

HIGH DENSITY LIPOPROTEINS FACILITATE HEPATITIS C VIRUS ENTRY THROUGH THE SCAVENGER RECEPTOR CLASS B TYPE I*

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Running title : HDL facilitate HCV entry

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The scavenger receptor class B type I (SR-BI) has recently been shown to interact with hepatitis C virus (HCV) envelope glycoprotein E2, suggesting that it might be involved at some step of HCV entry into host cells. However, due to the absence of a cell culture system to efficiently amplify HCV, it is not clear how SR-BI contributes to HCV entry. Here, we sought to determine how high-density lipoproteins (HDL), the natural ligand of SR-BI, affect HCV entry. By using the recently described infectious HCV pseudotyped particles (HCVpp) that display functional E1E2 glycoprotein complexes, we showed that HDL are able to markedly enhance HCVpp entry. We did not find any evidence of HDL association with HCVpp, suggesting that HCVpp do not enter into target cells using HDL as a carrier to bind to its receptor. Interestingly, lipid-free ApoA-I and ApoA-II, the major HDL apolipoproteins, were unable to enhance HCVpp infectivity. In addition, drugs inhibiting HDL cholesteryl transfer (BLT-2 and BLT-4) reduced HDL enhancement of HCVpp entry, suggesting a role for lipid transfer in facilitating HCVpp entry. Importantly, silencing of SR-BI expression in target cells by RNA interference markedly reduced HDL-mediated enhancement of HCVpp entry. Finally, enhancement of HCVpp entry was also suppressed when SR-BI-binding region on HCV glycoprotein E2 was deleted. Altogether, these data indicate that HDL-mediated enhancement of HCVpp entry involves a complex interplay between SR-BI, HDL and HCV envelope glycoproteins, and they highlight the active role of HDL in HCV entry.

Approximately 170 million individuals are infected worldwide by hepatitis C virus (HCV). HCV infection causes acute hepatitis,

which has a high probability of becoming chronic and, in the long term, can lead to cirrhosis and hepatocellular carcinoma (1). Owing to the serious consequences of its infection in humans, much effort is being made to understand the basic mechanisms of HCV lifecycle.

Due to the absence of an efficient cell culture system to replicate HCV, several laboratories have tried to develop surrogate models to study HCV entry (2). These models are based on the expression of HCV envelope glycoproteins E1 and E2, which form a noncovalent heterodimer. Several difficulties have been encountered in these approaches because HCV envelope glycoproteins are located in the endoplasmic reticulum (3), and their folding and assembly is very sensitive to mutations or deletions affecting the endoplasmic reticulum retention domains (4). Recently, infectious pseudotyped particles (HCVpp) that are assembled by displaying unmodified HCV envelope glycoproteins onto retroviral core particles have successfully been generated and now enable studies of HCV entry (5,6).

The cellular tropism of enveloped viruses is largely determined by selective interactions of viral envelope glycoproteins with specific cell-surface receptors. Several candidate receptors for HCV have recently been proposed. Molecules like the CD81 tetraspanin (7), the scavenger receptor class B type I (SR-BI)(8), the LDL receptor (9,10), and the asialoglycoprotein receptor (11) are potential candidate receptors for HCV, whereas the mannose binding lectins DC-SIGN and L-SIGN have been shown to function as HCV capture receptors but do not mediate viral entry into target cells (12-16). Among these molecules, there is mounting evidence that CD81 and SR-BI are necessary for HCV entry (5,6,17). Whereas the role of CD81 in HCV entry is now well documented (5,6,17-19), the contribution of SR-BI in HCV entry

needs to be further characterized. SR-BI is a 509-amino acid glycoprotein with two cytoplasmic C- and N-terminal domains separated by a large extracellular domain. SR-BI is a physiologically relevant lipoprotein receptor (20,21) responsible for selective uptake of cholesteryl ester from high-density lipoproteins (HDL). SR-BI mediated cholesteryl ester selective uptake is a two-step mechanism implying binding of lipoproteins to its extracellular domain followed by lipid exchange at the plasma membrane. SR-BI proteins are mainly expressed in the liver and steroidogenic tissues, where cholesterol selective uptake supplies cholesterol for biliary secretion and steroid hormone synthesis (22). HDL also play a key role in removing excess of cholesterol from peripheral tissues via the second HDL receptor, the ATP-binding cassette transporter A1 (ABCA1) receptor, and transporting it to liver cells to be taken by SR-BI.

Several studies indicate that HCV particles isolated from patients can be found in association with plasma lipoproteins like LDL and HDL (10,23). However, it is not clear how lipoproteins and their respective receptors contribute to HCV entry. Therefore, we sought to clarify the role of lipoproteins in HCV entry by using the HCVpp model. We show that HDL, but not LDL nor lipid-free HDL apolipoproteins, are able to markedly enhance entry of HCVpp. Drugs inhibiting transfer of HDL cholesteryl ester also reduce HCVpp entry. In addition, our data show that enhancement of HCVpp entry is dependent on SR-BI expression and on the presence of SR-BI binding region on HCV envelope glycoproteins. Altogether, these data reveal the active role of lipoproteins in HCV entry through a complex interplay between SR-BI, HDL and HCV envelope glycoproteins.

