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Characterization of Hepatitis C Virus Particle Subpopulations Reveals Multiple Usage of the Scavenger Receptor BI for Entry Steps^{*[5]}

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Background: SR-BI binds HCV E2 glycoprotein and lipoprotein components.

Results: HCV entry exploits several SR-BI properties and different viral and cellular determinants present on the viral particles.

Conclusion: SR-BI is a multifunctional entry factor essential for infectivity of HCV particles with different biophysical properties and host protein compositions.

Significance: Studying HCV subpopulations reveals differential entry steps and receptor usage.

Hepatitis C virus (HCV) particles assemble along the very low density lipoprotein pathway and are released from hepatocytes as entities varying in their degree of lipid and apolipoprotein (apo) association as well as buoyant densities. Little is known about the cell entry pathway of these different HCV particle subpopulations, which likely occurs by regulated spatiotemporal processes involving several cell surface molecules. One of these molecules is the scavenger receptor BI (SR-BI), a receptor for high density lipoprotein that can bind to the HCV glycoprotein E2. By studying the entry properties of infectious virus subpopulations differing in their buoyant densities, we show that these HCV particles utilize SR-BI in a manifold manner. First, SR-BI mediates primary attachment of HCV particles of intermediate density to cells. These initial interactions involve apolipoproteins, such as apolipoprotein E, present on the surface of HCV particles, but not the E2 glycoprotein, suggesting that lipoprotein components in the virion act as host-derived ligands for important entry factors such as SR-BI. Second, we found that in contrast to this initial attachment, SR-BI mediates entry of HCV particles independent of their buoyant density. This function of SR-BI does not depend on E2/SR-BI interaction but relies on the

lipid transfer activity of SR-BI, probably by facilitating entry steps along with other HCV entry co-factors. Finally, our results underscore a third function of SR-BI governed by specific residues in hypervariable region 1 of E2 leading to enhanced cell entry and depending on SR-BI ability to bind to E2.

With more than 130 million persistently infected individuals worldwide, hepatitis C virus (HCV)⁵ has a substantial impact on public health. HCV infection is characterized by its high propensity to persist with up to 80% of individuals failing to eliminate the virus. Infected patients have a high risk to develop severe liver damage such as cirrhosis and hepatocellular carcinoma with liver transplantation as the final treatment option. No vaccine against HCV is yet available, and despite the ongoing development of HCV-specific DAAs (direct-acting antivirals) such as protease inhibitors, it remains of utmost importance to pursue the development of novel DAAs that can target alternative steps of HCV life cycle to more efficiently cure HCV-infected individuals. HCV entry into the cell offers numerous targets for development of such antiviral treatments (for review, see Ref. 1).

HCV is an enveloped positive-stranded RNA virus of the *Flaviviridae* family. A hallmark of HCV particles is their peculiar heterogeneity, exhibiting diverse buoyant densities ranging from <1.06 to >1.25 g/ml in the blood of infected patients (2–6). Viral RNA detected in low density fractions is associated

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We dedicate this paper to the memory of Pr. Chantal Rabourdin-Combe.

[5] This article contains supplemental Figs. 1–7.

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⁵ The abbreviations used are: HCV, hepatitis C virus; HCVcc, cell culture-produced HCV; SR-BI, scavenger receptor BI; hSR-BI, human SR-BI; CLDN1, Claudin-1; OCLN, Occludin; sE2, soluble E2 glycoprotein; HCVpp and VSV-Gpp, HCV pseudoparticles; HVR1, hypervariable region-1; VSV, vesicular stomatitis virus; CE, cholesteryl ester; HS, human serum; hCD81, human CD81; GE, genome equivalent; MFI, mean fluorescent intensity; BLT, blocking lipid transfer; PL, phospholipids.

with lipoprotein components such as triglycerides and apolipoproteins (apo) (2, 3, 5). A lipo-viro-particle (LVP) model consisting of HCV core protein, RNA, and E1 and E2 glycoproteins incrustated into lipoprotein-like structure has been proposed (2, 7). Similar to *ex vivo* HCV particles, *in vitro* produced HCV particles, *i.e.* cell culture-produced HCV (HCVcc), exhibit a broad density profile with high specific infectivity associated with low density fractions (8–15). Additionally, recent evidence suggests that HCVcc particles contain apolipoproteins on their surface and that their lipid composition resembles that of low density and very low density lipoproteins (LDLs and VLDLs, respectively) (16–19). These cellular components likely influence HCV infectivity (17, 20–23). In line with these observations, HCV assembly and egress strongly depends on components of the VLDL synthesis machinery (20–22, 24, 25). The remarkable property of HCV to associate with lipoprotein components might affect receptor usage and entry route and appears to protect HCV from neutralizing antibodies by shielding of antigenic epitopes (for review, see Ref. 26).

Extensive studies *ex vivo* using HCV derived from infected patients (27–29) and *in vitro* using HCV models (12, 13, 30–34) suggest that viral entry into hepatocytes, the predominant target cells, is a complex process involving the viral E1 and E2 envelope glycoproteins and several host cell surface factors. Attachment of viral particles to the host cell are mediated by glycosaminoglycans (35, 36) and/or the LDL receptor (17, 27, 29, 37), although it is unclear whether such interactions subsequently lead to a productive infection. Then a set of four molecules needs to be concomitantly present on the cell surface to allow HCV entry, probably after this initial capture. These are the scavenger receptor BI (SR-BI) (38, 39), CD81 tetraspanin (40), Claudin-1 (CLDN1) (41), and Occludin (OCLN) (42, 43). Furthermore, host cell kinases have been shown to regulate HCV entry by promoting cell entry factor associations (44). HCV particles are internalized by clathrin-dependent endocytosis (45, 46). Subsequent release of the viral genome into the cytoplasm is thought to occur after low pH-induced fusion of the viral and the endosomal membranes, a process that is likely triggered by the viral envelope glycoproteins and upon their interaction with CD81 (36, 46–50). Yet, the exact roles of the HCV entry factors for cell attachment, internalization, and subsequent membrane fusion are still elusive.

The HCV entry factor SR-BI is a “multi-ligand” receptor that binds different classes of lipoproteins and thereby regulates the supply of cholesterol to the cell by its bidirectional lipid transfer function (51, 52). SR-BI was initially proposed as a HCV receptor based on its capacity to mediate binding of soluble HCV E2 glycoprotein (sE2) to human hepatic cells (38). Using cell entry models, *i.e.* HCV pseudoparticles (HCVpp) and HCVcc, we and others demonstrated that SR-BI is essential for viral entry (for review, see Ref. 26) involving an interplay between SR-BI and the hypervariable region-1 (HVR1), a 27-amino acid-residue-long region, located at the N terminus of E2. HCV entry is also modulated by SR-BI ligands (22, 23, 53–57). It is enhanced by high density lipoprotein (HDL) in a still poorly defined process that involves SR-BI lipid transfer function and HVR1 (23, 55–59) and that modulates neutralization efficiency by antibodies (60, 61).

Data obtained with CHO cells expressing the HCV entry factors suggest that SR-BI, but not CD81 or CLDN1 (41), could mediate HCVcc attachment to cells. However, results obtained with experiments analyzing the kinetics of HCV infection indicate that SR-BI also plays a role at a post-binding stage (62). Thus, the exact function(s) of SR-BI in the entry process of HCV particles is still enigmatic. Moreover, the impact of lipids and lipoproteins associated with viral particles on SR-BI-dependent entry has not been studied in detail.

By studying HCVcc subpopulations separated by density gradient centrifugation, we revealed three different functions of SR-BI that are essential for HCV cell entry. Taking advantage of rodent orthologs of SR-BI and HVR1 mutants in this study, we dissected the modalities by which SR-BI mediates these distinct functions.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Human Huh-7 (63), Huh-7.5 (kind gift of C. Rice), BRL3A rat hepatoma (ATCC CRL-1442), and 293T kidney (ATCC CRL-1573) cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Fu5AH rat hepatoma cells (64) were grown in Eagle’s minimal essential medium supplemented with 1% L-glutamine and 5% newborn calf serum. BLT-4 (65) and human HDL were purchased from Chembridge and Calbiochem, respectively.

Antibodies—For Western blot analysis, human SR-BI was detected with CLA-1 mAb (BD Pharmingen), rodent SR-BI with NB400–104 (Novus), β -actin with AC74 (Sigma), and HCV core with C7–50 (Santa Cruz). For FACS analysis, human SR-BI was stained with CLA-1, rodent SR-BI with GTX30467 polyAb (Genetex), human CD81 with JS81 mAb (BD Pharmingen), and human CLDN1 with 2H10D10 mAb (Zymed Laboratories Inc.). The 3/11 (34) and H52 (66) are E2-specific mAbs. A4 (67) is an E1-specific mAb. Anti-SR-BI antibodies were generated by genetic immunization and have been described elsewhere (62, 68).

Expression Constructs and Establishment of Cell Lines Expressing CLDN1, OCLN, CD81, and SR-BI wt/Mutants—Retroviral vectors expressing human CD81 (GenBank™ accession number: NM_004356), Claudin-1 (NM_021101), and SR-BI (Z22555) were described previously (58). Rat (NM_031329), mouse (NM_008756), or human OCLN (NM_002538) and rat (NM_031541), mouse SR-BI (NM_016741), or human/mouse chimeric SR-BI cDNAs were inserted in CNC murine leukemia virus vector backbones (a kind gift of M. Collins) harboring selectable marker genes for puromycin and G418, respectively. Construct details are available upon request. cDNAs encoding six human/mouse SR-BI chimeras were generated by PCR by swapping three SR-BI domains between amino acid positions 38–215, 216–398, and 399–432 (primer sequences are available upon request). Although the HHH and MMM SR-BI constructs refer to the wild-type human (H) and mouse (M) SR-BI molecules, respectively, the human/mouse SR-BI chimeras were denominated according to the origin of either SR-BI domain, *e.g.* HMM bears the domain 1 from human SR-BI and the domains 2 and 3 from murine SR-BI. cDNAs encoding mutant mouse SR-BI molecules, M158R and G420H/G424H, that exhibit reduced lipid transfer properties (69–71) were gen-

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erated. All mutants were sequenced to ensure that the clones possessed only the expected mutation. Retroviral vectors containing these cDNAs were produced from 293T cells as VSV-G-pseudotyped particles as described previously (72, 73). Stable expression of either receptor in target cells was obtained by transduction with vector particle-containing supernatants of 293T producer cells followed by antibiotic selection.

