expression of wild-type and glycine 420 mutant SR-BI receptors on HDL free cholesterol (FC) influx. COS-7 cells transiently expressing SR-BI or point mutant receptors were incubated at 37°C for 4 h with 10 μg/ml [^{3}H]cholesterol-labeled HDL_3 to measure FC influx. Values represent means ± SD of three replicates. Statistical significance was determined by one-way ANOVA with Bonferroni posttest for all groups (** $P < 0.001$) compared with wild-type SR-BI.

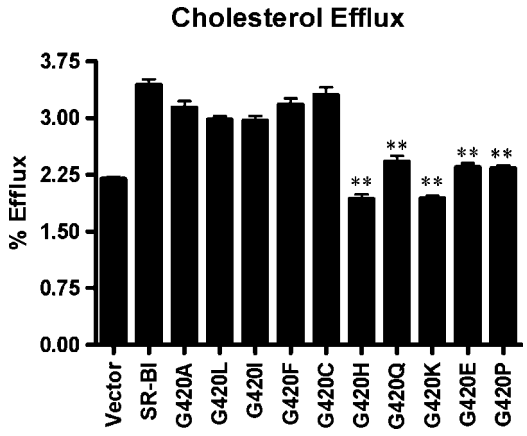

Fig. 6. Effects of the expression of wild-type and glycine 420 mutant SR-BI receptors on HDL cholesterol efflux. COS-7 cells transiently expressing SR-BI or point mutants were incubated overnight with [^{3}H]FC. Cholesterol efflux was determined after incubating cells with 50 μg/ml HDL_3 as the acceptor for 4 h at 37°C. Values represent means ± SD of three replicates. Statistical significance was determined by one-way ANOVA with Bonferroni posttest for all groups (** $P < 0.001$) compared with wild-type SR-BI.

DISCUSSION

Previously, we reported that changing glycine 420 to a histidine (G420H) had a profound effect on the ability SR-BI to deliver HDL CE in a way that allowed its efficient metabolism (42). Therefore, we hypothesized that glycine 420 plays a key structural role and that a histidine at this position would alter the interaction between this region of SR-BI and *1*) apoA-I on the HDL particle, *2*) the plasma membrane, or *3*) itself during homo-oligomer formation. This would result in a mutant receptor that is unable to form a productive complex with HDL particles for the proper delivery and metabolism of HDL CE. Because glycine 420 is highly conserved in evolution and appears on the uncharged/less polar side of a weakly amphipathic α-helix, we hypothesized that glycine 420 might play a dual role in the structure of SR-BI: *1*) it is uncharged, so it would keep the fairly hydrophobic nature of the less polar side of the α-helix; *2*) it is small and flexible, which may allow the α-helix to mediate conformational changes that may be necessary for HDL binding and membrane channel formation to occur; the latter is true for glycine residues that are found in GXXXG motifs within transmembrane domains (50, 51). To test these hypotheses, we substituted glycine 420 with amino acids possessing side chains that were charged, hydrophobic, polar, or bulky and tested the resulting mutants for their ability to support HDL binding, HDL cholesterol transport, and delivery of HDL CE for metabolism. Analysis of mutants with varying amino acid substitutions at glycine 420 in the extracellular domain of SR-BI revealed that they could be placed into four functional categories: *1*) hydrophobic, bulky, or short and polar; *2*) negatively charged; *3*) helix breaking/kinking; and *4*) positively charged or long and polar, with the potential for a positive charge depending on its environment within the protein. The descriptions of the amino acid side chains were determined by two references, Sweet and Eisenberg (52) and a recent review by Bowie (53), using OMH and partitioning from water to octanol as a means of determining the hydrophobicity of a given amino acid.

Substitution of glycine 420 with hydrophobic (G420L, G420I), bulky and hydrophobic (G420F), or short and polar (G420A, G420C) amino acids produced mutant receptors that when expressed in COS-7 cells exhibited no change in SR-BI function. Although cysteines have a dissociable proton and therefore have the potential for a negative charge, it is assumed from the data that the sulfur contained in its side chain is protonated and not free to react as a negative charge in its environmental context, or it would have had a discernible effect on receptor function. Even though glycine 420 is in register within a leucine zipper motif, it is so highly conserved as a glycine residue that it was surprising to observe that it could be replaced with a leucine without ramification to receptor function. It was also surprising that replacement of glycine 420 with much larger and bulkier residues, such as mutant G420I and G420F, had no effect on receptor function. This observation negates the hypothesis that this glycine residue needs to be small and flexible to allow conformational changes within the α-helix that might be necessary for HDL binding and membrane channel formation to occur, because it is clear that a large, bulky residue can replace it without disruption of HDL binding or HDL CE selective uptake. Alternatively, this residue may be highly conserved as a small and flexible glycine residue for another functional or structural reason that is not obvious from the assays we have used to date.

Substitution of glycine 420 with amino acids containing a negatively charged (G420E) or a helix breaking/kinking (G420P) side chain caused a significant reduction in HDL binding in cells expressing these mutant receptors. These substitutions either reduced the cell surface expression of the receptor or disrupted its ability to form a productive complex with the HDL particle. Despite the greatly reduced HDL binding and delivery of HDL CE to the cells, however, G420E and G420P mutant-expressing cells maintained the ability to deliver HDL CE for efficient hydrolysis.