Materials and Methods

Drugs, proteins and antibodies - BLT-2 and , BLT-4 (ChemBridge, Chicago) were used as previously described (24). ApoA-I (A0722) and ApoA-II (A0972) purified proteins were purchased from Sigma-Aldrich. E1 and E2 glycoproteins were detected with the A4 (25) and 3/11 (26)(kindly provided by J McKeating, Rockefeller University, NY) monoclonal antibodies (Mabs), respectively. Pseudoparticle core proteins were detected with an anti-capsid (MLV CA) antiserum (CRL-1912). Anti-SR-BI (ab396) and -ApoA-I (ab7613) polyclonal

antibodies were obtained from Abcam (Cambridge). The anti-transferrin receptor antibody (Zymed (San Francisco, CA) was used to check that similar amounts of protein were loaded.

Lipoproteins and lipoprotein depleted serum (LPDS) preparation - Human HDL₂ (density 1.09 to 1.11 g/ml), HDL₃ (density 1.13 to 1.18 g/ml) and LDL (density 1.050 to 1.063 g/ml) fractions from fresh human plasma and lipoprotein depleted serum (LPDS, density > 1.25 g/ml) from fetal bovine serum were isolated by KBr density gradient ultracentrifugation as previously described (27). Lipoproteins and LPDS fractions were extensively dialyzed against 0.1X PBS-150mM NaCl and sterile-filtered.

Cell cultures - 293T human embryo kidney cells (HEK293T) and Huh-7 human hepatoma (28) were grown in Dulbecco's modified essential medium (Invitrogen) supplemented with 10% fetal bovine serum.

Production of HCVpp and infection assays - Pseudotyped particles were produced as previously described (5) and plasmids were kindly provided by B Bartosch and FL Cosset (INSERM U412, Lyon, France). Briefly, 293T cells were co-transfected with a murine leukemia virus (MLV) Gag-Pol packaging construct, a MLV-based transfer vector encoding Luciferase (29) and envelope glycoprotein expressing vector phCMV-E1E2 (5) using a PEI Exgen 500-based protocol (Euromedex). For some experiments, a deletion has been made in the hypervariable region 1 (HVR1) present at the N-terminus of HCV glycoprotein E2 (phCMV-ΔHVR1). For this construct, the 106 bp fragment covering the NH2 terminus of E2 deleted of HVR1 region (amino acids 384 to 411) was supplied by Genaert (Regensburg, Germany) and subcloned into the phCMV-E2 plasmid (5). The phCMV-G and phCMV-RD114 (30) expression vectors encode the VSV-G protein and the feline endogenous virus RD114 glycoprotein, respectively. Pseudotyped particles harboring VSV-G or RD114 envelope glycoproteins onto MLV cores were used as controls in most of our experiments. Supernatants containing the pseudotyped particles were harvested 48 h after transfection, filtered through 0.45 μm pore-sized membranes, and 25 μl of HCVpp, VSV-Gpp (dilution 1:15) and RD114pp (dilution 1:30) were used in infection assays. Pseudotyped particles were added to 20,000 Huh-7 cells seeded the day before in 24-well plates and

incubated for 3 to 6 h at 37°C in indicated medium. Culture medium was then replaced by 4% LPDS and Luciferase assays were performed as indicated by the manufacturer 3 days later (Promega).

Immunoprecipitation assay - Immunoprecipitation assays were performed as previously described (31). Briefly, HDL (30ug/ml) pre-incubated in the presence or in absence of HCVpp at 37°C were immunoprecipitated using anti-ApoA-I antibodies. Immunoprecipitates were extensively washed with PBS, eluted in Laemmli buffer and analyzed by Western blotting using anti-ApoA-I, anti-Gag, anti-E1 and anti-E2 antibodies.

GNA pull down - After 3 hours of incubation in the presence or in absence of HDL (30ug/ml) at 37°C, HCVpp were pulled down with lectin from *galanthus nivalis* (GNA) immobilized by cross-linking on agarose beads (Sigma). Particles produced in the absence of envelope proteins were used as a negative control. After 1 hour of contact at 4°C, the beads were extensively washed with PBS, and pulled down proteins were eluted with 1M methyl- β -mannopyranoside. Eluted proteins were analyzed by Western blotting using anti-Gag, anti-E1, anti-E2 and anti-ApoA-I antibodies.

RNA interference assay - SiRNA pseudotyped particles were generated by co-transfecting 293T cells with three plasmids using a PEI Exgen 500-based protocol (Euromedex): a HIV-1 Gag-Pol packaging construct, a VSV-G envelop glycoprotein expressing vector, and a bicistronic lentiviral vector, FG12 (32), allowing the expression of a GFP marker protein to control the transduction efficiencies and the expression of the small interfering RNA (siRNA) directed against human SR-BI mRNA (33). The bicistronic lentiviral vectors containing the siRNA sequences (5'-GCAGCAGGUCCUUAAGAAC (siRNA-2) and 5'-GGACCCCUUGUGAAUCUC (siRNA-23)) were kindly provided by D Lavillette and FL Cosset (INSERM U412, Lyon, France). SiRNA2 has been shown to reduce SR-BI expression whereas siRNA23 did not and was therefore used as a negative control (34 in press). Huh-7 cells were infected with siRNA pseudotyped particles, trypsinated 3 days later, plated in 24-wells plates and infected 24 h later by HCVpp or VSV-Gpp as describes above.

Sucrose gradient fractionation - HCVpp and LPDS-HDL were analyzed by an overnight centrifugation in a 20–60% sucrose gradient at

35,000 rpm and 4°C in a Beckman SW 41 rotor. Fractions of 1 ml were collected and concentrated by ultracentrifugation through a 20% sucrose cushion in a TLA 100 Beckman rotor (42,000 rpm, 2 h). Viral pellets were suspended in 130 μ l Laemmli buffer and analyzed by Western blotting.

