Syndecan-1 Serves as the Major Receptor for Attachment of Hepatitis C Virus to the Surfaces of Hepatocytes

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Our recent studies demonstrated that apolipoprotein E mediates cell attachment of hepatitis C virus (HCV) through interactions with the cell surface heparan sulfate (HS). HS is known to covalently attach to core proteins to form heparan sulfate proteoglycans (HSPGs) on the cell surface. The HSPG core proteins include the membrane-spanning syndecans (SDCs), the lysophosphatidylinositol-linked glypicans (GPCs), the basement membrane proteoglycan perlecan (HSPG2), and agrin. In the present study, we have profiled each of the HSPG core proteins in HCV attachment. Substantial evidence derived from our studies demonstrates that SDC1 is the major receptor protein for HCV attachment. The knockdown of SDC1 expression by small interfering RNA (siRNA)-induced gene silence resulted in a significant reduction of HCV attachment to Huh-7.5 cells and stem cell-differentiated human hepatocytes. The silence of SDC2 expression also caused a modest decrease of HCV attachment. In contrast, the siRNA-mediated knockdown of other SDCs, GPCs, HSPG2, and agrin had no effect on HCV attachment. More importantly, ectopic expression of SDC1 was able to completely restore HCV attachment to Huh-7.5 cells in which the endogenous SDC1 expression was silenced by specific siRNAs. Interestingly, mouse SDC1 is also fully functional in mediating HCV attachment when expressed in the SDC1-deficient cells, consistent with recent reports that mouse hepatocytes are also susceptible to HCV infection when expressing other key HCV receptors. Collectively, our findings demonstrate that SDC1 serves as the major receptor protein for HCV attachment to cells, providing another potential target for discovery and development of antiviral drugs against HCV.

Hepatitis C virus (HCV) is a common cause of chronic liver diseases such as hepatitis, cirrhosis, and liver cancer. It is an enveloped RNA virus containing a single positive-sense RNA genome that encodes a polyprotein precursor of 3,000 amino acids (1). Upon translation, the viral polyprotein was cleaved by cellular peptidases, viral NS2/NS3 metalloprotease, and NS3/4A serine protease to produce mature HCV structural (C, E1, and E2) and nonstructural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (2, 3). The untranslated nucleotide sequences flanked at both the 5’ and 3’ ends of the HCV RNA genome are highly conserved and form complex secondary and tertiary structures serving as cis-acting RNA elements important for initiation of viral protein translation and/or viral RNA replication. Based on the common features of viral RNA genome organization, HCV is classified in the Hepacivirus genus of the Flaviviridae family (4, 5).

HCV enters cells via receptor-mediated endocytosis (6). A number of cell surface proteins were shown to interact with the viral envelope glycoproteins E1 and E2 (7). Human CD81 was identified as the first HCV receptor/coreceptor (8). Subsequently, many other cell surface proteins were found to be important for HCV cell entry (9), including the low-density lipoprotein receptor (LDLr) (10–12), scavenger receptor class B type 1 (SR-B1) (13, 14), claudins (CLDNs) (15–17), occludin (OCLN) (18, 19), den- dritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN) and liver/lymph node-specific SIGN (L-SIGN) (20–23), heparan sulfate proteoglycans (HSPGs) (24–26), asialoglycoprotein receptor (27), epithelial growth factor receptor (EGFR) and ephrin receptor A2 (28), and Niemann-Pick-C1-like-1 cholesterol absorption receptor (29). The exact roles and underlying molecular mechanisms of these individual cell surface proteins in HCV infection have not been defined. It is believed that each of these cellular proteins may function sequentially at different steps or stages of the virus entry process through distinct interactions with viral envelope proteins E1 and E2. A number of studies suggested that CD81, CLDN, and OCLN function at post-binding steps during HCV infection (15, 26, 30–33). Consistent with these findings, our recent studies demonstrated that the knockdown of LDLr, CD81, claudin-1, occludin, and SR-B1 expression in Huh-7.5 cells did not significantly affect HCV attachment but remarkably reduced HCV infection, also suggesting their importance at postattachment steps in HCV infection (32). More importantly, our previous studies demonstrated that the cellular apolipoprotein E (apoE) is an integral part of the HCV particle (35, 36). The structural nature of apoE was further confirmed by electronic microscopy studies demonstrating that apoE is located on the envelope of the HCV virion (37, 38). Additionally, findings derived from our previous studies demonstrate that apoE has dual functions in the HCV life cycle. The C-terminal portion of apoE is critical for virion assembly via specific interaction with NS5A (35, 36, 39, 40). Disruption of the apoE-NS5A interaction by deletion mutations of apoE resulted in ablation of HCV assembly (40). The importance of apoE in HCV production is also confirmed by a recent study demonstrating that apoE is the key factor for HCV particle formation.
only apolipoprotein required for HCV production in nonhepatic 293T cells (41). Apart from its role in HCV assembly, apoE also mediates HCV attachment through its N-terminal receptor-bind- ing domain, which binds the cell surface receptors HSPGs (32) (J. Jiang and G. Luo, unpublished results). Removal of HS from cell surface HSPGs by pretreatment of cells with heparinases could efficiently prevent HCV binding and infection (24–26, 32). HS is covalently attached to core proteins to form HSPGs. The HSPG core proteins include the membrane-spanning syndecans (SDCs), the lycosylphosphatidylinositol-linked glypicans (GPCs), the basement membrane proteoglycan perlecan (HSPG2), and agrin (AGRN) (42, 43). However, the core proteins of HSPGs required for HCV attachment in human hepatocytes have not been de- fined. In this study, we have profiled the importance of each of the HSPG core proteins in HCV attachment. Our findings demon- strate that syndecan-1 (SDC1) is the major receptor protein for HCV attachment to cell surface. Both human and mouse SDC1 are fully functional to mediate the attachment of HCV to target cells, providing another potential molecular target for discovery and development of anti-HCV drugs.