Down-regulation of SR-BI in Huh-7.5 Cell Line—The down-regulation of SR-BI in Huh-7.5 cells was achieved using small interfering RNA (shRNA)-expressing lentiviral vectors (target sequence GGCCTGTTCTGGAACCTTC) as previously described (50).

Binding and Cell Surface Staining Assays—Binding of sE2 was performed as previously described (61, 74). Briefly, sE2 harboring a His tag was incubated for 1 h at 37 °C with 10^6 target cells. The amount of cell-bound sE2 was determined by FACS analysis using 2 $\mu\text{g}/\text{ml}$ anti-His antibody (pentaHis, Qiagen) and using allophycocyanin-conjugated anti-mouse antibodies.

HDL was labeled using Amersham Biosciences Cy5 Mono-Reactive Dye Pack (GE Healthcare) following the manufacturer's protocol. Unbound Cy5 was removed by applying labeled HDL on illustra MicroSpin G-25 Columns (GE Healthcare). Cy5-HDL binding was performed for 1 h at 4 °C on 10^6 target cells.

The surface expression of human CD81, human CLDN1, and human or mouse SR-BI was quantified by FACS analysis from 10^6 live cells using antibodies added to cells for 1 h at 4 °C. After washing, the binding of antibody to the cell surface was detected using RPE (R-Phycoerythrin)- or allophycocyanin-conjugated anti-mouse antibodies.

Lipid Transfer Assays—Lipid efflux assays were performed as previously described (75). After plating, cells were labeled by incubation with [^3H]cholesterol (1 $\mu\text{Ci}/\text{ml}$) for 48 h. Subsequently cells were incubated for 24 h in the presence of BSA (0.5%) and newborn calf serum (25%) for Fu5AH or fetal bovine serum (25%) for BRL3a to allow equilibration of the label. After equilibration, cholesterol acceptors (20 μg of phospholipid/ml isolated HDL) were added in serum-free medium and incubated with cells for 4 h at 37 °C. Fractional cholesterol efflux (expressed as percentage) was calculated as the amount of the label recovered in the medium divided by the total label in each well (radioactivity in the medium + radioactivity in the cells) obtained after lipid extraction from cells in a mixture of 3:2 hexane-isopropyl alcohol (3:2 v/v). The background cholesterol efflux obtained in the absence of cholesterol acceptor was subtracted from the efflux values obtained with the test samples.

Selective HDL-CE (cholesteryl ester) uptake was performed as previously described (76). Cells were plated in 24-well tissue culture plates (10^5 cells/well). Two days after plating, cells were washed three times with PBS and once with serum-free medium. Cells were subsequently incubated in the presence of [^3H]CE-labeled HDL diluted in serum-free medium at 37 °C for 5 h. At the end of incubation, the medium was removed, and cells were washed 4 times with PBS and incubated in the presence of an excess of unlabeled HDL (100 μg of protein) for 30 min. Cells were then washed 4 times with PBS and solubilized with 200 μl of NaOH 0.2 N for 15 min at room temperature with gentle mixing. Protein content (20 μl) from each well was mea-

sured using the bicinchoninic acid protein reagent (Pierce). The radioactive content of 100 μl of each cell lysate was measured by liquid scintillation counting. Selective uptake was calculated from the known specific radioactivity of radiolabeled HDL-CE and is expressed in μg of HDL-CE/ μg of cell protein.

Production of HCVpp and HCVpp Entry Assays—The expression vectors for the E1E2 glycoproteins of HCV strain H77 (AF009606), for the HVR1 deletion mutant ($\Delta\text{G384-N411}$), and for the L399R point mutant in HVR1 were described previously (30, 39, 55). Viral pseudoparticles named HCVpp and VSV-Gpp harbored the glycoproteins of HCV and VSV, respectively, and were produced as described previously (30). Before harvest of viral particle-containing supernatants, producer cells were incubated in medium devoid of serum lipoprotein (Opti-MEM) for 24 h.

For infection assays, target cells were seeded 24 h before inoculation. 2 h before infection, target cells were preincubated in Opti-MEM devoid of serum lipoprotein. Then medium was removed, and dilutions of viral supernatants were added to the cells and incubated for 4 h. Where indicated, human serum (HS), prepared as described previously (55), or human HDL (Calbiochem) was added to the infection reactions at 6 $\mu\text{g}/\text{ml}$ of cholesterol. Supernatants were then removed, and the infected cells were kept in regular medium (DMEM, 10% FCS) for 72 h before analysis of the percentage of GFP-positive cells by FACS analysis (30). The infectious titers were expressed as GFP infection units per ml of HCVpp-containing medium. Background infection was controlled by using non-enveloped particles.

HCVcc Production and Cell Binding Assays—Plasmid pFK H77/JFH1/HQL displaying adaptive mutations (Y835H (in NS2), K1402Q (in NS3), and V2440L (in NS5A)) that enhance production and infectivity of HCVcc particles (77) was used to generate recombinant genomes containing the same H77 HCV E1E2 sequences, parental or HVR1-modified, used for HCVpp production. Generation of infectious HCV RNAs, production, and titration of HCVcc particles from Huh-7.5 cells was performed as previously reported (23, 58, 78).

For cell binding assay, BRL3A cells expressing or not expressing the HCV entry factors, seeded in 48-well plates were incubated with HCVcc particles (1×10^5 HCV RNA copies) for 2 h as indicated in figure legends. Cells were then washed three times with PBS, and total RNA from cell lysates was extracted using the RNeasy Mini kit (Qiagen) and reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). HCV and housekeeping gene RNA were quantified with HCV (5'-CTT-CACGCAGAAAGCGCCTA and 5'-CAAGCGCCCTAT-CAGGCAGT) or rat GAPDH (5'-GTTACCAGGGCTGCCT-TCTC and 5'-GGGTTTCCCGTTGATGACC) using the FastStart Universal SYBR Green Master kit (Roche Applied Science) on an Applied StepOne Real-Time PCR apparatus.

Iodixanol Density Gradient Fractionation—HCVcc particle-containing supernatants were passed through 0.45- μm pore-size filters and concentrated by Vivaspin molecular weight cut-off 100-kDa columns (Sartorius). 1 ml of concentrated particles was layered on top of a 0–30% continuous iodixanol gradient (Optiprep, Axis-Shield). Gradients were centrifuged for 16 h at 31,000 rpm in a SW41 TI swinging rotor at 4 °C using an Optima L-90 K Beckmann centrifuge. Twelve fractions of 1 ml

were collected from the top, subjected to another Vivaspin column centrifugation molecular weight cutoff 100 kDa to remove iodixanol, and then analyzed for virus infectivity, core protein amounts, viral RNA copy number, and receptor binding properties.

HCV core protein was quantified using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Wako Chemicals, Neuss, Germany). Viral RNAs were isolated from gradient fractions using Tri Reagent solution (Sigma) as recommended by the manufacturer. Quantification of purified RNAs was carried out as described above for cell binding assays.

Potassium Depletion—Endocytosis was blocked by potassium depletion as previously described (79). Briefly, cells were washed twice with PBS+ (PBS, 1 mM MgCl₂, 0.1 mM CaCl₂) followed by 1 wash and a short incubation of 5–7 min in hypotonic medium (PBS+/H₂O (1:3 v/v)). The cells were then washed 5–8 times in a medium containing 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 20 mM HEPES/KOH, pH 7.4, 0.5% DMSO, and 0.5% BSA followed by a 30-min incubation in the same medium. For HCVcc binding assays, HCVcc particles were added directly to the medium and processed as described above.

RESULTS

Attachment Properties of HCVcc Subpopulations to SR-BI—HCVcc has a heterogeneous profile of buoyant density and association to lipoprotein components (7, 19), which could differentially influence cell entry steps including the interaction with entry factors such as SR-BI. A previous report using human SR-BI (hSR-BI)- and human CD81 (hCD81)-expressing CHO cells suggested that hSR-BI but not hCD81 promotes binding of total HCVcc particles (41). Thus, using the same procedure (41), we investigated binding to SR-BI expressed in Huh-7.5-permissive cells of HCVcc subpopulations separated along their buoyant densities by iodixanol density gradients.

In agreement with recent reports (9, 14, 15, 19), HCVcc particles had more than 65% of their infectivity in fractions of densities of 1.08–1.13 g/ml, *i.e.* slightly shifted toward low densities as compared with the peak of HCV RNA level (1.10–1.16 g/ml) (Fig. 1A). In addition, in lower density fractions, *i.e.* below 1.08 g/ml, substantial amounts of infectious particles were detected, accounting for about 30% of total infectivity (Fig. 1A). When the same amounts of HCVcc particles, *i.e.* 1×10^5 HCV genome equivalent (GE) were compared for each density fractions, we found that HCVcc particle subpopulations had a variable capacity to bind Huh-7.5 cells, with a maximal binding detected for HCVcc particles of low density (*i.e.* 1.02–1.10 g/ml) (Fig. 1B). By modulating SR-BI levels through its overexpression or down-regulation (Fig. 1E), we found that SR-BI is a rate-limiting factor that preferentially permits the attachment of HCV particles of intermediate densities (*i.e.* above 1.10 g/ml; Fig. 1B). Indeed, SR-BI up-regulation increased by up to 10-fold the attachment of the latter HCVcc particles, which was competed by SR-BI blocking antibodies (Fig. 1C), whereas SR-BI down-regulation reduced their binding (Fig. 1B). Interestingly, up-regulation and down-regulation of SR-BI positively and negatively correlated, respectively, with the infectivity of HCVcc

(Fig. 1A). This effect was detected whatever the viral particle density, in contrast to the lack of impact of SR-BI expression modulation on binding of low density viral particles (Fig. 1B).