Immunoblotting analysis - Huh-7 cells lysates (lysis buffer Promega E153A) and viral pellets were separated on a 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked for 1h in PBS-I-M (PBS containing 0.1% Igepal and 5% non-fat dried milk) and then probed overnight with antibodies diluted in PBS-I-M. Antibody binding to protein was detected with secondary antibodies conjugated to horseradish peroxidase using enhanced chemiluminescence as recommended by the manufacturer (Amersham Biosciences).

RESULTS

HDL enhance HCVpp infectivity - Recent data are in favor of a potential role for SR-BI in HCV entry. Indeed, a soluble form of HCV envelope glycoprotein E2 has been shown to specifically interact with human SR-BI (8). In addition, antibodies directed against SR-BI strongly reduce the infectivity of HCV pseudotyped particles (17). Since HDL is a natural ligand for SR-BI, we wondered whether this lipoprotein might have some inhibitory effect on HCVpp entry by competing with HCVpp binding. We therefore compared the infectivity of HCVpp in the presence or absence of HDL. LDL, the other major human plasma lipoprotein, was used as a control because SR-BI is not a receptor for LDL. Surprisingly, we observed a threefold increase in the efficiency of HCVpp entry in the presence of HDL (Fig. 1A). This marked increase in HCVpp was specific of HDL, as no stimulating or enhancing effect was observed in the presence of LDL. In addition, HDL had no effect on VSV-G and RD114 pseudotyped particles infectivity (Fig. 1A and data not shown). Since these pseudotyped particles only differ in their envelope glycoproteins and thus in their receptor usage, these data indicate that HDL-mediated enhancement of infectivity is specific of the presence of HCV envelope glycoproteins on the pseudotyped particles. The active concentrations of HDL used in our experiments (1 to 30 ug/ml) were usually lower than that in normal human serum (approximately 1mg/ml); however, they

are in agreement with those generally used to study HDL binding, and cholesterol influx and efflux.

In contrast to HCVpp, VSV-Gpp were less infectious in the presence of LDL. This suggests that LDL might compete with VSV-G receptor or reduce its expression. Alternatively, LDL might reduce VSV-Gpp fusogenic activity by modifying the lipid composition at the site of entry.

Human HDL represent a mixture of lipoproteins that differ in size, composition and apolipoprotein content (35). HDL can be fractionated into subclasses by different techniques according to their physicochemical properties. Based on their density, HDL have been divided into two subfractions: HDL₂ (1.063-1.125 g/ml) and HDL₃ (1.125-1.21 g/ml). HDL₂, which are larger than HDL₃, preferentially bind to SR-BI and potentially display an increased cholesteryl ester uptake than HDL₃ (36). As shown in Fig.1B, both HDL₂ and HDL₃ subclasses enhanced HCVpp entry; however, the effect was stronger with HDL₃. These data do not strictly correlate with the cholesteryl ester uptake capacity of HDL₂ and HDL₃, but one cannot exclude a possible interaction of HCVpp with HDL at the cell surface involving a particular conformation of ApoA-I or a specific size. Altogether, our data indicate that HDL facilitate HCVpp entry.

HDL do not increase the level of expression of SR-BI - Since SR-BI is a putative receptor for HCV, a potential hypothesis to explain the enhancer effect of HDL on HCVpp infectivity is that HDL increases the level of expression of SR-BI. To evaluate the possible modulation of SR-BI expression by lipoproteins, we analyzed the level of expression of SR-BI in the presence or absence of HDL. As shown in the inset Fig.1A, the level of expression of SR-BI was not modified by the presence of lipoproteins, indicating that HDL enhancer effect on HCVpp infectivity is not related to an increase of SR-BI protein level in target cells.

HDL do not interact with HCVpp in culture medium - In the plasma of HCV-infected patients, HCV particles can be found in association with lipoproteins (10,23). We wanted therefore to determine whether HDL might work as a carrier that would be used by HCVpp to bind to the SR-BI receptor. HCVpp preincubated or not with HDL were analyzed by sedimentation through a sucrose gradient to determine whether HDL would modify the

sedimentation profile of HCVpp, which would be an indication that these viral particles interact with the lipoproteins. As shown in Fig.2, HCVpp produced in lipoproteins depleted serum LPDS (Fig.2A) or LPDS-HDL medium (Fig.2B) had the same sedimentation profile. They were indeed detected in fractions 7 to 9 (around 40% sucrose), where their constitutive proteins were detected (i.e., E1, E2, Gag). The sedimentation profile of ApoA-I protein, the major apolipoprotein of HDL, was similar in the presence or absence of HCVpp. Indeed, this protein was detected mainly in fractions 2 to 5 whether or not HCVpp were present (Fig.2C). These data show that HCVpp and HDL interaction in culture medium, if any, is not strong enough to allow the co-sedimentation of HDL and HCVpp on a sucrose gradient. Co-immunoprecipitation and pull-down assays were also performed to evaluate the possible interaction of HCVpp with HDL in a gentler way. Using anti-ApoA-I antibodies, HDL pre-incubated with HCVpp were immunoprecipitated, but no HCV envelope glycoproteins were coprecipitated with HDL (data not shown). On the other hand, HCVpp pre-incubated with HDL were pulled down with GNA, a lectin that is known to interact with E2 glycans (37). Whereas HCVpp were pulled down and specifically eluted, no ApoA-I protein was detected (data not shown).