Given that the region of SR-BI surrounding glycine 420 is part of a large α-helical domain and possibly part of SR-BI's HDL binding site (54), we hypothesized that it might be amphiphilic, as are many lipid-interacting peptides. Mutations at glycine 420 might affect the way in which this α-helix interacts with the cell membrane, thereby negating changes that the receptor produces in the plasma membrane. To test this hypothesis, we analyzed this region for the ability to form an amphipathic α-helix by calculating the hydrophobic moment of residues 412–439 with a program that uses the consensus scale of Eisenberg, Weiss, and Terwilliger (55). Whereas this program calculated a hydrophobic moment $<\mu_H>$ of 0.52 kcal/mol for melittin, a surface active toxin known to interact strongly with membranes via its amphiphilic α-helical structure, it calculated a $<\mu_H>$ of 0.15 kcal/mol for residues 412–439 of both mouse and human SR-BI. This result suggests that this α-helical region of SR-BI is weakly amphipathic but certainly not as amphipathic as the α-helical segments of apoA-I. In fact, 18A, an α-helix with properties common to all of the α-helical segments of apoA-I, has a $<\mu_H>$ of 0.57 kcal/mol (56), a value similar to the $<\mu_H>$ we calculated for melittin and much higher than the $<\mu_H>$ for residues 412–439 of SR-BI. To illustrate the fact that the extracellular part of this region is weakly amphipathic in nature, we represented this part of the α-helix in a helical wheel diagram (Fig. 1B). Because this region contains a heptad repeat of leucine residues, we chose a helical wheel diagram that is typical for leucine zipper motifs with ~3.6 amino acid residues per turn (Fig. 1B). As evident in this helical wheel diagram, one face of the helix is more charged than the other; however, some polar residues reside on the uncharged face of the helix (e.g., glycine), which makes it weakly amphipathic. In addition to the fact that amino acids 412–439 form an α-helix that is weakly amphipathic, the mean hydrophobicity of this region is fairly neutral: $<H>$ = 0.08 kcal/mol. If plotted on a hydrophobic moment plot ($<\mu_H>$ vs. $<H>$), as suggested by Eisenberg, Weiss, and Terwilliger (55), this segment

would lie between globular (soluble) and membrane (soluble in a nonpolar environment) proteins, suggesting that this region may be able to interact with membrane lipids under some circumstances or may be found in an overall hydrophobic environment near the cell membrane. However, the probability is low that it resides parallel to the membrane surface, as a highly amphiphilic peptide sequence would. Because it is unlikely that this α-helix is amphipathic in nature, this is probably not one of the mechanisms by which SR-BI affects the distribution of lipids in the plasma membrane.

Recently McMahon and Gallop (57) published a very insightful review of how proteins affect membrane structure. In that review, they list several mechanisms whereby proteins generate positive or negative membrane curvature and therefore affect cellular function: *1*) changing lipid composition; *2*) actively inserting an amphipathic α-helix; *3*) mediating cytoskeletal changes and altering microtubule motor activity; *4*) scaffolding by peripheral membrane proteins; and *5*) molding of membrane shape by membrane-spanning domains of integral membrane proteins, possibly by the shape of the membrane-spanning segments themselves or by oligomerization (57). Intriguingly, SR-BI has been shown to mediate two of these possibilities. First, SR-BI has the ability to change the composition of membrane lipids by increasing phospholipid species with long and unsaturated fatty acyl chains as well as increasing the amount of CE and FC within the cell (41, 42, 58). These changes decrease phosphatidylcholine-cholesterol interactions and enhance cholesterol desorption from the plasma membrane, which corresponds with the ability of SR-BI to facilitate the bidirectional flux of FC (58–61). In addition, it has been known for some time that cholesterol itself has profound effects on membrane structure that often correlate with changes in protein function. One example of this is the effect that SR-BI expression and its associated plasma membrane changes have on the function of endothelial nitric oxide synthase (62, 63). Second, SR-BI has been shown to homo-oligomerize (64–66). In fact, expression of SR-BI dimers correlates with changes in membrane topology, such as the formation of microvillar channels, the sites where selective HDL CE uptake is thought to occur (66–69). Finally, we propose that by changing the lipid composition of the plasma membrane and/or molding membrane shape by its two membrane-spanning domains, SR-BI is able to form a membrane domain that facilitates and/or enhances *1*) cholesterol oxidase sensitivity, *2*) bidirectional flux of FC, and *3*) delivery of HDL CE for hydrolysis. SR-BI, in fact, may organize membrane lipids in a manner that permits the access of a cytoplasmic CE hydrolase to the CE within the membrane bilayer, which would explain its ability to deliver HDL CE for metabolism. Furthermore, replacement of glycine 420 of SR-BI with a positively charged residue alters the structure of the receptor near the C-terminal transmembrane domain in such a way that it is no longer capable of mediating the requisite plasma membrane changes necessary for the proper delivery of HDL cholesterol, as was also observed in the FC flux studies. Whether the failure of the SR-BI glycine 420 positively charged mutants to enact membrane reorganization is related to a loss of the ability to change the composition of the membrane lipids or an inability to homo-oligomerize properly is not known at this time. However, this working model paves the way for more experimentation to dissect the effects of this region of the receptor on SR-BI-related membrane reorganization and cholesterol metabolism.

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