Next, to confirm and extend the significance of these results, we used BRL3A rat hepatocarcinoma cells devoid of endogenous SR-BI (58) that ectopically expressed, or not, hSR-BI to levels similar to the endogenous levels detected in Huh-7.5 cells (Fig. 1, *E versus G*). Similar to the observations made using Huh-7.5 cells, we found that a maximal binding to parental BRL3A cells was detected for HCVcc particles of low density (*i.e.* 1.02–1.10 g/ml) that allowed recovery of 10–20% of the input viral particles (10^5 HCV GE for each density fraction) (Fig. 1D). Importantly, although less than 1% of the input viral particles of intermediate densities (*i.e.* above 1.10 g/ml) could be recovered after incubation with BRL3A cells, expression of hSR-BI in these cells increased by 10–50-fold the recovery of these latter HCVcc particles (Fig. 1D), in agreement with results obtained with Huh-7.5 cells overexpressing SR-BI (Fig. 1B). SR-BI-mediated increased recovery of viral particles of intermediate density was also detected at 4 °C (Fig. 1F) (although less intensively than at 37 °C), indicating that hSR-BI promotes binding of virions. Consistently, SR-BI-mediated binding of intermediate density HCV particles was also detected when internalization was prevented in SR-BI-expressing BRL3A cells (Fig. 1H) through intracellular potassium depletion that inhibits clathrin-mediated endocytosis (80) and SR-BI internalization (79). In contrast, no change of binding profile upon hSR-BI expression could be detected for HCVcc particles of lower densities (*i.e.* below 1.10 g/ml).

Thus, altogether, these results indicated that the capacity of hSR-BI to interact with HCVcc particles specifically depends on their physicochemical properties and, in a non-exclusive manner, that binding of low density HCVcc particles is mediated by another cell surface receptor. Yet, SR-BI also seems to be involved in the infectivity of viral particles of these latter densities. We, therefore, sought to investigate the nature of the determinants that modulate HCV/SR-BI interaction.

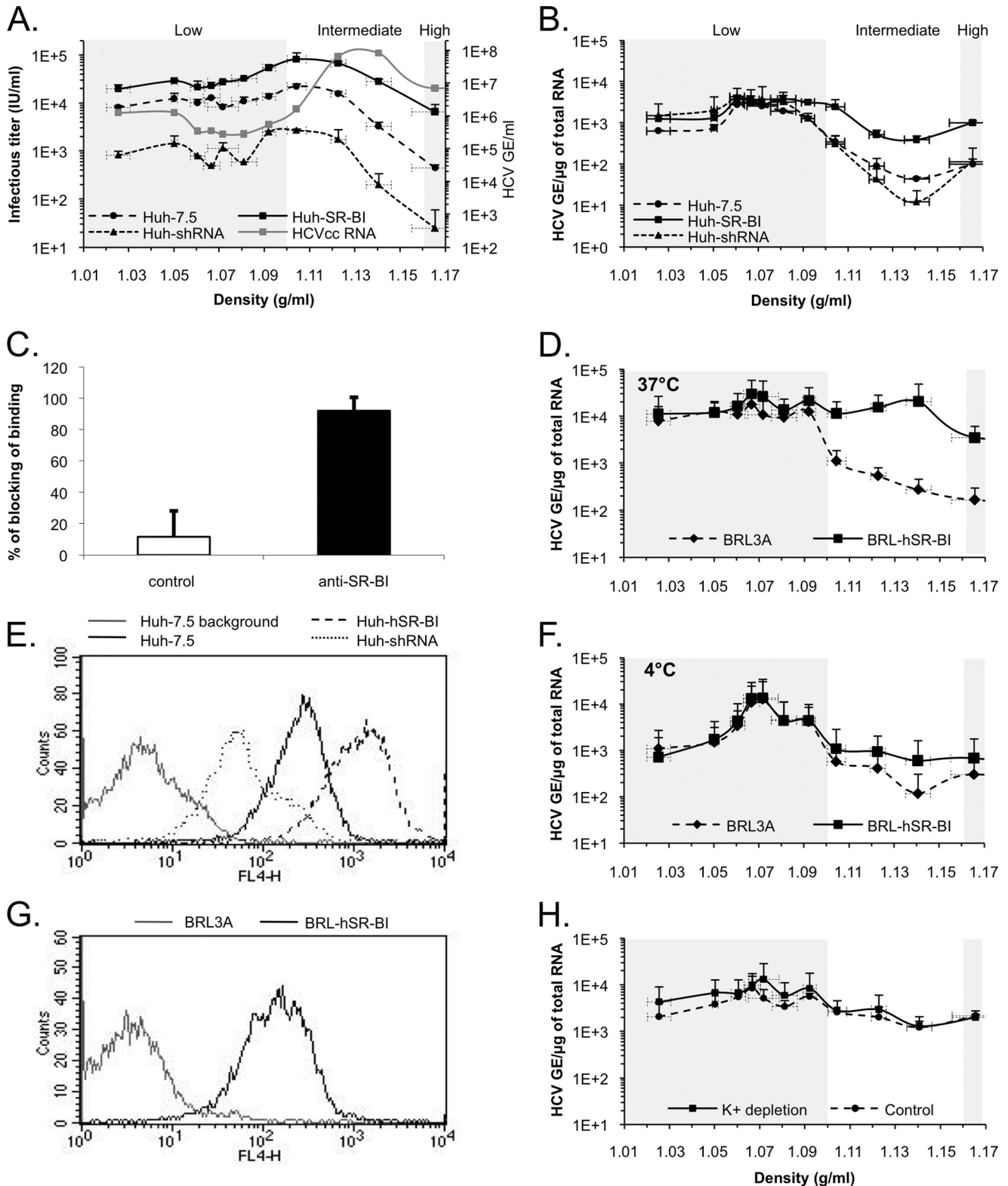
Study of Density-fractionated HCVcc Reveals That HVRI Modulates Particle Properties—HCV E2 binding to human SR-BI depends on the hypervariable region 1 (HVRI) (38, 79), a 27-amino acid-long region at the N terminus of E2. Here, we identified a conserved residue in HVRI, Leu-399, that upon leucine-to-arginine mutation completely abolished E2 binding to SR-BI, like HVRI deletion (supplemental Fig. 1A). To clarify the role of HCV E2 in SR-BI-mediated attachment of HCVcc particles, we sought to study HVRI modifications, *i.e.* through its deletion (Δ HVRI-HCVcc) or L399R mutation (L399R-HCVcc). We found that whereas supernatant levels of HCV RNA and core protein were similar for HVRI-mutant and parental HCVcc (Fig. 2, A and B, *left panels*), either HVRI modification had effects on HCVcc infectivity with a reduction of 40-fold for Δ HVRI-HCVcc, consistently with previous reports (14, 81), and 3-fold for L399R-HCVcc (Fig. 2C, *left panel*).

Then different parameters of HCVcc density subpopulations of the above-mentioned HVRI mutants were analyzed (Fig. 2, *right panels*). First, we investigated physical particle release of HCVcc populations separated along their buoyant densities. Parental HCVcc and HVRI-HCVcc mutants exhibited a major

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peak of physical particles sedimenting at densities of 1.10–1.16 g/ml and containing more than 90% of viral RNA and 95% of core protein (range, $1.5\text{--}3.2 \times 10^8$ genome equivalent/ml and $4.1\text{--}9.8 \times 10^5$ fmol/ml at the peak density of 1.12–1.13 g/ml) (Fig. 2, A and B). In line with studies of others (10, 11, 14), viral particles could also be detected at higher densities (to up to 1.26

g/ml, data not shown) and at lower densities (to up to 1.02 g/ml; Fig. 2, A and B). Yet, $\sim 10\text{--}60$ -fold less physical particles could be detected for Δ HVR1-HCVcc as compared with parental HCVcc in the low density fractions (*i.e.* 1.02–1.10 g/ml) (Fig. 2, A and B). In contrast, as compared with parental HCVcc, L399R-HCVcc had ~ 10 -fold higher levels of physical particles in fractions with den-



