Identification of transferrin receptor 1 as a hepatitis C virus entry factor

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Hepatitis C virus (HCV) is a liver tropic pathogen that affects \textasciitilde170 million people worldwide and causes liver pathologies including fibrosis, cirrhosis, steatosis, iron overload, and hepatocellular carcinoma. As part of a project initially directed at understanding how HCV may disrupt cellular iron homeostasis, we found that HCV alters expression of the iron uptake receptor transferrin receptor 1 (TfR1). After further investigation, we found that TfR1 mediates HCV entry. Specifically, functional studies showed that TfR1 knockdown and antibody blocking inhibit HCV cell culture (HCVcc) infection. Blocking cell surface TfR1 also inhibited HCV pseudoparticle (HCVpp) infection, demonstrating that TfR1 acts at the level of HCV glycoprotein-dependent entry. Likewise, a TfR1 small-molecule inhibitor that causes internalization of surface TfR1 resulted in a decrease in HCVcc and HCVpp infection. In kinetic studies, TfR1 antibody blocking lost its inhibitory activity after anti-CD81 blocking, suggesting that TfR1 acts during HCV entry at a postbinding step after CD81. In contrast, viral spread assays indicated that HCV cell-to-cell spread is less dependent on TfR1. Interestingly, silencing of the TfR1 trafficking protein, a TfR-1 specific adaptor protein required for TfR1 internalization, also inhibited HCVcc infection. On the basis of these results, we conclude that TfR1 plays a role in HCV infection at the level of glycoprotein-mediated entry, acts after CD81, and possibly is involved in HCV particle internalization.

Interestingly, TfR1 has been identified as an entry receptor for several viruses, including the New World arenaviruses, Machupo virus (MACV) and Junin virus, mouse mammary tumor virus (MMTV), canine parvovirus, and feline panleukopenia virus (10–12). HCV entry is a multistep process that uses multiple host molecules. Glycosaminoglycans (13, 14), liver/lymph node-specific intercellular adhesion molecule 3-grabbing integrin (15, 16), and the low-density lipoprotein receptor (LDL-R) (17, 18) are thought to facilitate initial attachment, followed by interactions with scavenger receptor class B type 1 (SRBI) (19, 20), the tetraspanin CD81 (21, 22), two tight junction proteins [claudin 1 (CLDN1) (23) and occludin (OCLN) (24)], and the cholesterol uptake receptor Niemann-Pick C1-Like 1 (NPC1L1) (25). In addition, receptor tyrosine kinases epidermal growth factor receptor and ephrin receptor A2 have been identified as HCV entry cofactors (26).

Because TfR1 expression was altered by HCV infection, we investigated whether TfR1 is involved in HCV infection, using siRNA knockdown, antibody blocking, and small-molecule targeting of TfR1. After observing the inhibition of HCV cell culture (HCVcc) infection initiation in response to these treatments, we assayed whether TfR1 is required for envelope (E1/E2) glycoprotein-dependent HCV pseudoparticle (pp) entry and found that similar to the TfR1-dependent MACVpp, blocking TfR1 prevented HCVpp entry. Further functional analysis suggests that TfR1 interacts with the viral particle at a postbinding step subsequent to the interaction of CD81 and may be involved in HCV internalization.

Results

TfR1 is Down-Regulated During HCV Infection. Because HCV patients develop hepatic iron overload (4–6), we initially were interested in using the HCVcc infection system to determine whether HCV infection of nongrowing Huh7 cell cultures results in alterations in host iron metabolism genes. Therefore, nongrowing Huh7 cells were prepared as previously described (27, 28) and then mock-infected or infected with HCVcc at a multiplicity of infection (MOI) of 0.5. Expression of different iron genes was monitored over time. Of the genes analyzed, the most significant and consistent change observed was in TfR1 mRNA levels, which were reduced by 10 h postinfection (p.i.) (Fig. 1A). Because TfR1 is regulated posttranscriptionally, we examined TfR1 protein levels in mock- and HCVcc-infected Huh7 cells by Western blot (Fig. 1B). Infection was confirmed by detection of the viral nonstructural (NS)3 protein. Although the level of β-actin was similar in infected and mock-infected cells, TfR1 levels were reduced in HCV-infected cells by 5 d p.i. and remained down until the end of the experiment at day 14 p.i. (Fig. 1B). To

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TfR1 knockdown cells relative to controls when assessed 4 and 5 days posttransfection by RT-qPCR, indicating that TfR1 knockdown had not affected HCV replication in this constitutive HCV replication system (Fig. 2D).