MATERIALS AND METHODS

Viruses and cell culture. The cell culture-adapted HCV of genotype 2a (JFH1) was grown in Huh-7.5 cells, as used in our earlier work (43, 78). This cell culture–grown HCV is designated HCVcc. The genotype 1b HCV was obtained from hepatitis C patients (Teragenix, Fort Lauderdale, FL) and was generously provided by Hengli Tang (44). This clinical 1b isolate is designated HCV1b. Huh-7.5 and adenovirus packaging cell line AD293 cells (Agilent Technologies) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 0.1 mM streptomycin at 37°C in a 5% CO2 incubator. Human embryonic stem cell (hESC) line WA09 (H9) was obtained from the WiCell Research Institute and maintained on Geltrix-coated culture plates in Stem Pro medium (Invitrogen, Carlsbad, CA). hESC–differentiated human hepatocytes (DHHS) were prepared and maintained as described previously (44).

Antibodies and synthetic siRNAs. HCV NSSA monoclonal antibody (MAB) (9E10) was provided by Charlie Rice. Rabbit anti-NSSA polyclonal antibody and goat anti-rabbit secondary antibody conjugated with Alexa 488 were from Virogen and Invitrogen, respectively. β-Actin monoclonal antibody (AC15) was from Sigma-Aldrich. Human SDC1 monoclonal and polyclonal antibodies (sc-12765, 553712, and 550804) were pur- chased from Santa Cruz and BD Biosciences, respectively. SDC2–specific monoclonal antibody (2965) was from R&D Systems. NS3-specific monoclonal antibody was produced in the lab as previously described (45). CD81 MAB (clone 5A6) was from Santa Cruz. Claudin-1 monoclonal antibody and occludin–specific rabbit polyclonal antibody were from Invitrogen. SR-B1 rabbit polyclonal antibody (EP15565) was from Abcam. Horseradish peroxidase–conjugated goat anti-mouse IgG was from Pierce. Cellular gene-specific small interfering RNAs (siRNAs) (SmartPool and individual siRNAs) and nonspecific control (NSC) siRNA were synthesized by Dharmacon.

Silencing of cellular gene expression by RNA interference (RNAi). Cell culture plates (12- and 24-well plates) were coated with 50 μg/ml of collagen (Huh-7.5) or Geltrix (DHHS). Huh-7.5 cells and DHHSs were seeded overnight at 37°C in an incubator and then transfected with vari- ous concentrations (0, 2, 10, and 50 nM) of siRNAs using RNAiMax transfection reagent (Invitrogen), as previously described (32). An NSC siRNA was used as a negative control. At 48 h posttransfection (p.t.), the mRNA and protein levels of targeted cellular genes were determined by a quantitative reverse transcription-PCR (qRT-PCR) method and Western blotting using the housekeeping gene glyceraldehyde-3-phosphate dehy- drogenase (GAPDH) and β-actin as internal controls, respectively.

HCV attachment and infection assays. At 48 h after siRNA transfection, Huh-7.5 cells and DHHSs in 12-well cell culture plates were incubated with HCVcc at a multiplicity of infection (MOI) of 10 on ice for 2 h or at 37°C for 1 h or with HCV1b at 37°C for 2 h (attachment assay). The unbound HCV was removed by washing with 1× phosphate-buffered saline (PBS) three times. Total RNA was extracted with RNAzol reagent (Molecular Research Center) and was used for quantification of HCV virion RNA (vRNA) by a real-time qRT-PCR method. HCV infection was carried out by incubation of HCVcc with Huh-7.5 cells at 37°C for 1.5 h, and the HCV-infected cells were washed with PBS and incubated with fresh DMEM at 37°C for 24 h (single-cycle growth). The cell culture supernatants were collected for determination of infectious HCV titers by limiting dilution, whereas the HCV-infected Huh-7.5 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer for detection of HCV NS5A and cellular proteins by Western blotting using specific monoclonal antibodies.

Quantification of HCV vRNA and cellular gene mRNAs by one-step or two-step qRT-PCR method. The levels of HCV vRNA and cellular gene mRNAs were determined by either one-step qRT-PCR or the two- step RT and qPCR method using specific primers and probes (available on request) and the StepOnePlus real-time PCR system (Applied Biosys- tems). The one-step real-time qRT-PCR was carried out using the following program: 50°C for 10 min and 95°C for 5 min followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. For two-step qRT-PCR, cDNAs were first synthesized from total RNA using Moloney murine leukemia virus (M- MuLV) reverse transcriptase (New England Biolabs) for GPCs, HSPG2, and AGRN, and then qPCR was carried out as follows: 10 min at 95°C and 40 cycles at 95°C for 15 s and 60°C for 1 min. GAPDH was used as a control to normalize total amounts of RNAs used in qRT-PCR.

Western blotting. The protein concentration of cell lysates was deter- mined using a protein assay reagent (Bio-Rad). Twenty micrograms of total protein for each sample was resolved by 10% SDS-PAGE and trans- fferred to a nitrocellulose membrane. The membrane was incubated with either primary antibodies specific to SDC1, NSSA, and β-actin and then with secondary peroxidase–conjugated anti-mouse antibody, followed by enhanced chemiluminescence (ECL) staining.

Immunofluorescence assay (IFA). HCV-infected cells were fixed with 4% paraformaldehyde solution and permeabilized with 0.1% Triton X-100, as previously described (46). The NS3 protein in the HCV-infected Huh-7.5 cells was reacted with an NS3–specific monoclonal antibody and visualized with the secondary donkey anti-mouse IgG conjugated with Alexa Fluor 594 fluorescent (Molecular Probes). The NSSA in the HCV- infected DHHSs was stained with a rabbit anti-NSSA polyclonal antibody, followed by secondary Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes). Cell nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI) present in the antifade and antiphotobleaching mounting me- dium (Vector Laboratories).