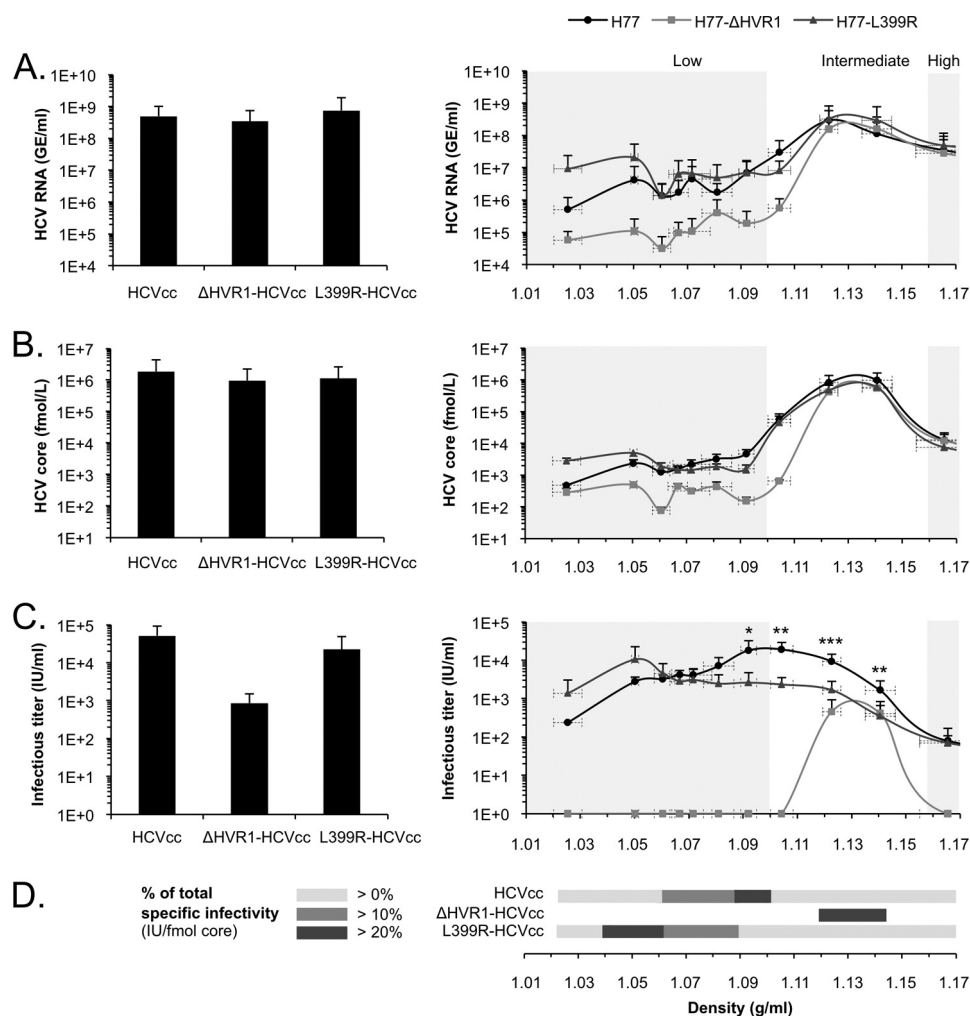


FIGURE 2. Biophysical properties of HCVcc particles. A–C, HCVcc particles harboring wt (HCVcc), HVR1-deleted (Δ HVR1-HCVcc), or L399R-mutated (L399R-HCVcc) H77-E1E2 glycoproteins were harvested 48 h after transfection of Huh7.5 cells and were analyzed from filtered cell culture supernatants (left panels) or after separation of viral subpopulations into 12 fractions by using continuous iodixanol density gradient centrifugation (right panels). HCV RNA GE (genome equivalent) (A) and HCV core protein (B) amounts contained in each fraction were determined by qRT-PCR and ELISA, respectively (mean \pm S.D.; $n = 3$). C, HCV infectious titers were determined as focus-forming units/ml. In the right panels, values are plotted against the density of the respective fractions. Statistical analysis was performed by a paired Student's *t* test: *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$. D, specific infectivity of wt HCVcc, Δ HVR1-HCVcc, and L399R-HCVcc was calculated as infectivity per fmol core protein and is presented as percentage of total specific infectivity in individual fractions.

sities of 1.02–1.05 g/ml but similar amounts in the other fractions (i.e. 1.05–1.16 g/ml) (Fig. 2, A and B). Altogether, these results suggested that HVR1 modulates the assembly, stability, and/or lipoprotein-association of low density particles.

Next, we analyzed the distribution of infectivity of HCVcc subpopulations for the above-mentioned HVR1 mutants (Fig. 2C, right panel). As for the Δ HVR1-HCVcc particles, virtually all infectivity was detected in fractions of densities of 1.10–1.16

FIGURE 1. HCVcc particle attachment to SR-BI. A, HCV infectious titers of virus populations separated by using a continuous iodixanol density gradient centrifugation were determined on human SR-BI-overexpressing (solid line, Huh-SR-BI) or SR-BI-down-regulated (dotted line, Huh-shRNA) Huh-7.5 cells, as compared with control Huh-7.5 cells (dashed line, Huh-7.5). Results are expressed as focus-forming units/ml and compared with HCV RNA (GE, genome equivalent) of each virus population (gray line, HCVcc RNA) determined by RT-qPCR. B, shown is attachment of HCVcc subpopulation to human SR-BI-overexpressing (solid line, Huh-SR-BI) or SR-BI-down-regulated (dotted line, Huh-shRNA) Huh-7.5 cells as compared with control Huh-7.5 cells (dashed line, Huh-7.5). Equal amounts of fractionated HCVcc particles, i.e. 1×10^5 HCV GE for each density fractions, were incubated to the cells. The bound HCVcc particles were assessed by detection of HCV RNA in lysates of virus-incubated cells and normalized to the human GAPDH mRNA levels (mean \pm S.D.; $n = 3$). C, shown are the results of blocking of binding of HCVcc particles of intermediate densities (1.12–1.14 g/ml) to human SR-BI-overexpressing Huh-7.5 cells using SR-BI polyclonal antibodies (preincubation of the cells with 100-fold dilution of anti-SR-BI serum) and compared with control serum. The results of binding inhibition are displayed as percentage of blocking of binding compared with untreated cells. Bound HCV RNA was quantified as described in B (mean \pm S.D.; $n = 2$). D, equal amounts of fractionated HCVcc particles, corresponding to 1×10^5 HCV GE contained in fractions of densities specified on the x axis, were allowed to attach on BRL3A cells expressing (dashed line, BRL-hSR-BI) or not (solid line, BRL3A) human SR-BI for 2 h at 37 °C. Bound HCV RNA was quantified as described in B (mean \pm S.D.; $n = 3$). E, cell surface expression of SR-BI in human SR-BI-overexpressing (dashed line, Huh-SR-BI), SR-BI-down-regulated (dotted line, Huh-shRNA), or control Huh-7.5 cells (solid line, Huh-7.5) by immunostaining is shown. Staining with secondary antibody only provided the background of fluorescence (gray line, Huh-7.5 background). F, equal amounts of fractionated HCVcc particles (1×10^5 HCV GE per fraction) were allowed to attach on BRL3A cells expressing (dashed line, BRL-hSR-BI) or not (solid line, BRL3A) human SR-BI for 2 h at 4 °C. Bound HCV RNA was quantified as described in B (mean \pm S.D.; $n = 3$). G, cell surface expression of SR-BI in BRL3A cells ectopically expressing, or not, human SR-BI by immunostaining is shown. H, BRL3A cells expressing human SR-BI were treated (solid line) or not (dashed line) with potassium-depleted medium and subsequently incubated for 2 h at 37 °C with equal amounts of fractionated HCVcc particles (1×10^5 HCV GE). Bound HCV RNA was quantified as described in B (mean \pm S.D.; $n = 2$).

SR-BI Dependency of HCV Subpopulations

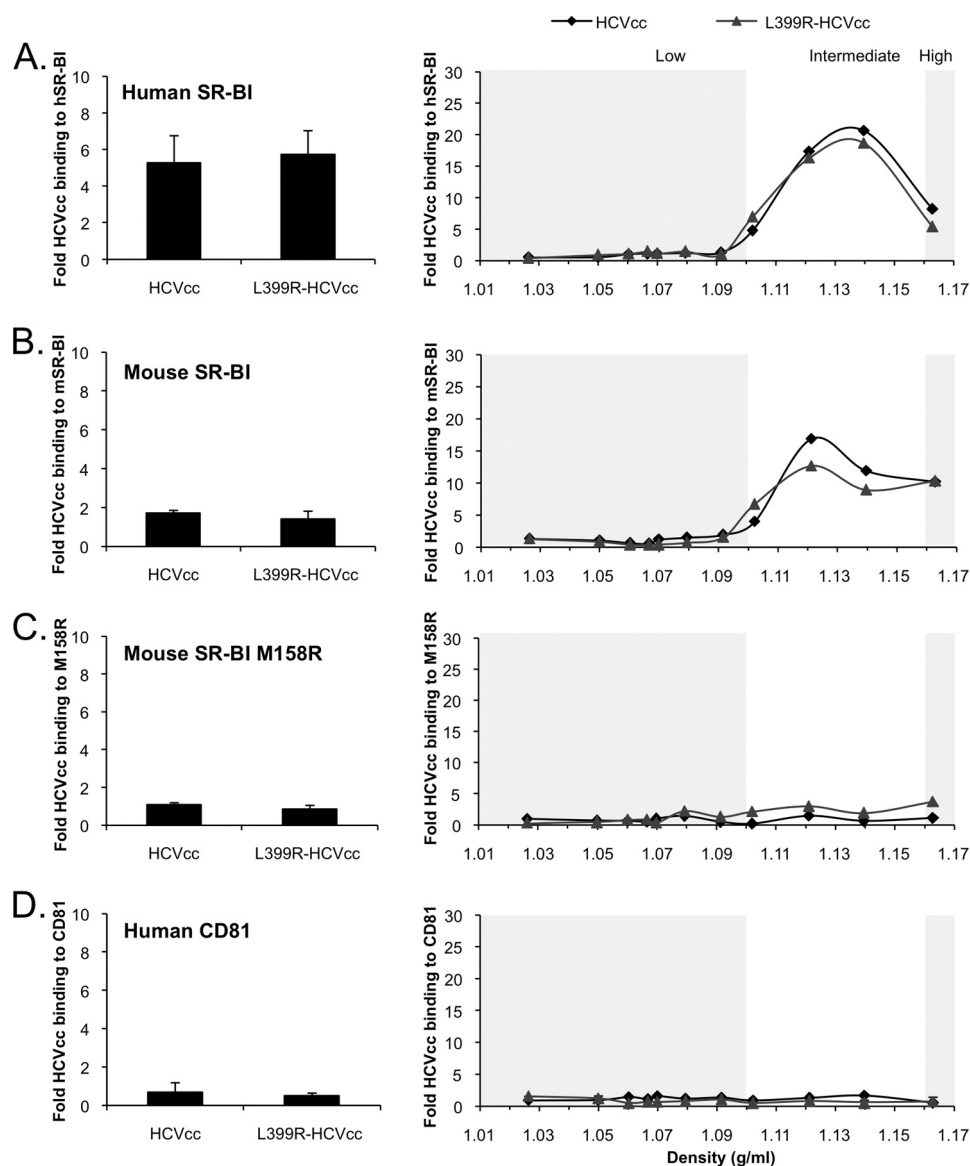


FIGURE 3. Cell attachment properties of HCVcc particles of different densities. *A–D*, shown is cell attachment of wt HCVcc and L399R-HCVcc particles to BRL3A cells expressing human SR-BI (*A*), mouse SR-BI (*B*), mouse M158R SR-BI mutant (*C*), or human CD81 (*D*). Shown is -fold binding of HCVcc particles to these cells in comparison to parental BRL3A cells, as assessed by detection in lysates of virus-incubated cells of viral RNA by qRT-PCR that was normalized to the rat GAPDH mRNA levels. The *left panels* show the binding of non-fractionated viral particles to BRL3A cells expressing the indicated receptors (mean \pm S.D.; $n = 3$), whereas the *right panels* show the binding of viral particles in individual density fractions (results are representative of three independent experiments).