Blocking or Down-Regulation of Cell Surface TfR1 Inhibits HCV Infection Initiation. Because TfR1 knockdown appeared to inhibit an early step of HCV infection before replication, we investigated whether blocking surface TfR1 inhibited HCV infection initiation. Initially, we performed blocking experiments in which antibodies were added to cells before or after infection initiation. Huh7 cells were preincubated for 1 hour with irrelevant mouse IgG negative control, anti-CD81 positive control, or anti-TfR1 before inoculation with HCVcc. Alternatively, the same antibodies were added when the viral inoculum was removed at 10 hours postinfection. At 24 and 48 hours postinfection, cellular RNA was harvested and HCV RNA was measured by RT-qPCR. Similar to cells preincubated with antibody to the known HCV entry receptor, CD81, cultures preincubated with anti-TfR1 had less intracellular HCV RNA relative to the isotype control (Fig. 3A). In contrast, HCV RNA levels were comparable to the IgG-treated control when the same antibodies were added to cultures 10 hours postinfection (Fig. 3B). Although the lack of HCV inhibition in cultures treated with antibody 10 hours postinfection indicates that the antibody treatment was not affecting HCV replication, to confirm TfR1 antibody treatment was not initiating signaling that effects HCV replication or other downstream events, we also performed antibody blocking experiments with the stable HCV sgJFH-1 replicon cell line, measuring HCV RNA levels by RT-qPCR at 24 and 48 hours postantibody treatment, and confirmed no effect on HCV replicon levels (Fig. 3C).

To confirm that the reduction in HCV observed after preincubation with TfR1 antibody was specific, we performed analogous experiments using a TfR1 inhibitor, ferristatin, which binds to and causes internalization and degradation of cell surface TfR1.

**TIR1 siRNA Knockdown Inhibits HCVcc Infection.** As an initial experiment to determine whether TfR1 is functionally involved in HCV infection, we assessed the effect of TfR1 knockdown. Huh7 cells were transfected with an irrelevant control siRNA or TfR1-specific siRNA. A greater than 95% decrease in TfR1 mRNA was observed in TfR1 knockdown cells compared with scrambled siRNA control cells 4–8 days posttransfection (Fig. 2A), resulting in down-regulation of TfR1 protein (Fig. S1). Transfected cells were inoculated with JFH-1 HCVcc at 4 days posttransfection at an MOI of 0.05 and HCV infection kinetics were assessed by monitoring intracellular HCV RNA by real-time quantitative PCR (RT-qPCR). Consistent with TfR1 playing a role in some aspect of infection, HCV RNA levels were 30-fold lower in TfR1 knockdown cells (1.8 × 10^{6}) compared with control cells (5.5 × 10^{6}) 2 days posttransfection (Fig. 2B). However, amplification kinetics of the HCV RNA present in the TfR1 knockdown cells at day 2 paralleled that of the HCV RNA in scrambled siRNA transfected control cells between day 2 and 4, suggesting that perhaps inhibition occurred early, with subsequent intracellular viral RNA replication not being affected.

**TIR1 siRNA Knockdown Does Not Affect HCV Replication.** To directly determine whether TfR1 knockdown affects HCV replication, we performed siRNA knockdown, with the same siRNAs mentioned earlier in Huh7 cells stably replicating subgenomic (sg) JFH-1 HCV RNA. TfR1 mRNA levels were reduced by 95% compared with controls by day 4 posttransfection (Fig. 2C); however, steady-state sgJFH-1 RNA levels were not altered in

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**Fig. 1.** TIR1 is down-regulated during HCV infection. Huh7 cultures were mock-infected or infected with HCVcc. (A) TIR1 and GAPDH mRNA was measured at the indicated times by RT-qPCR. TIR1 levels relative to GAPDH are expressed as fold change compared with uninfected cells (n = 8; average ± SD). (B) Western blot of TIR1, NS3, and β-actin protein. (C) Confocal images of TIR1 and E2 in mock- and HCVcc-infected cells day 8 p.i. Fixed cells were stained for TIR1 (red), HCV E2 (green), and Hoechst (blue). (Scale bar = 20 μm.) (D) Flow cytometric analysis of cell surface TIR1 in mock- and HCVcc-infected cells 5 days posttransfection. Cells were stained with mouse anti-TFIR1 followed by a PE-conjugated anti-mouse secondary antibody. Data are representative of at least 3 independent experiments.