In another approach to determine whether HDL can be a carrier to target HCVpp to their receptor, we analyzed HDL enhancement of infectivity by adding HDL during or after adsorption of HCVpp to host cells at 4°C. If HDL are a carrier to target HCVpp to their receptor, there should be no enhancement of infectivity if HDL are added after HCVpp binding. Importantly, a fivefold increase in HCVpp infectivity was observed when HDL were added after binding of HCVpp to Huh-7 cells, whereas co-binding of HCVpp and HDL only induced a 1.7-fold increase in HCVpp infectivity (Fig.3). This indicates that HDL enhancement of infectivity occurs after HCVpp binding to their target cells.

Altogether, these data indicate that HDL and HCVpp do not interact before binding to the cell surface, and that HDL are not a carrier to target HCVpp to their receptor.

Lipid-free ApoA-I and ApoA-II are unable to enhance HCVpp entry - HDL contain lipids and apolipoproteins. ApoA-I and ApoA-II are the major protein components of these particles and

are therefore SR-BI ligands. To determine if ligand binding on SR-BI is sufficient to enhance the efficiency of HCVpp entry, we evaluated the effect of HDL versus lipid-free ApoA-I and ApoA-II proteins. Although ApoA-I and ApoA-II are known to have a lower affinity for SR-BI compared to their lipoprotein complex form (38), they are nevertheless natural SR-BI ligands. In contrast to native HDL, ApoA-I and ApoA-II were unable to enhance HCVpp entry (Fig. 4). In addition, neither ApoA-I nor ApoA-II competed with HDL for enhancement of HCVpp infectivity (data not shown). These data indicate that lipid-free ApoA-I and ApoA-II are unable to enhance HCVpp entry.

Lipid transfer inhibitors affect HDL-mediated enhancement of HCVpp infectivity - SR-BI is a lipoprotein receptor responsible for selective uptake of cholesteryl ester from HDL (22), and inhibitors of lipid transfer mediated by SR-BI have recently been described (24). In order to evaluate the implication of the cholesteryl ester transfer properties of SR-BI on HCVpp entry, we analyzed the effects of two drugs, block lipid transport 2 and 4 (BLT-2 and BLT-4) (24) on HDL-mediated enhancement of HCVpp infectivity. BLT-2 and BLT-4 have recently been discovered on the basis of their capacity to inhibit cholesteryl ester transfer via SR-BI (24).

In the absence of HDL, BLT-2 reduced HCVpp and VSV-Gpp infectivity (Fig.5, A and C). Indeed, up to 44% of reduction in HCVpp infectivity was observed in the presence of BLT-2. No increase in cell mortality was observed in the presence of the drug (data not shown). This suggests that BLT-2 has a general effect on pseudoparticle infectivity. However, BLT-2 induced a stronger inhibition of HCVpp infectivity in the presence of HDL. Indeed, the HDL facilitating effect on HCVpp infectivity was reduced by 60% when the drug was present.

In the absence of HDL, BLT-4 had only a slight effect on VSV-Gpp and HCVpp infectivity. Indeed, a slight increase of infectivity was observed for VSV-Gpp in the presence of BLT-4 (Fig.5C), whereas a slight reduction of HCVpp infectivity was obtained (Fig.5B). Importantly, the HDL facilitating effect on HCVpp infectivity was strongly reduced in the presence of BLT-4. Indeed, the HDL facilitating effect decreased in the presence of increasing amount of BLT-4, and at the concentration of 10 μ M of BLT-4, HDL had no more facilitating effect on HCVpp infectivity (Fig.5B). These data highlight the relationship

between cholesterol transfer and HCV entry. It is worth noting that the level of expression of SR-BI was not modified by the presence of the drug (data not shown), indicating that the inhibitory effect of these two drugs is not due to modulation of SR-BI protein expression. Although the mechanism of cholesterol transfer inhibition by these drugs is largely unknown, lipid transfer inhibition is linked to the increase of HDL affinity to SR-BI (24). This reduction of HDL turnover may explain the reduced cholesteryl ester transfer via SR-BI and consequently the reduction of HCVpp entry. Altogether, these data suggest that HDL-mediated enhancement of HCVpp entry is at least in part related to SR-BI lipid transfer activity.

SR-BI down-expression reduced the stimulatory effect of HDL on HCVpp entry - To further investigate the role of SR-BI in HDL-mediated enhancement of HCVpp entry, we silenced its expression in Huh-7 cells using siRNA (34). Two siRNAs that target different regions of SR-BI mRNA were expressed in target cells via a VSV-G pseudotyped HIV-1-based retroviral vector that also encodes the GFP marker protein. Upon infection with this vector, about 95% Huh-7 cells expressed the GFP marker (data not shown). siRNA2 down-regulated SR-BI expression to 32% compared to the negative control siRNA23, as shown by Western-blot analysis (inset Fig. 6). The silencing of SR-BI expression did not inhibit the infectivity of pseudotyped particles harboring VSV-G glycoproteins, which bind a non-related receptor (data not shown). The level of HCVpp infectivity was similar with siRNA23 and siRNA2 target cells. However, HDL stimulation was markedly reduced in siRNA2 target cells compared to siRNA23 control cells (Fig.6). In addition, inhibition of HCVpp entry was more pronounced with low HDL concentrations. These data correlate well with the reduction of SR-BI protein expression level observed by Western blot (inset Fig.6), and thus indicate that HDL-mediated enhancement of HCVpp infectivity is dependent on SR-BI expression.