g/ml (Fig. 2*C*), containing most physical particles. No infectivity could be detected in low density fractions, presumably due to strongly reduced levels of physical particles in these fractions (Fig. 2, *A* and *B*). Nonetheless, this contrasted with the L399R-HCVcc particles for which ~80% of the infectivity remained in these low density fractions (Fig. 2*C*) and particularly in those of lowest densities (1.02–1.05 g/ml). Importantly, the L399R-HCVcc viruses had 5–10-fold reduced infectivity titers than the parental HCVcc particles in the peak infectivity fractions of the latter virus ($p < 0.05$), *i.e.* 1.08–1.13 g/ml (Fig. 2*C*), despite similar amounts of physical particles in such fractions (Fig. 2, *A* and *B*). Altogether, these results indicated that HVR1 determines at least in part infectivity and perhaps lipid association of HCVcc particles. Viruses harboring wt *versus* L399R-mutated E2 glycoproteins were then compared in the subsequent experiments to analyze SR-BI involvement in HCV entry.

Intermediate density HCVcc Particles Use SR-BI for Cell Attachment via Their Lipoprotein Components—We then sought to determine the importance of E2/SR-BI interaction in mediating attachment of the different virus populations. First, we found that, unexpectedly, SR-BI induced attachment of HCVcc particles of intermediate density for both wt viruses (Figs. 1*B* and 3*A*) and modified viruses harboring the L399R mutation in HVR1 (Fig. 3*A*) that abrogated soluble E2/SR-BI binding (supplemental Fig. 1*A*). Second, no significant attachment to cells expressing CD81 could be detected for parental and HVR1-mutated HCVcc particles contained in all density gradient fractions (Fig. 3*D*), although individually, the recombinant HVR1-modified E2 proteins had higher capacity to bind CD81 than wt E2 (supplemental Fig. 1*A*). Third, the mouse SR-BI ortholog, which does not allow binding of HCV E2 (38, 82), also mediated attachment of wt

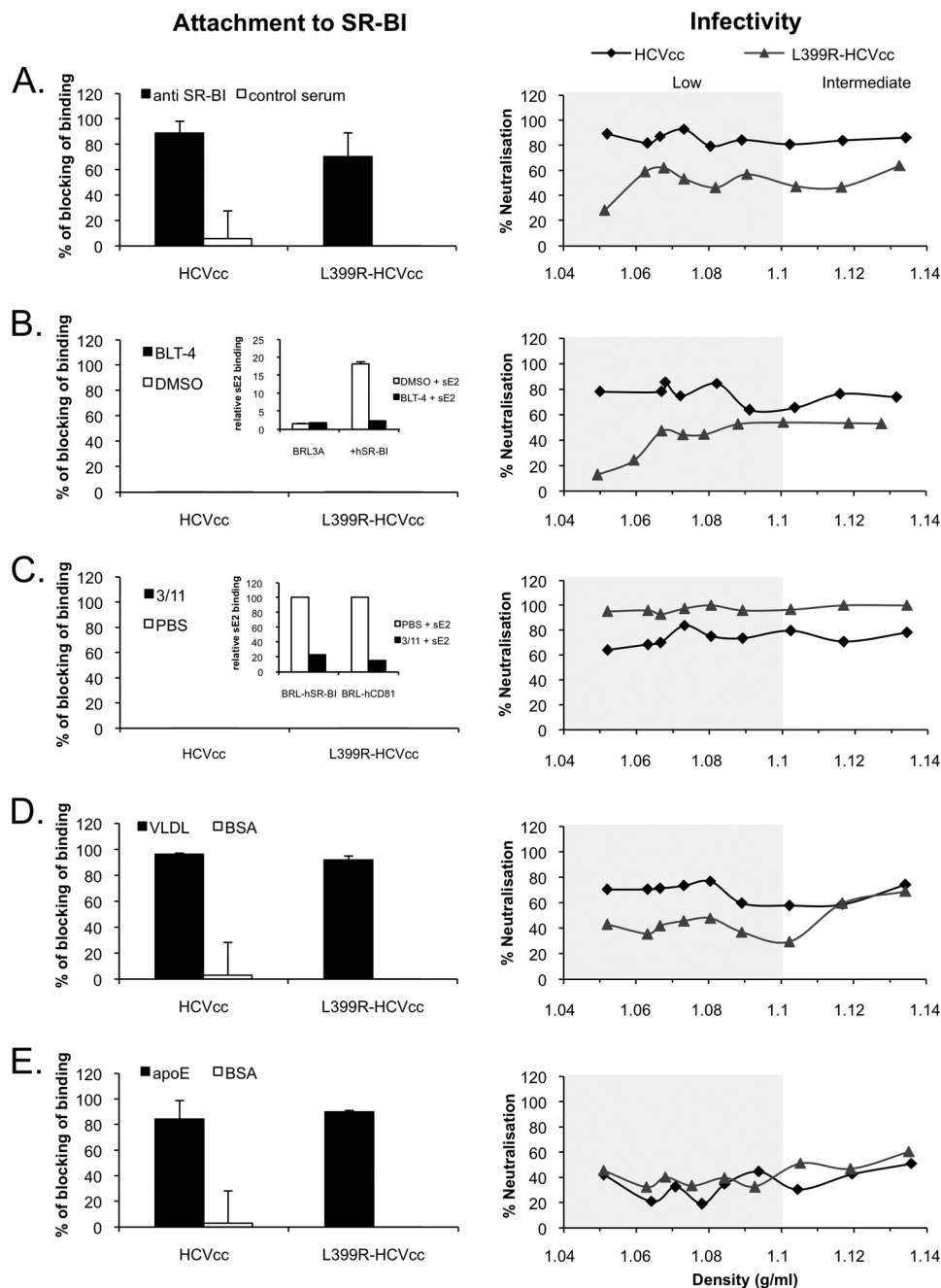


FIGURE 4. Blocking of HCVcc binding and infectivity. *A–E, left panels*, shown are the results of blocking of binding of HCVcc particles of intermediate densities (1.12–1.14 g/ml) to human SR-BI in the presence of anti-SR-BI or control serum (100-fold dilution) (*A*) or in the presence of BLT-4 (50 μ M) or the DMSO diluent (*B*) by preincubating viral particles with mAb 3/11 against HCV E2 (20 μ g/ml) or PBS diluent (*C*) or by preincubating cells with VLDL (50 μ g/ml) (*D*) or purified apoE (50 μ g/ml) (*E*). The results are displayed as the percentage of blocking of binding to hSR-BI-expressing BRL3A cells incubated with the indicated compounds compared with untreated cells (mean \pm S.D.; $n = 3$). The *insets* in the *left panels* of *B* and *C* show the results of blocking of sE2 binding to BRL3A cells expressing hSR-BI or hCD81 with the same concentration of the BLT-4 compound or 3/11 antibody used for blocking of HCVcc binding. *A–E, right panels*, neutralization of infectivity of viral particles of the indicated buoyant densities with anti-SR-BI or control serum (100-fold dilution) (*A*) or with BLT-4 (50 μ M) (*B*) by preincubating viral particles with mAb 3/11 (20 μ g/ml) (*C*) or by preincubating cells with VLDL (50 μ g/ml) (*D*) or with purified apoE (50 μ g/ml) (*E*). The results of infectivity inhibition are displayed as the percentage of neutralization relative to the infectivity in Huh-7.5 cells determined in the absence of inhibitor. The results are representative of five independent experiments.

and L399R-mutated HCVcc intermediate density particles (Fig. 3*B*).

Confirming that SR-BI specifically interacts with HCVcc particles of intermediate densities, we found that SR-BI blocking antibodies almost completely inhibited SR-BI-mediated attachment of wt and L399R-mutated HCVcc particles to SR-BI-expressing BRL3A cells (Fig. 4*A, left panel*). Yet we found that this HCVcc/

SR-BI interaction was not mediated by the HCV E2 glycoprotein as it was abrogated neither by the HCV E2 glycoprotein as it was abrogated neither by BLT-4 (Fig. 4*B, left panel*), a small molecule SR-BI inhibitor (65), nor by E2-specific antibodies (Fig. 4*C, left panel*), both of which block sE2 binding to SR-BI (58) (Fig. 4, *B* and *C, insets*). Altogether, these results indicated that HCV E2 is not at play for HCVcc attachment to SR-BI, which thus involves alternative virus surface component(s).