Determination of infectious HCV titer. Infectious HCV in cell culture supernatants was titrated by a limiting dilution. The serially di- luted HCV was used to infect Huh-7.5 cells in 96-well plates. After 2 h of incubation at 37°C, 100 μl fresh DMEM containing 1% methylcellulose was added into each well. At 48 h postinfection (p.i.), focus-forming units (FFU) were determined by IFA staining using an NS3-specific monoclonal antibody as described previously (36). Infectious HCV titer was calcu- lated based on the average number of NS3-positive FFU/ml in triplicate assays.

Construction of recombinant adenoviruses expressing syndecans. To ectopically express human and mouse SDC1 that is resistant to RNA interference (RNAi), three silent nucleotide mutations were introduced into the siRNA–targeting region. Thus, the SDC1–specific siRNA used in this study would silence only endogenous but not ectopic SDC1 expres- sion. Silent mutations were introduced into human and mouse SDC1 genes by two overlapping PCR methods using synthetic oligonucleotide primers. PCR DNA fragments were cloned into pShuttle-CMV/hsDC1

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and pShuttle-CMV/mSDC1 vectors between the restriction enzyme sites KpnI and HindIII. The plasmid DNAs were confirmed by sequencing analysis at the Northwestern University Biotechnology Laboratory. Resulting SDC1 mutants were designated pShuttle-CMV/hSDC1m and pShuttle-CMV/mSDC1m. Recombinant adenoviruses expressing siRNA-resistant human and mouse SDC1 were produced using the AdEasy XL system, as previously described (32). Recombinant adenoviruses were prepared in large quantities and were purified using a Virapur Adeno Virakit from Virapur, aliquoted, and stored at −80°C in a freezer. Infectious titers of recombinant adenoviruses were determined by limiting dilution.

Ectopic expression of SDC1 in Huh-7.5 cells with silencing of endogenous SDC1. Huh-7.5 cells in 12-well plates were transfected with 0.2 nmol of SDC1-specific siRNA, whose target sequence is CCAAAACAGGAGGAAUUCUC, and NSC siRNA at 37°C for 24 h. The siRNA-transfected cells were infected with recombinant adenoviruses expressing hSDC1m or mSDC1m at an MOI of 10 at 37°C for 6 h. The unbound adenoviruses were removed, and fresh DMEM was added to each well. At 24 h p.i., Huh-7.5 cells were infected with HCVcc at an MOI of 10 at 37°C for 1 h. The unbound HCV was removed by washing cells with 1× PBS three times. Total RNA was extracted with RNAzol reagent and was used for determination of HCV virnal levels and SDC1 mRNA levels by qRT-PCR methods.

Statistical analysis. All data were analyzed by one-way analysis of variance (ANOVA) using SPSS 20.0 software. A P value of <0.05 was considered a statistically significant difference.

RESULTS

Profiling of the HSPG core proteins in HCV attachment. Previous studies by others suggested that HSPGs are important for HCV infection in cell culture (24–26). Our recent studies demonstrated that apoE mediates HCV attachment through interaction with the cell surface heparan sulfate (HS) (32). HS is covalently attached to core proteins of HSPGs, which include SDCs, GPCs, HSPG2, and AGRN, serving as core proteins for HSPG synthesis and modification. To determine the importance of each of these HSPG core proteins in HCV attachment, we carried out an initial profiling of HSPG core protein genes using synthetic siRNAs specific to each of the SDC, GPC, HSPG2, and AGRN mRNAs. A mix of 4 individual (SmartPool) siRNAs targeting different regions of each mRNA was used to specifically silence the expression of each mRNA in Huh-7.5 cells. The knockdown of gene expression was determined by quantifying the levels of mRNAs using a real-time qRT-PCR method and specific primers. The mRNAs encoding 12 HSPG core proteins were efficiently reduced in a dose-dependent manner compared to that of nonspecific control siRNA (Fig. 1). Specific siRNAs at a 50 nM concentration resulted in more than 90% reduction of SDC1, SDC2, SDC3, and SDC4 mRNAs, respectively (Fig. 1A). Likewise,
the levels of SDC1 and SDC2 proteins were proportionally decreased by increasing concentrations of siRNAs, resulting in >80% reduction of SDC1 and undetectable SDC2 expression at 50 nM specific siRNAs (see Fig. 3A). Similarly, the levels of GPC1, GPC2, GPC3, GPC4, GPC5, GPC6, and HSPG2 mRNAs were decreased by >80% at a 50 nM siRNA concentration. To a lesser extent, AGRN mRNA was lowered by 60% at 50 nM siRNAs (Fig. 1B).

The effects of siRNA-mediated knockdown of HSPG core protein expression on HCVcc attachment were subsequently determined. At 48 h after siRNA transfection, Huh-7.5 cells were incubated with HCVcc on ice for 2 h, under which conditions HCV can bind but is unable to enter cells. Unbound virus was completely removed by extensive washing. The mRNA of cell-attached HCVcc was then extracted and quantified by a real-time qRT-PCR method using specific primers and probes. Interestingly, knockdown of SDC1 expression remarkably decreased the levels of HCV vRNA in a dose-dependent manner. The SDC1-specific siRNAs at 2, 10, and 50 nM concentrations resulted in about 50%, 75%, and 85% reductions of HCV vRNA, respectively (Fig. 1C). The degree of reduction of HCV attachment is consistent with the knockdown of SDC1 expression at corresponding siRNA concentrations (Fig. 1A). SDC2 siRNAs also caused a modest but significant decrease of HCV vRNA with 50% reduction of HCVcc attachment at 50 nM siRNAs. However, SDC3 and SDC4 siRNAs did not significantly affect the levels of HCV vRNA (Fig. 1C). None of the GPC, HSPG2, and AGRN siRNAs had significant inhibition of HCVcc attachment, although they efficiently silenced the expression of their target genes, similarly to SDCs (Fig. 1B and D).