HDL enhancement of HCVpp entry is suppressed by HVR1 deletion on E2 glycoprotein - Hypervariable Region 1 (HVR-1) is a 27-amino acid long segment located at the amino-terminal end of E2. Deletion of the HVR1 domain of a recombinant soluble E2 protein has been shown to reduce its interaction with SR-BI (8). The same deletion also reduces HCVpp

infectivity (17). We thus wanted to evaluate the effect of HDL on HCVpp harboring HVR-1-deleted E2 proteins (Δ HVR1-HCVpp). In the presence of HDL, infectivity of Δ HVR-1 mutant slightly increased to a maximum of 130% (Fig.7). However, this was very low compared to HCVpp containing unmodified envelope glycoproteins. Indeed, a 350% increase was observed in the presence of 30ug/ml of HDL for HCVpp. These data indicate that SR-BI/E2 interaction via HVR-1 domain is likely essential for HDL-mediated enhancement of HCVpp infectivity.

DISCUSSION

To infect a target cell, a virus particle must proceed through a multistep entry process, which involves binding to a receptor and sometimes to a co-receptor. For some viruses, the initial steps of entry can be very complex and involve the successive use of multiple attachment factors and receptors (39). In the case of HCV, several putative receptors have been proposed (40), suggesting that HCV entry might also potentially involve successive interactions. In this report, we investigated the role of one of the putative HCV receptor, SR-BI, in viral entry. We sought to determine how HDL, the natural ligand of SR-BI, affect HCV entry. Our data demonstrate an active role for HDL and its physiologically relevant receptor, SR-BI, in facilitating HCV entry. To our knowledge, this is the first description of the active involvement of a lipoprotein in the entry step of a virus.

To date, among several putative HCV receptors, only CD81 and SR-BI have been shown to play a role in HCVpp entry in hepatoma cell lines (5,6,17,18,34). Whereas the role of CD81 in HCV entry is now well documented (5,6,17-19), the contribution of SR-BI in HCV entry is less characterized. Although HCV glycoprotein E2 can interact with human SR-BI (8) and antibodies directed against SR-BI reduce HCVpp infectivity (17), our data show that HDL is not a competitor for HCV entry. Because SR-BI displays a complex structure that includes different domains corresponding to several properties (ligand binding and lipid transfer) (41), this might suggest that the binding sites of HDL and HCVpp are located on separate domains of SR-BI. However, HCVpp and HDL are particles that have a size of approximately 70 nm and 10 nm, respectively, and binding of HDL to SR-BI might therefore potentially hinder

the recognition of SR-BI by HCVpp. Alternatively, since interaction between HDL and SR-BI does not lead to internalization and is transient due to a loss of affinity after cholesteryl ester uptake (38,42), HCVpp binding to SR-BI might occur after HDL release from its receptor. This would explain the absence of competition of HDL for HCV entry.

We did not observe any change in the sedimentation profile of HCVpp when HDL were preincubated with the viral particles. This contrasts with previous reports indicating that HCV-like particles or native HCV virions interact with various classes of lipoproteins (10,23,43). We cannot exclude that due to differences in their structure and/or assembly, HCVpp might exhibit some differences in their capacity to interact with lipoproteins. Interestingly, no direct interaction between HDL and HCVpp was observed in culture medium, and HCVpp infectivity was greatly enhanced when HDL were added to HCVpp pre-bound to target cells. This suggests that HDL and HCVpp do not interact before binding to the cell surface, and that HDL are not a carrier to target HCVpp to their receptor. However, we cannot exclude that HDL-HCVpp interactions occur after binding of HCVpp and/or HDL to SR-BI.

The mechanism of SR-BI-mediated selective cholesterol uptake and intracellular transport differs fundamentally from the pathway of the receptor-mediated endocytosis through clathrin-coated pits used by the LDL receptor to deliver cholesteryl esters from LDL to cells (22). Indeed, the cholesterol delivered by HDL is the result of a productive binding mechanism: cholesteryl ester-rich HDL bind to SR-BI receptor, deliver cholesteryl ester to cells, and then dissociate from their receptors due to a loss of affinity (38,42). On the basis of these particular SR-BI properties, our data highlight the link between HDL productive binding on SR-BI and HCVpp entry. First, lipid-free ApoA-I and ApoA-II proteins, the major HDL apolipoproteins responsible for HDL binding to SR-BI, have no effect on HCVpp entry, despite their weak but real binding capacity to SR-BI (44,45). In fact, the functional role of HDL in lipid transfer is dependent on the proper orientation of ApoA-I associated with lipids (46). Secondly, drugs inhibiting the cholesterol transfer mediated by HDL via SR-BI (BLT-2 and BLT-4) affected HDL-mediated enhancement of HCVpp entry, reinforcing the hypothesis that HCVpp entry might be

dependent on the lipid exchange activity of SR-BI. Although the mechanism of cholesterol influx mediated by HDL is largely unknown, several reports indicate that the cholesterol transfer is essential for regulating the properties of cell membranes, probably by maintaining a proper sphingolipid ratio (47). Local changes in lipid composition might therefore potentially affect the efficiency of fusion between HCV envelope and the cell membrane (48). Finally, HDL enhancement of HCVpp entry is largely dependent on SR-BI expression as demonstrated by the suppression of the HDL enhancer effect after SR-BI silencing.