SR-BI Dependency of HCV Subpopulations

TABLE 1
SR-BI functions and determinants required for HCV entry into cells

Function	Determinant	HCV subpopulation
Attachment	VLDL components (apoE)	1.10–1.16 g/ml (intermediate density)
	E2-independent HVR1-independent	
Access	SR-BI lipid transfer-dependent	1.02–1.16 g/ml (low and intermediate density)
	E2/SR-BI binding-independent HVR1-independent	
Enhancement	SR-BI lipid transfer-dependent	1.08–1.12 g/ml (lower intermediate density)
	E2/SR-BI binding-dependent	
	HVR1-dependent HDL component (apoC-I)	

Previous studies have underscored the association of HCV particles with β -lipoprotein components such as apoB, apoC-I, or apoE (17, 22, 28), which may contribute to HCV/SR-BI interaction. Importantly, no attachment of HCVcc particles occurred to cells expressing the M158R-mutated mouse SR-BI (Fig. 3C) that does not bind lipoproteins (70, 71) (supplemental Fig. 4C), suggesting that a lipoprotein component(s) could influence attachment to SR-BI of HCVcc particles. Consistently, purified VLDL and apoE strongly inhibited attachment of intermediate density HCVcc particles to SR-BI-expressing BRL3A cells (Fig. 4, D and E, left panels). Again, strictly analogous results were obtained for HCVcc particles carrying the L399R mutation, as compared with wt virus (Fig. 4, left panels), further indicating that a host factor rather than a viral ligand mediates attachment of intermediate density HCVcc particles to SR-BI.

Then we investigated the impact of these latter molecules on HCVcc infectivity (Fig. 4, right panels). Consistently with the inhibition of SR-BI-mediated attachment, both purified VLDL and apoE inhibited entry of HCVcc of intermediate density in a dose-dependent manner (Fig. 4, D and E, right panels, and supplemental Fig. 2, D and E). In addition, either molecule also impaired cell entry of the other HCVcc subpopulations (Fig. 4, D and E, right panels, and supplemental Fig. 2, D and E), suggesting that lipoprotein components also permit attachment and/or penetration of HCVcc via SR-BI and/or alternative lipoprotein receptor(s).

SR-BI Mediates Entry of All HCVcc Subpopulations via Its Lipid Transfer Activity and Independent of E2/SR-BI Binding—Importantly, we found that SR-BI antibodies blocked infectivity in Huh-7.5 cells of both parental and HVR1-mutated HCVcc particles of intermediate density (>1.10 g/ml), as expected due to the SR-BI role in attachment of the latter viral particles (Figs. 1 and 2). Importantly, these antibodies also blocked infectivity of low density HCVcc particles (<1.10 g/ml) (Fig. 4A, right panel, and supplemental Fig. 2A), which do not attach to SR-BI (Figs. 1 and 2). This confirmed our assumption (Fig. 1, A and B) that, besides mediating cell attachment, an additional function of SR-BI, which we designated “access function” (Table 1), allows cell entry of all HCVcc subpopulations in a manner independent of E2/SR-BI binding and of HCVcc/SR-BI attachment. Yet, HCV E2-blocking antibodies neutralized infectivity of HCVcc particles of all densities (Fig. 4C, right panel, and supplemental Fig. 2C), suggesting that the HCV surface glycoproteins mediate cell entry of all HCVcc subpopulations, although they are not involved in HCVcc attachment to cells. Furthermore, we found that BLT-4, a small inhibitor of

the lipid transfer activity of SR-BI (65) (supplemental Fig. 3), inhibited HCVcc infectivity of all HCVcc populations (Fig. 4B, right panel) in a manner correlating to the levels of lipid transfer inhibition (supplemental Figs. 2B and 3), indicating that the physiological activity of SR-BI may exert this access function of SR-BI by other means than by mediating attachment of HCVcc particles.

Thus, aiming to discriminate the nature of either “attachment” versus “access” SR-BI functions, we took advantage of the HCVpp assay, which allows the study of entry factors using receptor-complementation assays (42, 58) independently of the lipoprotein components associated to HCVcc particles. As previously described (42, 82), although it does not bind HCV E2 (38, 82) (Fig. 5A), mouse SR-BI fully supported HCVpp entry with an efficiency similar to human SR-BI as shown here (Fig. 5B) in BRL3A cells co-expressing human CD81, CLDN1, and endogenous rat Occludin (BRL-hCD81-hCLDN1 cells). Consistently, despite loss of E2/SR-BI binding (Fig. 5A, supplemental Fig. 1A), L399R-HCVpp depended on SR-BI for entry into cells as BRL-hCD81-hCLDN1 cells became susceptible only upon expression of either mouse or human SR-BI (Fig. 5B). Finally, we found that BLT-4 inhibited HCVpp entry in BRL-hCD81-hCLDN1 expressing either human or mouse SR-BI by about 70–90% (similar to the inhibition of HCVpp entry on Huh-7 cells) (Fig. 5C), in line with the levels of lipid transfer inhibition achieved by BLT-4 in these cells (Fig. 5C and supplemental Fig. 3). Because HCV E2 does not bind mouse SR-BI, this result indicated that the SR-BI lipid transfer-dependent function could be involved in its capacity to mediate HCV entry independently of E2/SR-BI interaction.

To corroborate these results, we performed infection assays using mutants of mouse SR-BI that are impaired in their lipid transfer activities (M158R or G420H-424H) (69, 70) (Fig. 5D and supplemental Fig. 4). In BRL-hCD81-hCLDN1 cells expressing either mouse mutant, we found that the levels of HCVpp infection were significantly reduced by about 60–80% as compared with parental mouse SR-BI (Fig. 5D), which correlated well with a 3–4-fold decrease in their lipid transfer function (Fig. 5D and supplemental Fig. 4). Altogether, these results indicated that the function of SR-BI used during HCV entry of all HCVcc populations (access function) is HCV E2/SR-BI binding-independent but requires the lipid transfer activity of SR-BI.

The Interaction between HCV E2 and SR-BI Triggers a Third Function of SR-BI That Leads to Enhanced HCV Entry—Thus far we described two functions of SR-BI important for HCV cell entry, an attachment function and an access function, which are both HCV E2/SR-BI binding-independent (Table 1). Then we wanted to investigate the potential role of HCV E2 binding to SR-BI. First, when we introduced in HCVcc genome the L399R mutation in HVR1 that abrogates HCV E2/SR-BI binding, we found that these HCVcc particles exhibited a decreased sensitivity to neutralization by anti-SR-BI antibodies (Fig. 4A, right panel, and supplemental Fig. 2A) or by BLT-4 (Fig. 4B, right panel, and supplemental Fig. 2B), as compared with parental HCVcc. Second, we observed that these HVR1-mutated HCVcc particles, compared with wt viruses, had a 5–10-fold decrease of infectivity for the viral particle populations that corresponded to

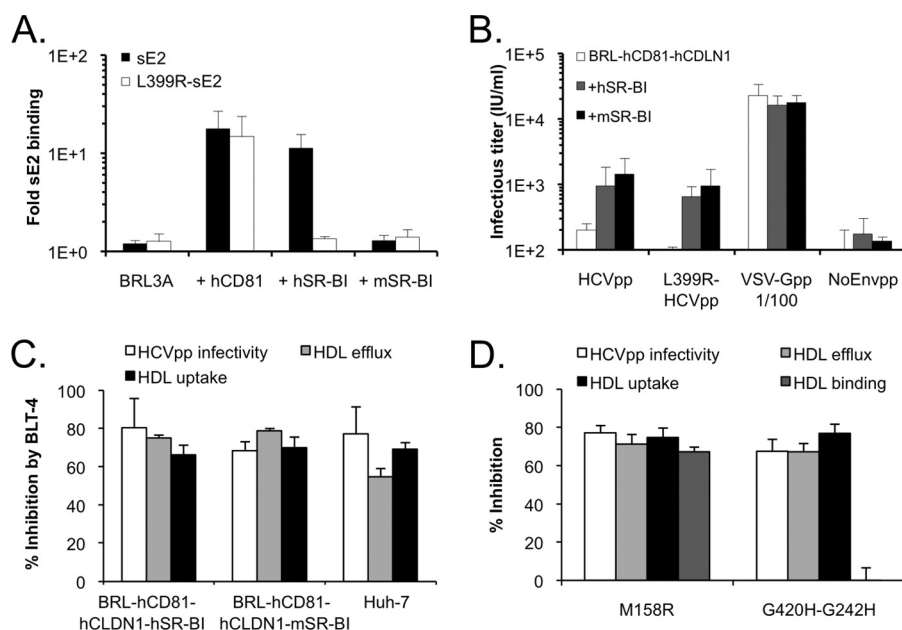


FIGURE 5. Inhibition of HCV entry by blocking lipid transfer activity of SR-BI. *A*, binding of His-tagged soluble wt (sE2) or L399R-mutated (L399R-sE2) H77-E2 glycoprotein to BRL3A cells ectopically expressing CD81 or the indicated SR-BI orthologs was detected by flow cytometry. The results are expressed as -fold of mean fluorescent intensity relative to the mean fluorescent intensity (MFI) determined in the absence of sE2 (mean \pm S.D.; $n = 3$). *B*, results are shown of HCV entry assays in Huh-7 cells or in BRL-hCD81-hCLDN1 rat hepatocarcinoma cells ectopically expressing, or not, human or mouse SR-BI (hSR-BI and mSR-BI, respectively) along with human CD81, human CLDN1, and endogenous OCLN. Cells were inoculated with HCVpp harboring wt (HCVpp) or L399R-mutated (L399R-HCVpp) H77-E1E2 glycoproteins, control virus particles harboring VSV-G glycoprotein (VSV-Gpp, diluted 1:100), or no glycoprotein (noENVpp), and infectivity titers were determined 72 h after inoculation. Results represent average infectious titers, expressed as infectious unit (IU) per ml (mean \pm S.D.; $n = 3$). *C*, shown are the results of inhibition of SR-BI-dependent HCVpp entry, cholesterol efflux (using 50 μ g/ml HDL-PL), and HDL-CE uptake (using 60 μ g/ml HDL-PL) in BRL-hCD81-hCLDN1 cells ectopically expressing hSR-BI or mSR-BI as indicated and in Huh-7 cells treated with 50 μ M of BLT-4 relative to DMSO-only, set at 100% (mean \pm S.D.; $n = 3$). *D*, shown are the results of SR-BI-dependent HCVpp entry assays, cholesterol efflux (using 50 μ g/ml HDL-PL), HDL-CE uptake (using 60 μ g/ml HDL-PL), and Cy5-labeled HDL binding (using 5 μ g/ml HDL) in BRL-hCD81-hCLDN1 cells ectopically expressing mutated mouse SR-BI M158R or G420H/G424H as indicated. Shown are the percentages of inhibition as compared with cells expressing wild-type mouse SR-BI (mean \pm S.D.; $n = 3$).

the highest infectivity levels of parental HCVcc (*i.e.* 1.08–1.13 g/ml) (Fig. 2C) and that contained the highest amounts of E1E2 glycoproteins (supplemental Fig. 5). Altogether, these results suggested the possibility that E2/SR-BI interaction leads to an enhanced infectivity for HCVcc particles.