Effects of the siRNA-mediated silencing of SDC1 and SDC2 expression on HCV attachment and infection. To confirm the importance of SDC1 and SDC2 in HCV attachment, we carried out HCVcc attachment and infection experiments at 37°C. Similarly to that observed at 4°C, knockdown of SDC1 expression efficiently prevented HCVcc from attaching to Huh-7.5 cells when tested at 37°C, decreasing HCVcc attachment by more than 70% at 50 nM siRNAs (Fig. 2). However, knockdown of SDC2 had only a modest (<30%) effect on HCV attachment. The silencing of SDC3 and SDC4 expression did not affect HCV attachment. These results are in line with the data obtained from an HCVcc attachment assay at 4°C (Fig. 1C). Next, we determined the effects of the siRNA-induced SDC1 and SDC2 knockdown on HCV infection. Huh-7.5 cells were transfected with various amounts of SDC1 siRNAs, SDC2 siRNAs, or NSC siRNA and then infected with HCVcc at 37°C for 1.5 h. At 24 h p.i., the levels of SDC1, SDC2, and HCV NS5A proteins were determined by Western blotting. Also, the infectious HCV titers in the cell culture supernatants were quantified by limiting dilution. The levels of SDC1 and SDC2 expression were proportionally decreased with increasing amounts of specific siRNAs, which caused a >80% reduction of SDC1 expression and an undetectable level of SDC2 at 50 nM concentrations of corresponding siRNAs. More importantly, the silencing of SDC1 expression resulted in a dose-dependent reduction of NS5A, which is in parallel with the decrease of SDC1 expression (Fig. 3A). When Huh-7.5 cells were infected with HCV and then cotransfected with SDC1 siRNAs, the levels of NS5A remained unchanged, although SDC1 was efficiently silenced, suggesting that SDC1 siRNA did not affect viral RNA replication (data not shown). However, the levels of NS5A were not significantly affected by SDC2 siRNAs at 2 and 10 nM concentrations, even though SDC2 expression was lowered by 60% and 74%, respectively. SDC2 siRNA at a 50 nM concentration moderately reduced the level of NS5A (Fig. 3A), which is consistent with its effect on HCV vRNA (Fig. 2). Similarly, infectious HCV titers were reduced by SDC1-specific siRNA in a dose-dependent manner, resulting in a nearly 100-fold reduction at a 50 nM concentration of siRNAs (Fig. 3B). Again, the silence of SDC2 expression caused only a modest reduction at 50 nM but not at 2 nM and 10 nM concentrations (Fig. 3B), similarly to NS5A reduction (Fig. 3A). As expected, neither SDC1 and SDC2 expression nor HCV infection was affected by NSC siRNA (Fig. 3). Taken together, these data suggest that SDC1 likely serves as the major receptor protein for HCV attachment during virus infection.

To further validate the physiological relevance of SDC1 in HCV attachment, we used a clinical isolate of genotype 1b HCV (HCV1b) obtained from hepatitis C patients together with DHHs in an HCV attachment assay. Unlike Huh-7.5 cells, DHHs resemble primary human hepatocytes (PHHs) and are susceptible to infection by clinical HCV isolates, as demonstrated previously by others (44, 47). To confirm that DHHs prepared in our own lab were susceptible to HCV infection, DHHs at day 10 were infected with HCVcc. NS5A in the HCVcc-infected DHHs was stained using an NS5A-specific polyclonal antibody. Similarly to previous findings (44), most DHHs were permissive to HCV infection (Fig. 4A). However, a minor fraction of cells did not stain positively for NS5A, suggesting that these cells were not fully differentiated to render them permissive to HCV infection (Fig. 4A), consistent with previous observations (44). Subsequently, the effects of siRNA-mediated SDC1 silencing on HCV1b attachment were determined. Like Huh-7.5 cells (Fig. 1C and Fig. 2), SDC1 mRNA in DHHs was proportionally silenced by increasing concentrations of specific siRNAs with an about 80% reduction of SDC1 mRNA at 50 nM SDC1-specific siRNA (Fig. 4B). Consequently, knockdown of SDC1 expression potently suppressed the attachment of HCV1b to DHHs, resulting in more than 70% reduction of HCV1b attachment to DHHs at a 50 nM concentration.
and HCV1b attachment in DHHs (Fig. 4). Collectively, these findings demonstrate that SDC1 is important for HCV attachment during virus infection.

Restoration of HCV attachment by ectopic expression of SDC1. To exclude possible off-target effects associated with RNA interference (RNAi), we sought to determine whether ectopic expression of SDC1 would restore HCV attachment to Huh-7.5 cells defective in endogenous SDC1 expression. The endogenous SDC1 expression was silenced by transfection with a synthetic siRNA, which was found to most potently silence SDC1 expression among 4 individual SmartPool siRNAs profiled (Fig. 5 and data not shown). Unlike SmartPool siRNAs, the individual siRNA was less able to induce degradation of endogenous SDC1 mRNA. Therefore, higher concentrations of the SDC1 siRNA were required to efficiently silence endogenous SDC1 expression, with an about 80% reduction of SDC1 mRNA and protein at a 200 nM concentration (Fig. 5). To ectopically express the siRNA-resistant SDC1, three synonymous nucleotide mutations were introduced into the siRNA-targeting region of either the human or the mouse SDC1 gene. These mutations do not cause any change of amino acids but instead create nucleotide mismatches between SDC1 mRNA target and siRNA sequences. Therefore, the SDC1-specific siRNA could silence endogenous but not ectopic SDC1 expression. Recombinant adenoviruses were constructed to ectopically express human and mouse SDC1s. A nonspecific control siRNA (siNSC) and a recombinant adenovirus expressing a lacZ gene (LacZ) were used as negative controls in these experiments (Fig. 5). Huh-7.5 cells were transfected with SDC1-specific siRNA and then infected with recombinant adenoviruses. After 24 h, HCV attachment experiments were carried out at 37°C for 1 h. Upon extensive washing to remove unbound HCV, total RNAs were extracted from the HCVcc-treated cells. The levels of both SDC1 mRNA and HCV vRNA were determined by real-time qRT-PCR methods. Results show that SDC1 was ectopically expressed in the siRNA-treated Huh-7.5 cells as shown by mRNA quantification (Fig. 5C) as well as SDC1 protein expression detected by Western blotting (Fig. 5D). More significantly, ectopic expression of both human and mouse SDC1s in Huh-7.5 cells fully restored HCV attachment, which otherwise was inhibited by knockdown of endogenous SDC1 expression (Fig. 5E). In fact, overexpression of human SDC1 significantly increased the susceptibility of Huh-7.5 cells to HCVcc attachment (Fig. 5E). Additionally, infectious HCV titers in the culture supernatants were also completely restored (data not shown). Altogether, these data demonstrate that SDC1 functions as the major receptor for HCV attachment.