**Fig. 2.** TIR1 knockdown inhibits HCVcc infection but not HCV replication. (A) TIR1 mRNA levels in Huh7 cells transfected with control (siCon) or TIR1-specific (siTIR1) siRNA expressed as a percentage of the copies in siCon-transfected cells. (B) At 4 days posttransfection, Huh7 cells were infected with HCVcc at an MOI of 0.05. Intracellular HCV RNA levels were measured at the indicated times postinfection. (C) TIR1 mRNA in sgJFH-1 replicon cells transfected with control or TIR1-specific siRNA. (D) sgJFH-1 HCV RNA levels in cells transfected with control or TIR1-specific siRNA. At the indicated times post-siRNA transfection, cellular RNA was harvested. TIR1 mRNA, HCV RNA, and GAPDH mRNA levels were quantified by RT-qPCR. Average HCV levels normalized to GAPDH are graphed ± SD (n = 2). Significant differences relative to controls (one-way analysis of variance and Tukey’s post hoc test) are denoted as *P < 0.05 or **P < 0.01. Data are representative of at least 3 independent experiments.

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TfR1 Enhances but is Not Absolutely Required for HCV Cell-to-Cell Spread. Although HCV infection is initiated by extracellular viral binding to cell surface receptors, intracellular amplification, HCV can then enter uninfected cells via direct cell-to-cell spread. Hence, as part of the efforts to determine the role of TfR1 plays in HCV cell entry, we assessed whether TfR1 is required for HCV cell-to-cell spread by performing foci spread assays. The first round of HCV infection was initiated by inoculating cultures with 0.01 foci-forming units (FFU)/mL HCVcc. At 20 h p.i., antibodies against CD81, CLDN1, TfR1, or an isotype control antibody were added to the culture medium. In addition, all cultures were incubated with anti-E2 at a concentration that neutralizes the virus and prevents cell-free viral spread. Cells were fixed at 72 h p.i., foci were quantified using Confocal microscopy and counted. The number of HCV-positive cells per foci was counted, and the average across all 3 experiments is shown in Fig. S4).

(29). After initial dosing experiments determined a suitable, nontoxic dose (Fig. S3A), HuH7 cells were pretreated for 1 h with 50 μM ferristatin before HCVcc infection. As a measure of infection, intracellular HCV RNA levels were measured by RT-qPCR and are graphed as average genome copies per microgram cellular RNA normalized to GAPDH mRNA ± SD. The average across all 3 experiments is shown in Fig. S4A. Consistent with a block in HCV infection initiation, there were reduced numbers of foci in cultures pretreated with ferristatin. As expected, there was no decrease in entry of any of the pseudotyped viruses after preincubation with the isotype control antibody, but entry of JFH-1 HCVpp was significantly reduced after preincubation with anti-TfR1 compared to the inhibition observed with anti-CD81 (Fig. S3 and D). We also tested whether blocking TfR1 inhibited HCVpp expressing E1/E2 from different HCV genotypes (GT1a clone H77; GT1b clone Con1). Similar to the GT2a HCV JFpp, H77pp and Con1pp entry was inhibited to a similar extent when either TfR1 or CD81 was blocked (Fig. 3D).

TfR1 Participates in E1/E2-Dependent HCVpp Entry. To determine whether TfR1 acts at the level of E1/E2-mediated entry, we performed analogous TfR1 inhibition experiments using a pp system, which consists of recombinant HIV luciferase reporter particles pseudotyped with the E1 and E2 glycoproteins from the HCV JFH-1 virus or the glycoproteins of control viruses. As previous studies demonstrated that MACV uses TfR1 as an entry receptor, whereas entry of the Old World arenavirus Lymphocytomegalo-meningitis virus (LCMV) is independent of TfR1 (11), particles pseudotyped with the glycoproteins of these viruses were used as positive and negative controls for TfR1-dependence, respectively. In addition, a vesicular stomatitis virus (VSV)pp-negative control was included. HuH7 cells were left untreated or preincubated with a control mouse isotype antibody, anti-CD81, or anti-TfR1 and then inoculated with the different pseudotype viruses. Seventy-two hours postinoculation, cells were fixed and stained with an anti-E2 antibody to quantify the number of HCV-positive cells per foci. The average across all 3 experiments is shown in Fig. 4.

Fig. 3. Anti-TfR1 inhibits HCV infection initiation and E1/E2-dependent HCVPp entry. (A) HuH7 cells were preincubated for 1 h with irrelevant isotype control, anti-CD81, or anti-TfR1 and then inoculated with HCVcc at an MOI of 0.01. (B) HuH7 cells were infected with HCVcc at an MOI of 0.01. At 10 h postinoculation, virus was removed and cultures were maintained in the presence of the respective antibodies. Intracellular HCV RNA levels were measured by RT-qPCR and are graphed as average genome copies per microgram cellular RNA normalized to GAPDH mRNA ± SD (n = 3). (C) HuH7 cells were left untreated or preincubated for 1 h with isotype control IgG, anti-CD81, or anti-TfR1 and then inoculated with JFH-1 HCVpp, MACVpp, LCMVpp, or VSVpp. At 72 h postinfection, cell lysate was collected. Luciferase activity was measured from duplicate samples and is expressed as average relative light units (RLUs) ± SD. (D) HuH7 cells were treated similar to in C and infected with pps displaying E1/E2 from different HCV genotypes. Significant differences relative to controls (one-way analysis of variance and Tukey’s post hoc test) are denoted as *P < 0.05 or **P < 0.01. Data are representative of at least 3 experiments.