Infection enhancement of HCV infectivity has been reported before by us and others (55–59, 61). Particularly, HDL from HS stimulate post binding entry of HCVpp and HCVcc particles in a manner that is governed by HVR1 and is dependent on the lipid transfer function of SR-BI. Our results in this report further indicate that infection enhancement is likely related to HCV E2/SR-BI binding, as cell entry of HVR1-HCVpp mutants harboring E2 glycoproteins that do not bind SR-BI (supplemental Fig. 1A) was not enhanced by HDL or HS (supplemental Fig. 1B). Consistently, HDL enhanced infectivity of wt HCVcc but not of L399R-HCVcc particles (supplemental Fig. 6). Finally, although mouse SR-BI expressed in BRL3A cells mediated efficient HDL binding and lipid transfer (supplemental Fig. 4), it did not allow enhancement of HCV infection in the presence of HDL or HS, in contrast to human SR-BI (Fig. 6A and supplemental Fig. 1B). Interestingly the absence of HDL-mediated infection enhancement uncovered an inhibitory activity of lipoproteins, possibly due to oxidized lipids (53). Altogether, these results indicated that although rodent and human SR-BI promote HCV entry through their access function, only human SR-BI features both capacities to mediate HCV E2 binding and to induce HCV entry enhancement in the presence of HDL.

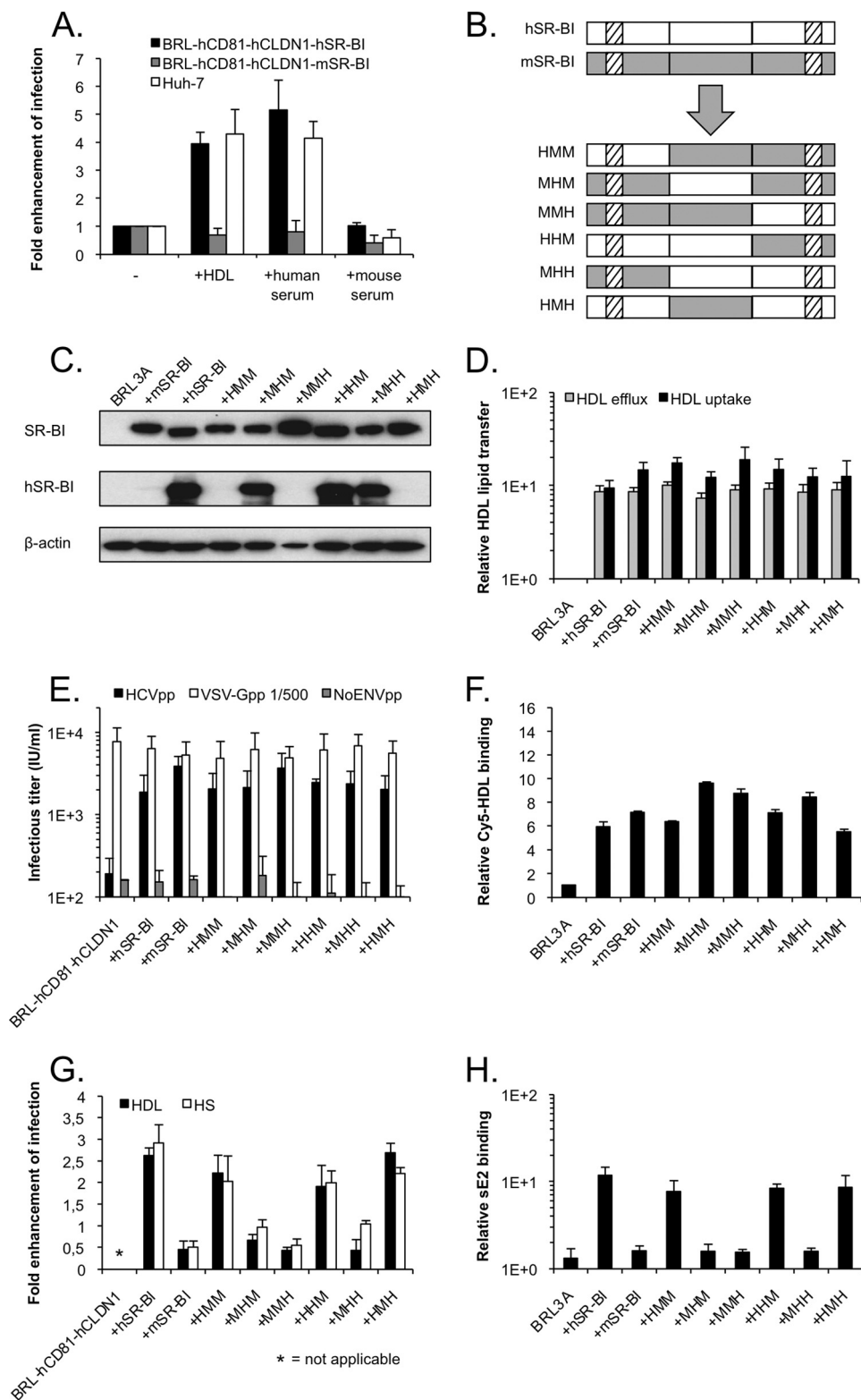
To establish the molecular basis of the difference between the access function of SR-BI and SR-BI/HDL-mediated entry enhancement, we generated a set of SR-BI chimeras by swapping segments between human and mouse orthologs. Using SR-BI sequence comparisons as well as structural features predictions, we delineated three domains in the SR-BI ectodomain, *i.e.* between amino acid positions 38 and 215, 216 and 398, and 399 and 432. We then generated six human/mouse SR-BI chimeras (Fig. 6B) that were readily expressed in BRL3A cells (Fig. 6C) and fully functional for lipid transfer (Fig. 6D, supplemental Fig. 7). Although they all supported efficient HCVpp entry upon expression in BRL-hCD81-hCLDN1 cells (Fig. 6E) and displayed full HDL binding capacities (Fig. 6F), only those chimeras that harbored the first domain of human SR-BI supported HCV entry enhancement by HDL or HS (Fig. 6G). As shown in Fig. 6H, we found that the first domain of human SR-BI harbored binding sites for HCV E2, as only when this domain was present in a chimeric human/mouse SR-BI molecule could we demonstrate sE2 binding with an efficiency comparable with that of wt human SR-BI. Hence, the ability of these chimeric molecules to bind HCV E2 correlated with their capacity to induce HDL-mediated infection enhancement. Altogether, these results suggested that HDL-mediated stimulation requires HCV E2 binding to SR-BI, which thus characterizes this third function of SR-BI (referred to below as the enhancement function, Table 1), present in human SR-BI and distinct from the access function detected with either SR-BI orthologs.

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DISCUSSION

Here we show that SR-BI is a multifunctional entry factor essential for infectivity of HCV particles with different biophysical properties and host protein compositions. The analysis of HCVcc populations separated by density gradient revealed that during HCV entry, SR-BI exerts both direct and indirect inter-

actions with different components on the viral particles. Thus, we propose three distinct HCV entry functions for SR-BI (Table 1): a primary attachment function, an access function, and an enhancement function that, respectively, lead to the capture of HCV particles from the extracellular milieu, their functional entry into cells, and the stimulation of their infectivity. Intrigu-



ingly, we found that although the binding of HCV E2 to SR-BI is required for the latter SR-BI function at a post-attachment step both the attachment and access functions are independent of HCV E2/SR-BI interaction. Finally, our results highlight a critical role of HVR1 residues and of SR-BI domain I for E2/SR-BI binding and, consequently, the implementation of the enhancement function.

SR-BI is a multiligand receptor that mediates the binding and lipid transfer from different classes of lipoproteins (51, 52). Because of their low density, lipid composition, and association to apolipoproteins, plasma-derived HCV as well as HCVcc particles resemble VLDL particles in the form of lipo-viro-particles (for review, see Ref. 7) and, as for HCVcc particles retrieved from our buoyant density gradients, fall in different subpopulations of low (<1.10 g/ml), intermediate (1.10–1.16 g/ml) and high (>1.16 g/ml) densities.

It was previously proposed that SR-BI could act as a receptor of HCV entry through a direct E2/SR-BI interaction (38, 39). However, here we found that the initial attachment of HCVcc particles to SR-BI is independent of E2 but, rather, is mediated by lipoprotein components, such as apoE. Indeed, a point mutation of HVR1 leading to complete loss of E2/SR-BI binding did not interfere with HCVcc/SR-BI attachment. Consistently, we found that HCVcc capture by SR-BI cannot be inhibited by E2/SR-BI binding blockers such as E2 antibodies or BLT-4, which further excludes a role for HCV E2 in the attachment function of SR-BI. Moreover, no attachment of HCVcc particles occurred on the lipoprotein binding-deficient M158R SR-BI mutant. The preferential attachment of HCV particles of intermediate density to SR-BI via a lipoprotein component is in agreement with the observation that several apolipoproteins (apoB, -E, -C-I) are predominantly incorporated into HCVcc particles of intermediate densities (17, 19) and that apoE plays a crucial role during HCV assembly and infectivity (17, 20–22, 83–86).