HCV envelope glycoprotein E2 has been demonstrated to be a ligand for SR-BI and the HVR-1 region of E2 is essential for E2 binding

to SR-BI (8). The absence of enhancement of infectivity when HCVpp harboring HVR-1-deleted E2 proteins were incubated in the presence of HDL indicates that, in addition to HDL-SR-BI interaction, a direct contact between HCVpp and SR-BI is also necessary for HDL-mediated enhancement of HCVpp entry. In conclusion, we have demonstrated that HDL play an active role in HCV entry and that HDL-mediated enhancement of HCV entry involves a complex interplay between SR-BI, HDL and HCV envelope glycoproteins. These findings contribute to improve our understanding of the tightly regulated steps of HCV entry and will be helpful in the design of novel antiviral therapies targeting HCV entry.

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FOOTNOTES

§ J Dubuisson and Ngoc Vu-Dac ontributed equally to this study.

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¹The abbreviations used are: ABCA1; ATP-binding cassette transporter A1; ApoA-I, apolipoprotein AI; ApoA-II, apolipoprotein AII; HCV, hepatitis C virus; HCVpp, HCV pseudotyped particles; HDL, high density lipoprotein; HIV; human immunodeficiency virus; HVR-1, hypervariable region 1; GNA lectin, *Galanthus nivalus* lectin; LDL, low density lipoprotein; LPDS, lipoprotein depleted serum; MLV, murine leukemia virus; siRNA, small interfering RNA; SR-BI, scavenger receptor class B type I; VSV, vesicular stomatitis virus.

FIGURE LEGENDS

Fig. 1. HDL specifically enhance HCVpp infectivity. Human hepatoma cells Huh-7 were infected for 6 hours with pseudotyped particles harboring HCV E1E2 envelope glycoproteins (HCVpp) or VSV-G envelope glycoprotein (VSV-Gpp) in DMEM medium supplemented with 4% lipoprotein depleted serum (LPDS) and 30ug/ml of human HDL or LDL (A) or with 4% LPDS and increasing amounts of HDL₂ or HDL₃ (B). The inset in panel (A) shows a representative Western blot of total SR-BI cellular level from Huh-7 cells cultured in the presence of lipoproteins. The results are presented as percentages of the infectivity relative to infectivity of HCVpp or VSV-Gpp in LPDS medium. Results are reported as the mean +/- standard deviations of three independent experiments. Pseudotyped particles produced in the absence of envelope protein were used as a control. The mean luciferase activity of such pseudotyped particles produced in the absence of envelope proteins represented less than 2% of the activity measured for HCVpp and no HDL-mediated enhancement was observed for this control (data not shown). HDL had no effect on pseudotyped particles harboring no E1E2 glycoproteins (data not shown).

Fig. 2. HDL do not interact with HCVpp. HCVpp pre-incubated or not with HDL as well as HDL alone were analyzed by sedimentation through 20-60% sucrose gradients. Sedimentations of HCVpp produced in LPDS are presented in panel (A), HCVpp pre-incubated with 30ug/ml HDL in panel (B) and HDL alone in panel (C). Fractions were collected from top to bottom, concentrated by ultracentrifugation through a 20% sucrose cushion, and protein content was analyzed by Western blotting using anti-E1 (A4), anti-E2 (3/11) anti-Gag (CRL1912) and anti-ApoA-I (A0722) antibodies.

Fig. 3. HDL facilitation on HCVpp entry is a post-binding event. HDL enhancement of HCVpp entry was analyzed by adding HDL during or after HCVpp binding. (A) HCVpp mixed or not with 15 ug/ml HDL were incubated with Huh-7 cells for 1 hour at +4°C. After virus adsorption, cells were washed with DMEM and further incubated for 3 hours at 37°C in LPDS 4%. Cells were then further incubated and processed as described in the “experimental procedures”. (B) HCVpp were incubated with Huh-7 cells for 1 hour at +4°C. After virus adsorption, cell were washed with DMEM and further incubated for 3 hours at 37°C with 15ug/ml of HDL. Cells were then further incubated in the absence of HDL and processed as described in the “experimental procedures”.

Fig. 4. Lipid-free ApoA-I and ApoA-II are unable to enhance HCVpp entry. Huh-7 cells were infected with HCVpp in DMEM medium supplemented with LPDS and various amounts of HDL or purified ApoA-I and ApoA-II proteins. The results are presented as percentages of the infectivity relative to infectivity of HCVpp in LPDS medium. Results are reported as the mean +/- standard deviations of three independent experiments.

Fig. 5. BLT-2 and BLT-4 affect HCVpp infectivity. Huh-7 cells were infected for 6 hours with HCVpp in LPDS medium or in LPDS-HDL medium (30 ug/ml) in the presence of various amounts of

BLT-2 (A) or BLT-4 (B). VSV-Gpp were used as a control to evaluate the effect of BLT-2 and BLT-4 on viral entry (C). The results are presented as percentages of the infectivity relative to infectivity of HCVpp in LPDS medium. Results are reported as the mean of three independent experiments. Standard deviations varied between 0 and 18%.