Effect of SDC1 silencing on the expression of CD81, CLDN1, OCLN, and SR-B1. To exclude a possible effect of SDC1 siRNAs on the expression of key HCV receptors such as CD81, CLDN1, OCLN, and SR-B1, we determined their respective expression levels by Western blotting in the SDC1 siRNA-transfected Huh-7.5 cells in the same way as that shown in Fig. 3A. None of these key HCV receptors was significantly affected by the siRNA-mediated knockdown of SDC1 expression (Fig. 6), suggesting that reduction of HCV attachment by silencing SDC1 expression was not due to its effect on CD81, CLDN1, OCLN, and SR-B1 expression. In fact, our previous studies demonstrated that the aforementioned HCV receptors do not play a significant role in HCV attachment, although they are critically important for HCV infection at postattachment steps (32).

DISCUSSION
A number of previous studies suggested that HSPGs serve as receptors to mediate initial binding of many different viruses to the cell surface, including but not limited to herpes simplex virus 1 (HSV-1); human papillomavirus (HPV); hepatitis B, C, and E viruses; rotavirus; and respiratory syncytial virus (48–64). Compelling evidence accumulated over the years suggests that the heparan sulfate covalently attached to core proteins of HSPGs is required for mediating virus attachment to the cell surface. In the case of HCV, either removal of HS from the cell surface by heparinases or addition of heparin and its derivatives could efficiently
block HCV infection (24–26, 32). However, the importance and underlying mechanism of the HSPG core proteins in HCV attachment have not been experimentally examined. In the present study, we have profiled each of the 12 HSPG core proteins using gene-specific siRNAs and an in vitro HCV attachment assay. Findings derived from our experiments suggest that SDC1 acts as the major receptor protein for binding of HCV to hepatocytes. Knockdown of SDC1 expression by specific siRNAs in Huh-7.5 and DHH cells blocked HCV attachment by over 80% (Fig. 1C, 2, and 4). Consistently, the siRNA-induced silence of SDC1 expression also resulted in reduction of HCV infection and production (Fig. 3). More significantly, the siRNA-induced downregulation of SDC1 expression also blocked the binding of a clinical HCV of genotype 1b to DHH cells (Fig. 4), suggesting that SDC1 likely functions as the receptor for HCV attachment in vivo. DHHs resemble primary human hepatocytes in many aspects as revealed by recent studies (44, 47). More importantly, ectopic expression of SDC1 not only fully restored but actually enhanced the susceptibility of Huh-7.5 cells to HCV attachment (Fig. 5D). It should be noted that knockdown of SDC2 expression also resulted in a modest but not dose-dependent inhibition of HCV attachment (Fig. 1C and 2). This may explain why the binding of HCV to the cell surface was not completely blocked by knockdown of SDC1 expression per se. However, other HSPG core proteins such as SDC3, SDC4, GPCs, HSPG2, and ARGN do not appear to play a significant role in HCV attachment, since knockdown of their expression had little effect on HCV binding (Fig. 1 and 2). Therefore, it is most likely that SDC1 proteoglycan serves as the major receptor for mediating initial binding of HCV to hepatocytes before sequential interactions with other key receptors and/or coreceptors. Future studies are warranted to corroborate the physiological significance of SDC1 in HCV infection in vivo. A fully humanized mouse model of HCV infection and replication made it possible to determine the biological relevance of SDC1 in HCV infection using wild-type and SDC1-knockout mice (65). Our results clearly show that ectopic expression of a mouse SDC1 was able to fully restore the susceptibility of Huh-7.5 cells to HCV binding, consistent with the susceptibility of mouse hepatocytes to HCV infection in vivo (65). It is noteworthy that SDC1 expression was detected in both mouse hepatoma cell line Hepa 1-6 and DHHs at about 40% of that in Huh-7.5 cells, consistent with the important role of SDC1 in HCV attachment in the cell types tested in this study (data not shown).

Why SDC1 but no other HSPG core proteins are preferentially
utilized by HCV for initial attachment to hepatocytes remains unknown. Previous studies found that different viruses favor distinct syndecans as attachment receptors. For instance, dengue virus, another member of the *Flaviviridae* family, prefers SDC2 proteoglycan as its binding receptor (66). For human immunodeficiency virus type 1 (HIV-1), SDC3 was found to be a major attachment receptor on dendritic cells (DCs) (67). However, SDC1, SDC2, SDC3, and SDC4 could all serve as HIV-1 attachment receptors when ectopically expressed in nonpermissive cells (68). In the case of HPV, SDC1 appeared to favorably increase cell permissiveness to the binding of virus-like particles and authentic virions compared with SDC4 and GPC1 expressed ectopically (69). Thus, the choice of different HSPG core proteins as attachment receptors for different viruses is complex and is
likely influenced by multiple factors. The levels of HSPG core protein expression among different cell types may contribute to their usage as attachment receptors by different viruses. More importantly, the HS attachment was found to be critically important for the function of SDCs (70). There are three highly conserved sites available for HS attachment at the N-terminal ectodomain of SDCs. HS attachment to each of these serine residues individually or in different combinations at different cell types may determine the tropism of SDCs as attachment receptors for different viruses. The SDC1 detected in mouse and human hepatocytes appears as a homogenous proteoglycan with a mass of >80 kDa, suggesting that it is highly sulfated in hepatocytes (Fig. 5B and D). We have tested a couple of commercially available SDC1-specific monoclonal and polyclonal antibodies in the HCV attachment assay. However, none of the antibodies examined had any effect on HCV attachment (data not shown). This could be due to their lack of blocking activity. Alternatively, HS moieties attached to SDC1 are the determinants for binding of protein ligands. It is known that specific interaction between HS and protein ligands depends on unique oligosaccharide sequences containing iduronic acid, N-sulfated glucosamine residues, and O-sulfated sugars attached to the core proteins (71). The saccharides can be modified by N and O sulfation at different positions in the cell (72). It was found that 3-O-sulfation of specific glucosamine residues in HS is important for HSV-1 binding (73). Therefore, various derivatives of HS add another complex to the selection of SDCs as attachment receptors for different viruses. Whether the ectodomain amino acid residues of SDCs play any role in virus attachment has not been determined. The amino acid residues of ectodomains are divergent between SDC1, SDC2, SDC3, and SDC4, although their C-terminal membrane-anchorage domains are highly conserved. However, human and mouse SDC1 proteins are nearly identical, differing in only three residues. This may explain why both human and mouse SDC1 have the same capacity of mediating HCV attachment to hepatocytes (Fig. 5).