Overall, our data are in agreement with a previous study indicating that the cellular uptake of HCV particles derived from the plasma of HCV-infected patients can be mediated by SR-BI through its interaction with lipoprotein components harbored by the virus (28). Although there may be differences between HCVcc and patient-derived particles, *e.g.* in their apolipoprotein composition of viral subpopulations of different densities,

as HCVcc-producing hepatocarcinoma cells are impaired at late steps of VLDL biogenesis and production (7, 87, 88), our study further extends these previous results (28) and underscores the notion that distinct capture molecules are used by the different HCV subpopulations to induce attachment to target cells. Although attachment of HCVcc of intermediate density, particularly, seems to be mediated by SR-BI, we found that the infectivity of low density HCVcc particles also involves a VLDL-derived component as it could be competed by VLDL and/or by purified apoE. This may involve cell attachment of these latter viral particles to molecules such as glycosaminoglycans (35, 36, 86), lipoprotein lipase (89), and/or LDL receptor (17, 27, 29, 37) that all bind VLDL.

We found that the access function of SR-BI that mediates HCV entry into cells is independent of E2/SR-BI binding. Indeed, entry of HCV particles bearing HVR1 modifications abrogating sE2/SR-BI binding remained dependent on the presence of SR-BI. Likewise, mouse SR-BI mediated full HCV entry as compared with its human counterpart, in agreement with previous reports (42, 82, 90). Thus, our results indicated that a SR-BI property other than E2 binding must be at play during HCV entry.

SR-BI mediates the bidirectional flux of free and esterified cholesterol between cells and lipoproteins (51, 52). Here we found that blocking the lipid transfer activity of mouse SR-BI by small chemical inhibitors (BLTs) (65) decreased HCVpp entry. Likewise, point mutants of mouse SR-BI abrogating lipid transfer (69–71) decreased HCVpp entry as compared with wt mouse SR-BI. Finally, blocking of lipid transfer of human SR-BI by either anti-SR-BI antibodies or BLT-4 decreased the infectivity of HCVcc particles of the different buoyant densities, including those of low density that do not initially attach SR-BI.

Although the access function promoted by SR-BI relies on the lipid transfer activity of SR-BI but does not depend on E2/SR-BI binding, it does depend on E2/CD81 interaction, as shown in this report by sensitivity to antibodies that block E2/CD81 interaction. This is in line with recent studies revealing that, whether or not SR-BI bears HCV E2 binding capacity, HCV entry remains dependent on human CD81 (as well as on Occludin and Claudin-1) (42, 58). A possibility is that the lipid transfer function of SR-BI facilitates the formation of an HCV receptor complex, perhaps by augmenting the rate of recruit-

FIGURE 6. HCV entry into cells expressing human/mouse SR-BI chimeras. *A*, HCV entry assays using H77-HCVpp (produced in serum-free medium) in the presence of 6 μ g/ml cholesterol-HDL or 2.5% human or mouse serum and BRL3A cells expressing human or mouse SR-BI along with human CD81 and human CLDN1. Huh-7 cells were used as a reference. Results show the -fold increases of infection determined by calculating the ratios between average infectious titers determined in the presence or absence (-) of HDL or serum (mean \pm S.D.; $n = 3$). No changes of infectivity of VSV-Gpp control particles were detected under these experimental conditions (data not shown). *B*, a schematic representation of human/mouse SR-BI chimeras shows the three domains that were swapped between amino acid positions 38 and 215, 216 and 398, and 399 and 432. The *gray boxes* represent mouse sequences, whereas the *white boxes* represent the human sequences. The *dashed boxes* represent the SR-BI trans-membrane domains. *M*, mouse; *H*, human. *C*, shown is a comparison of expression levels of human/mouse SR-BI chimeras by Western blot analysis using SRBI (400–104) or human SR-BI-specific (CLA-1 mAb) antibodies. Actin staining (AC74 mAb) was used as loading control. *D*, shown are the results of SR-BI-dependent free cholesterol efflux (using 50 μ g/ml HDL-PL) and HDL-CE uptake (using 60 μ g/ml HDL-PL) in BRL3A cells expressing wild-type or chimeric human/mouse SR-BI relative to parental BRL3A (mean \pm S.D.; $n = 3$). *E*, shown are the results of HCV entry assays into BRL-hCD81-hCLDN1 cells ectopically expressing parental human or mouse SR-BI or chimeric human/mouse SR-BI molecules by using H77-HCVpp, VSV-Gpp (500-fold dilution in medium), or non-enveloped particles (*noENVpp*). Results represent average infectious titers, expressed as infection units/ml (mean \pm S.D.; $n = 3$). *F*, shown is binding of Cy5-labeled HDL (using 5 μ g/ml HDL) to BRL3A cells expressing parental or chimeric human/mouse SR-BI. Results are expressed as -fold of MFI relative to the MFI determined in the absence of Cy5-labeled HDL (mean \pm S.D.; $n = 3$). *G*, shown are HCV entry assays using H77-HCVpp in the presence of 6 μ g/ml cholesterol-HDL or 2.5% HS. Results show the -fold increases of infection determined by calculating the ratios between average infectious titers measured in the presence or absence of HDL or HS (mean \pm S.D.; $n = 3$). No changes of infectivity with VSV-Gpp control particles were detected under these experimental conditions (data not shown). *H*, shown are results of binding of His-tagged soluble H77-E2 (sE2) to BRL3A cells ectopically expressing the indicated SR-BI orthologs or chimeras or to cells expressing human CD81 as compared with parental cells. Results are expressed as -fold MFI relative to the MFI determined in the absence of sE2 (mean \pm S.D.; $n = 3$).

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ment of the CD81 tetraspanin and the other entry co-factors at virion-binding sites and/or the internalization of HCV/CD81 complexes via the modulation of the “tetraspanin web,” which is regulated by cholesterol (91). In this respect it is interesting that CD81 and SR-BI function cooperatively to initiate HCV infection (62, 92) and that HCV entry is dependent on membrane cholesterol (92). In contrast to the attachment and access functions of SR-BI, which both are independent of E2/SR-BI interaction, E2 binding to SR-BI triggers a third entry function of SR-BI at a post-attachment step that leads to stimulation of cell entry (enhancement function).

Compared with HVR1-mutated viruses, whose E2 glycoproteins do not bind SR-BI, wt HCVcc particles of all densities exhibited a higher sensitivity to lipid transfer inhibitors (*e.g.* SR-BI antibodies and BLTs), which could be interpreted as a higher SR-BI dependence for entry. Thus, these results indicated a role for E2/SR-BI interaction for the entry process at a post-binding step. Consistently, as compared with the HVR1-mutated viruses, we found that wt HCVcc displayed a >10-fold higher infectivity for HCVcc particles of intermediate density that exhibited the highest infectivity (1.08–1.13 g/ml) and that contained the highest levels of E1E2 glycoproteins. Hence, for HCVcc of both low and intermediate densities, the interplay between SR-BI, E2, and/or HVR1 seems to trigger an important SR-BI function leading to enhanced cell entry.

Previous studies revealed that HCVpp entry is specifically enhanced by HDL in a manner dependent on SR-BI lipid transfer function (55, 56, 58, 60, 61). Here, by studying human/mouse SR-BI chimeras and their HCV entry properties, we found that HDL-mediated HCV infection enhancement correlates to E2/SR-BI binding, which itself is modulated by specific HVR1 residues. The close vicinity of HCV particle and a specific lipid transfer activity adjacent to the cell entry site could stimulate infectivity by different manners. Cholesterol transfer from SR-BI-bound lipo-viro-particles or lipoproteins could be important to facilitate post-attachment events at the site of entry into cells. Local enhanced membrane fluidity due to an elevated cholesterol/lipid content could favor crucial HCV cell entry events such as receptor movements (93) and interactions (68, 94), particle internalization (61, 95), and finally fusion with the host membrane (23, 47, 96). Furthermore, acceleration or facilitation of these crucial entry steps might help the virus to escape the host humoral immune response (60, 61) and to establish persistence (26).

In conclusion, we demonstrate that SR-BI is an entry factor with unparalleled functions. Its usage by HCV subpopulations to enter the cell is manifold and, intriguingly, involves different components on the viral particle (Table 1). First, we show that the initial attachment of HCV particles is independent of E2 and, at least for particles of intermediate densities, occurs via SR-BI. The nature of the attachment factor for HCV particles of low density was not identified in this study but is likely a molecule that binds VLDL, as deduced from VLDL and apoE competition in infection assays. Thus, in agreement with several previous studies (17, 22, 84), lipoprotein components provide the viral particle mimics of host-derived ligands for important capture molecules such as, *e.g.* LDL receptor and SR-BI. By targeting these explicit cholesterol receptors, HCV might opti-

mize replication *in vivo* as they are abundantly expressed on hepatocytes, which themselves harbor the VLDL assembly machinery that is important for HCV particle production. Second, besides mediating attachment, SR-BI induces entry and/or entry enhancement of HCV particles. We surmise that, whether they were initially captured by SR-BI or by alternative attachment molecules, the viral particles could be “passed on” to the next entry step, which would allow HCV E2 to directly interact with CD81 and/or SR-BI in an entry complex potentially containing the other entry co-factors. Why such direct interactions cannot be detected during the primary attachment step of the viral particles remains unclear; yet, one possibility is that modifications of HCV particles occur during/after their capture on lipoprotein receptor(s), allowing HCV E1E2 to become accessible for further interactions with CD81 and SR-BI. Although HCV E2/CD81 interaction was recently found to prime HCV for low pH-dependent fusion (49), direct interactions between HCV E2 and SR-BI enhance infectivity of the particle at post-attachment levels (26).

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