Fig. 6. SR-BI down-expression reduces the stimulatory effect of HDL on HCVpp entry. Huh-7 cells expressing retroviral delivery vectors siRNA2 or siRNA23 (control) were infected with HCVpp in DMEM medium supplemented with various amounts of HDL for 3 hours. The inset shows a representative Western blot of total SR-BI cellular level from Huh-7 expressing siRNA2 or siRNA23 (control). The results are presented as percentages of the infectivity relative to infectivity of HCVpp in LPDS medium. Results are reported as the mean +/- standard deviations of three independent experiments.

Fig. 7. HDL enhancement of HCVpp entry is suppressed by HVR-1 deletion on E2 glycoprotein. Huh-7 cells were infected with HCVpp harboring wild type or HVR-1-deleted E2 glycoproteins (HCVpp Δ HRV-1) in LPDS medium or in LPDS containing various amounts of HDL. The results are presented as percentages of the infectivity relative to infectivity in LPDS medium. Results are reported as the mean +/- standard deviations of three independent experiments. The infectious titer Δ HVR-1-HCVpp was approximately four times lower compared to HCVpp titer.

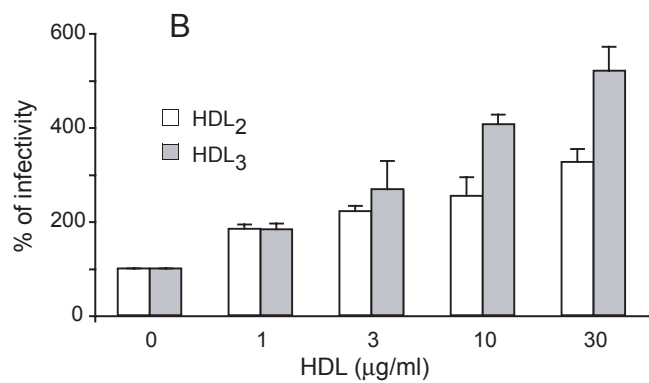
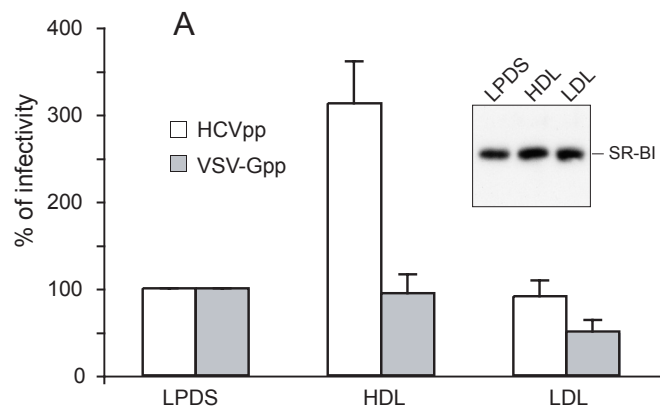