It was suggested that HCV envelope protein E2 mediates the initial binding of HCV to the cell surface through interactions with HSPGs (24). However, substantial evidence derived from our studies and studies by others demonstrates that apoE anchored on the envelope of HCV mediates HCV attachment by binding to the cell surface HSPGs (32–36, 40, 74). apoE- but not HCV E2-specific monoclonal antibody was found to efficiently block HCV attachment (32). Additionally, short peptides derived from the apoE receptor-binding region could potently block HCV attachment to cells (32, 74). Similarly, mutations at the apoE receptor-binding domain resulting in defects in HCV infectivity were found to inactivate the ability of apoE to bind to heparin as shown by an in vitro heparin pulldown assay (32). It is believed that the 2-O-sulfated groups of the iduronic acid monosaccharides or the N- and 6-O-sulfo groups of the glucosamine sulfate monosaccharides are important for apoE binding (75). However, which GAG and GAG modification are required for apoE binding remains unknown. Future studies are warranted to determine the importance of HS side chains in HCV attachment.

Identification of SDC1 as the major receptor for HCV attachment provides a potential molecular target for antiviral drug discovery and development against HCV. The proof of principle for SDC1 as a viable target was first demonstrated by the findings that a recombinant SDC1-Fc fusion protein expressed in CHO cells could potently block HCV infection of T cells, macrophages, and dendritic cells as well as HSV-1 infection (76). Recently, 3-O-sulfonated HS octasaccharides were synthesized and were shown to have antiviral activity inhibiting HSV-1 infection (77). It is anticipated that recombinant SDCs, HS mimetics, and/or small-molecule inhibitors can be developed as prophylactic and/or therapeutic agents for different viruses.

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REFERENCES

1. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362.
2. Lindenbach BD, Rice CM. 2005. Unravelling hepatitis C virus replication from genome to function. Nature 436:933–938.
3. Lindenbach BD, Thiel HJ, Rice CM. 2007. Flaviviridae: the viruses and their replication, 5th ed, vol 1. Lippincott-Raven, Philadelphia, PA.
4. Pringle CR. 1999. Virus taxonomy—1999. The universal system of virus taxonomy, updated to include the new proposals ratified by the International Committee on Taxonomy of Viruses during 1998. Arch. Virol. 144:421–429.
5. Robertson B, Myers G, Howard C, Brettin T, Buhk J, Gaschen B, Gojobori T, Maertens G, Mizokami M, Nairn O, Netesov S, Nishioka K, Shin-i T, Simmonds P, Smith D, Stuyver L, Weiner A. 1998. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. International Committee on Virus Taxonomy. Arch. Virol. 143:2493–2503.
6. Blanchard E, Belouzard S, Goussain L, Wakita T, Dubuisson J, Wychowska C, Roulle Y. 2006. Hepatitis C virus entry depends on clathrin-mediated endocytosis. J. Virol. 80:6964–6972.
7. Bartosch B, Cosset FL. 2006. Cell entry of hepatitis C virus. Virology 348:1–12.
8. Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petraccia R, Weiner AJ, Houghton M, Rosa D, Grandi G, Aigrignani S. 1998. Binding of hepatitis C virus to CD81. Science 282:938–941.
9. Coquereul L, Voisset G, Dubuisson J. 2006. Hepatitis C virus entry: potential receptors and their biological functions. J. Gen. Virol. 87:1075–1084.
10. Agnellu V, Abel G, Elbashar M, Knight GB, Zhang QX. 1999. Hepatitis C virus and other flavivirus viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. U. S. A. 96:12766–12771.
11. Monazahian M, Bohme I, Bonk S, Koch A, Scholz C, Grethe S, Thomsen R. 1999. Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. J. Med. Virol. 57:223–229.
12. Owen DM, Huang H, Ye J, Gale M, Jr. 2009. Apolipoprotein E on hepatitis C virion facilitates infection through interaction with low-density lipoprotein receptor. Virology 394:99–108.
13. Scarrelli E, Ansuini H, Cerino R, Scarselli E, Agnello V, Abel G, Elfahal M, Knight GB, Zhang QX. 1999. Hepatitis C virus and other flavivirus viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. U. S. A. 96:12766–12771.
14. Weiser AJ, Houghton M, Rosa D, Grandi G, Aigrignani S. 1998. Binding of hepatitis C virus to CD81. Science 282:938–941.
15. Coquereul L, Voisset G, Dubuisson J, Wychowska C, Roulle Y. 2006. Hepatitis C virus entry: potential receptors and their biological functions. J. Gen. Virol. 87:1075–1084.
16. Monazahian M, Bohme I, Bonk S, Koch A, Scholz C, Grethe S, Thomsen R. 1999. Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. J. Med. Virol. 57:223–229.
2007. Claudin-6 and claudin-9 function as additional coreceptors for hepatitis C virus. J. Virol. 81:12465–12471.

18. Liu S, Yang W, Shen L, Turner JR, Coyne CB, Wang T. 2009. Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. J. Virol. 83:2101–2114.