Fig 1

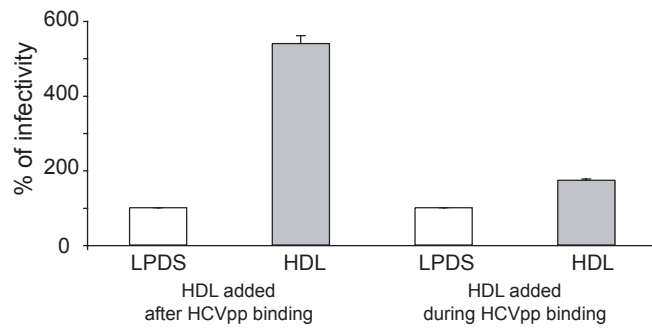


Fig 3

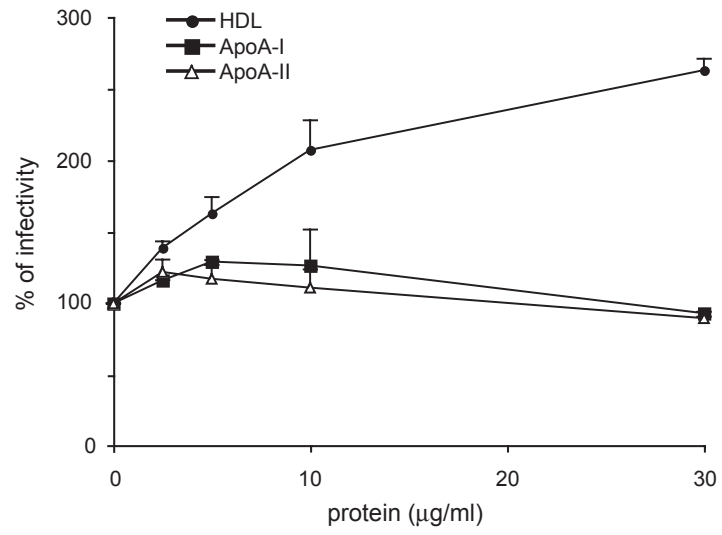


Fig 4

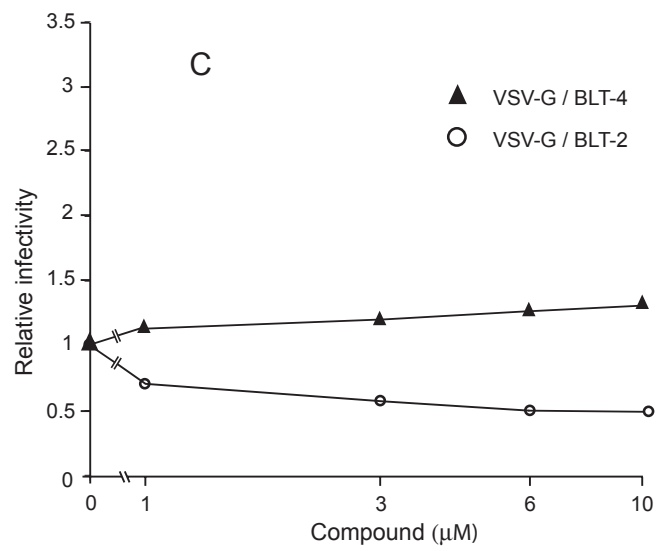
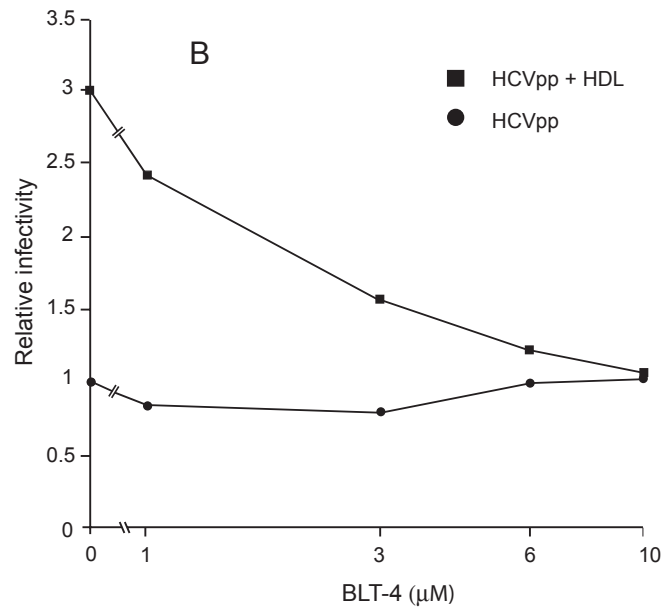
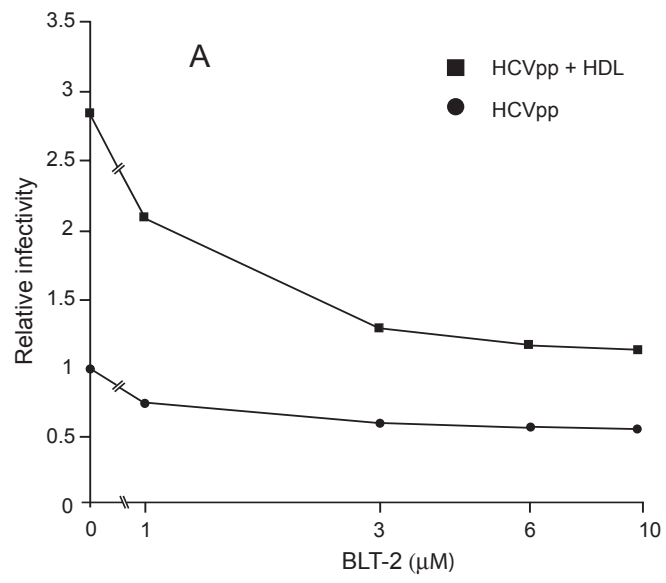


Fig 5

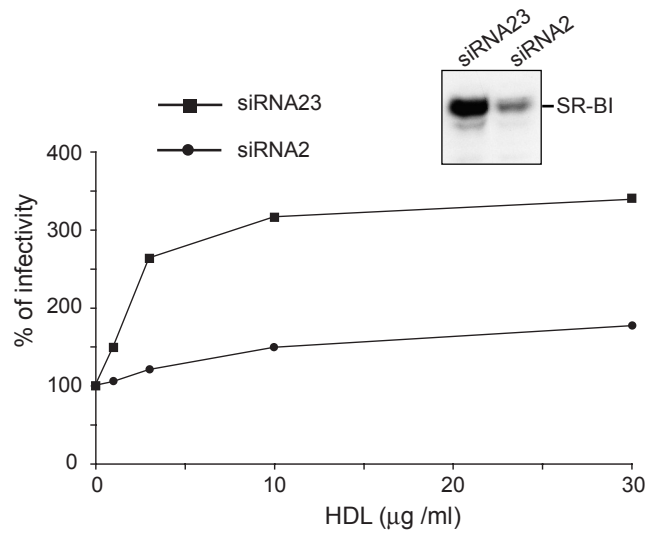


Fig 6

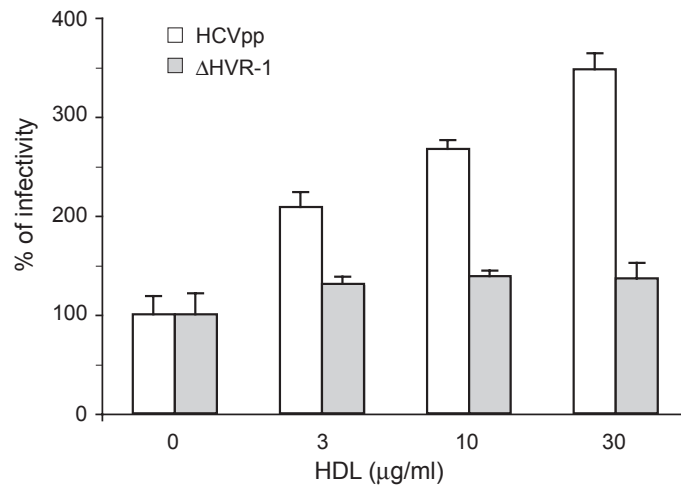


Fig 7