19. Ploss A, Evans MJ, Gay singskaya VA, Panis M, You H, de Jong YP, Rice CM. 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature 457:882–886.

20. Cormier EG, Durso RJ, Tsamis F, Boussembre L, Manix C, Olson WC, Gardner JP, Dragic T. 2004. L-SIGN (CD209L) and DC-SIGN (CD209) mediate transfection of liver cells by hepatitis C virus. Proc. Natl. Acad. Sci. U. S. A. 101:14067–14072.

21. Lozach PY, Amara A, Bartosch B, Virelizier JL, Arenzana-Seisdedos F, Allmeyer R. 2004. C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus particle-like particles. J. Biol. Chem. 279:32035–32045.

22. Barth H, Scharer C, Adah MI, Zhang F, Linhardt RJ, Toyoda H, Kinoshita-Toyoda A, Toida T, Van Kuppevelt TH, Depla E, Von Weizsacker F, Blum HE, Baumert TF. 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J. Biol. Chem. 278:20358–20366.

23. Koutsoudakis G, Kaul A, Steinmann E, Kallis S, Lohmann V, Pietrzyk M, Pohlmann S. 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J. Virol. 80:5308–5320.

24. Morikawa K, Zhao Z, Date T, Miyamoto M, Murayama A, Akazawa D, Tanabe J, Sone S, Wakita T. 2007. The roles of CD81 and glycosaminoglycans in the adsorption and uptake of infectious HCV particles. J. Virol. 81:714–723.

25. Saunier B, Triayani M, Ulilainich I, Maruvada P, Yen P, Kohn LD. 2003. Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes. J. Virol. 77:546–559.

26. Luberger J, Zeisel MB, Xiao F, Thummann C, Fofana I, Zona L, Davis C, Mee CJ, Turek M, Gorke S, Royer C, Fischer B, Zahid MN, Lavillette D, Fresquet J, Cosset FL, Rothenberg SM, Pietschmann T, Patel AH, Pessanha M, Dangel M, Raffelsberger W, Zhang F, Magueure G, Pfeffer S, Long G, Baumert TF. 2011. EGFR and EphA2 are host factors for hepatitis C virus infection of stem cell-derived hepatocytes. J. Virol. 85:11925–11932.

27. Schwartz RE, Trehan K, Boornot P, Rani M, Krijnse-Locker J, Bartenschlager R. 2004. C-type lectins L-SIGN and DC-SIGN cap-heparin and cell-surface glycosaminoglycans on human keratinocytes. J. Biol. Chem. 279:10524–10531.

28. Lu S, Kuo PJ, Fairley KA. 1999. Human blood group antigens are ligands for the hepatitis C virus envelope glycoprotein E2. J. Biol. Chem. 275:17960–17968.

29. Lu S, Kuo PJ, Fairley KA. 1999. Human blood group antigens are ligands for the hepatitis C virus envelope glycoprotein E2. J. Biol. Chem. 275:17960–17968.

30. Gazzaz-Pina P, Dryden KA, Boyd B, Wood MR, Law M, Yeager M, Chisari FV. 2010. Ultrastructural and biophysical characterization of hepatitis C virus particles produced in cell culture. J. Virol. 84:10999–11009.

31. Merz A, Long G, Hiet MS, Brugger B, Chlanda P, Andre P, Wieland F, Krijnse-Locker J, Bartenschlager R. 2010. Biochemical and morphological properties of hepatitis C virus particles and determination of their lipidome. J. Biol. Chem. 285:3018–3023.

32. Benda WJ, Krieger SE, Dimitrova M, Zeisel MB, Parrot N, Mubepur J, Hildt E, Luo G, Maclachlan J, Baumert TF, Schuster C. 2010. Apolipoprotein E interacts with hepatitis C virus nonstructural protein 5A and determines assembly of infectious particles. Hepatology 51:43–53.

33. Cun W, Jiang J, Luo G. 2010. The C-terminal alpha-helix domain of apolipoprotein E is required for interaction with nonstructural protein 5A and assembly of hepatitis C virus. J. Virol. 84:11352–11361.

34. Da Costa D, Turek M, Felmlee DJ, Girardi E, Pfeffer S, Long G, Bartenschlager R, Zeisel MB, Baumert TF. 2012. Reconstitution of the entire hepatitis C virus life cycle in nonhepatic cells. J. Virol. 86:11919–11925.

35. Yoneyama K, Bernfield M. 1994. Core protein structure and sequence determine the site and presence of heparan sulfate and chondroitin sulfate on Sindbis virus. J. Biol. Chem. 269:13204–13209.

36. Carlsson P, Presto J, Spillmann D, Lindahl U, Kjellen L. 2008. Heparin/heparan sulfate biosynthesis: processive formation of N-sulfated domains. J. Biol. Chem. 283:20008–20014.

37. Xu W, Robotham JM, Lee D, Dalton S, Kneteman NM, Gilbert DM, Tang H. 2012. Productive hepatitis C virus infection of stem cell-derived hepatocytes reveals a critical transition to viral permissiveness during differentiation. PLoS Pathog. 8:e1002617. doi:10.1371/journal.ppat.1002617.

38. Cai Z, Zhang C, Chang KS, Jiang J, Ahn BC, Wakita T, Liang TJ, Luo G. 2005. Robust production of infectious hepatitis C virus (HCV) from stably HCV cDNA-transfected human hepatoma cells. J. Virol. 79:13963–13973.

39. El-Hage N, Luo G. 2003. Replication of hepatitis C virus RNA occurs in a membrane-bound replication complex containing nonstructural viral proteins and RNA. J. Virol. 77:2759–2770.

40. Schwartz RE, Trehan K, Andrus L, Sheahan TP, Ploss A, Duncan SA, Rice CM, Bhattachrya SN. 2012. Modeling hepatitis C virus infection using human induced pluripotent stem cells. Proc. Natl. Acad. Sci. U. S. A. 109:2544–2548.

41. Byrnes AP, Griffin DE. 1998. Binding of Sindbis virus to cell surface heparan sulfate. J. Virol. 72:7349–7356.

42. Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ. 2003. Apolipoprotein E interacts with hepatitis C virus nonstructural protein 5A and determines assembly of infectious particles. J. Virol. 85:1074–1083.

43. Joyce JG, Tung JS, Przybyciek CT, Cook JC, Lehman ED, Sands JA, Jansen KU, Keller PM. 1999. The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. J. Biol. Chem. 274:5810–5822.

44. Korn A, Schmidt K, Leher C, Muller OJ, Wobus CE, Bettinger K, Von der Lieth CW, King JA, Kleinenschmidt JA. 2003. Identification of a heparin-binding motif on adenovirus-associated virus type 2 capsids. J. Virol. 77:11072–11081.

45. Krustat T, Strecket HJ. 1997. Heparin-dependent attachment of respiratory syncytial virus (RSV) to host cells. Arch. Virol. 142:1247–1254.

46. Olshiro Y, Murakami T, Matsuda K, Nishikawa K, Yoshida K, Shi et al.
Yamamoto N. 1996. Role of cell surface glycosaminoglycans of human T cells in human immunodeficiency virus type-1 (HIV-1) infection. Microbiol. Immunol. 40:827–835.

58. Reference deleted.

59. Selinka HC, Giroglou T, Sapp M. 2002. Analysis of the infectious entry pathway of human papillomavirus type 33 pseudoviruses. Virology 299:279–287.

60. Shieh MT, WuDunn D, Montgomery RJ, Esko JD, Spear PG. 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J. Cell Biol. 116:1273–1281.

61. Spear PG, Shieh MT, Herold BC, WuDunn D, Koshy TI. 1992. Heparan sulfate glycosaminoglycans as primary cell surface receptors for herpes simplex virus. Adv. Exp. Med. Biol. 313:341–353.

62. Summerford C, Samulski RJ. 1998. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J. Virol. 72:1438–1445.

63. Trybala E, Bergstrom T, Spillmann D, Svennerholm B, Flynn SJ, Ryan P. 1998. Interaction between pseudorabies virus and heparin/heparan sulfate. Pseudorabies virus mutants differ in their interaction with heparin/heparan sulfate when altered for specific glycoprotein C heparin-binding domain. J. Biol. Chem. 273:5047–5052.

64. WuDunn D, Spear PG. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. 63:52–58.

65. Dorner M, Horwitz JA, Robbins JB, Barry WT, Feng Q, Mu K, Jones CT, Schoggins JW, Catanese MT, Burton DR, Law M, Rice CW, Ploss A. 2011. A genetically humanized mouse model for hepatitis C virus infection. Nature 474:208–211.

66. Okamoto K, Kinoshita H, Parquet MC, Raekiansyah M, Kimura D, Yui K, Islam MA, Hasebe F, Morita K. 2012. Dengue virus strain DEN2 16681 utilizes a specific glycochain of syndecan-2 proteoglycan as a receptor. J. Gen. Virol. 93:761–770.

67. de Witte L, Bobardt M, Chatterji U, Degeest G, David G, Geijtenbeek TB, Gallay P. 2007. Syndecan-3 is a dendritic cell-specific attachment receptor for HIV-1. Proc. Natl. Acad. Sci. U. S. A. 104:19464–19469.

68. Bobardt MD, Saphire AC, Hung HC, Yu X, Van der Schueren B, Zhang Z, David G, Gallay PA. 2003. Syndecan captures, protects, and transmits HIV to T lymphocytes. Immunity 18:27–39.

69. Shafti-Keramat S, Handisurya A, Kriehuber E, Meneguzzi G, Slupetzky K, Kirnbauer R. 2003. Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses. J. Virol. 77:13125–13135.

70. Langford JK, Stanley MJ, Cao D, Sanderson RD. 1998. Multiple heparan sulfate chains are required for optimal syndecan-1 function. J. Biol. Chem. 273:29965–29971.

71. Hallak LK, Kwlas SA, Peeples ME. 2007. Interaction between respiratory syncytial virus and glycosaminoglycans, including heparan sulfate. Methods Mol. Biol. 379:15–34.

72. Gotte M, Spillmann D, Yip GW, Versteeg E, Echtermeyer FG, van Kuppevelt TH, Kiesel L. 2008. Changes in heparan sulfate are associated with delayed wound repair, altered cell migration, adhesion and contractility in the galactosyltransferase I (beta4GalT-7) deficient form of Ehlers-Danlos syndrome. Hum. Mol. Genet. 17:996–1009.

73. Shukla D, Liu J, Blaiklock P, Shworak NW, Bai X, Esko JD, Cohen GH, Eisenberg RJ, Rosenberg RD, Spear PG. 1999. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell 99:13–22.

74. Liu S, McCormick KD, Zhao W, Zhao T, Fan D, Wang T. 2012. Human apolipoprotein E peptides inhibit hepatitis C virus entry by blocking virus binding. Hepatology 56:484–491.

75. Libeu CP, Lund-Katz S, Phillips MC, Wehli S, Hernaiz MJ, Capila I, Linhardt RJ, Raffai RL, Newhouse YM, Zhou F, Weisgraber KH. 2001. New insights into the heparan sulfate proteoglycan-binding activity of apolipoprotein E. J. Biol. Chem. 276:39138–39144.

76. Bobardt MD, Chatterji U, Schaffer L, de Witte L, Gallay PA. 2010. Syndecan-Fc hybrid molecule as a potent in vitro microbicidal anti-HIV-1 agent. Antimicrob. Agents Chemother. 54:2753–2766.

77. Hu YP, Lin SY, Huang CY, Zulueta MM, Liu YJ, Chang W, Hung SC. 2011. Synthesis of 3-O-sulfonated heparan sulfate octasaccharides that inhibit the herpes simplex virus type 1 host-cell interaction. Nat. Chem. 3:557–563.

78. Jiang J, Luo G. 2012. Cell culture adaptive mutations promote viral protein–protein interactions and morphogenesis of infectious hepatitis C virus. J. Virol. 86:8987–8997.