Fig. 4. TfR1 enhances but is not absolutely required for HCV cell-to-cell spread. Although HCV infection is initiated by extracellular viral binding to cell surface receptors, intracellular amplification, HCV can then enter uninfected cells via direct cell-to-cell spread. Hence, as part of the efforts to determine the role TfR1 plays in HCV cell entry, we assessed whether TfR1 is required for HCV cell-to-cell spread by performing foci spread assays. The first round of HCV infection was initiated by inoculating cultures with 0.01 foci-forming units (FFU)/mL HCVcc. At 20 h p.i., antibodies against CD81, CLDN1, TfR1, or an isotype control antibody were added to the cell medium. In addition, all cultures were incubated with anti-E2 at a concentration that neutralizes the virus and prevents cell-free viral spread. Cells were fixed at 72 h p.i., foci were detected by staining for HCV viral protein E2, and the number of HCV-positive cells per foci was counted (Fig. 4 and Fig. S4). Under these conditions, in which the cell number increases ~1.5-fold during the assay, a focus containing 4 or more E2-positive cells is taken as evidence of cell-to-cell spread, as a focus containing fewer than 4 cells could result from cell division during the assay. In the experiment shown in Fig. 4, when cultures were left untreated or incubated with the anti-isotype control antibody, more than 80% of HCV foci contained 4 or more E2-positive cells. In contrast, and consistent with 4 or more cells being indicative of HCV cell-to-cell spread, TfR1 blockade resulted in a significant reduction in the number of foci containing 4 or more E2-positive cells. This result is consistent with previous studies demonstrating that TfR1 enhances but is not absolutely required for HCV cell-to-cell spread. As expected, there was no decrease in entry of any of the pseudotyped viruses after preincubation with the isotype control antibody, but entry of JFH-1 HCVpp was significantly reduced after preincubation with anti-TfR1 compared to the inhibition observed with anti-CD81 (Fig. S3 and D).
of HCV spread, we observed 93% of foci containing fewer than 4 E2-positive cells in cultures incubated with anti-CLDN1, as previous reports have shown CLDN1 is required for HCV cell-to-cell spread (30). Although there was a statistically significant reduction of HCV cell-to-cell spread observed in ferritin-treated cultures, greater than 30% of foci observed still contained 4 or more E2-positive cells, suggesting that although TIR1 may enhance HCV cell-to-cell spread, it is not absolutely required. Notably, intermediate levels of HCV cell-to-cell spread were observed in multiple experiments in the presence of ferritin or antibody against TIR1 (Fig. S4).

**TIR1 Acts After CD81 in HCV Entry.** To determine when TIR1 acts during entry relative to other HCV entry factors, we used a previously published antibody time-of-addition strategy (23, 31, 32). The strategy is based on the principle that blocking antibodies lose their inhibitory activity when applied after the targeted protein has already served its function. Thus, cells were inoculated with HCVcc at 4 °C to allow virus binding. Cells were then moved to 37 °C to allow entry to proceed. Antibodies to CD81, TIR1, or isotype control IgG were added to parallel cultures before virus binding or after virus binding at hour intervals after the temperature shift. Exactly as previous groups have observed (31, 32), when normalized to the IgG control at each time, anti-CD81 lost its inhibitory effect by 2 h postbinding. In contrast, addition of anti-TIR1 inhibited HCV by more than 50% until 4 h after the temperature shift, indicating that TIR1 functions in HCV entry at a step after CD81 (Fig. 5A).

**HCV Particle Binds to TIR1.** Because the HCVpp data indicate that TIR1 is involved in E1/E2-mediated particle uptake, we performed binding studies to determine whether the HCV particle binds to TIR1. For this, CHO cells were transfected with expression plasmids encoding human SRBI, CD81, or TIR1. Clones were selected, initially screened by RT-qPCR for high transgene mRNA levels, and then chosen for binding studies based on detectable surface expression of the respective human receptor. Binding experiments were performed by inoculating cell clones with HCVcc at 4 °C for 1 h to allow virus binding. Cells were then washed, and lysis buffer was added to measure viral RNA bound to cell surface by RT-qPCR. Although not a robust assay, analogous to previous reports, we observed a threefold increase in HCVcc binding to CHO cells expressing human SRBI than to parental CHO cells, and this binding was more pronounced than that detected on CHO cells expressing CD81. Likewise, CHO cells expressing TIR1 exhibited greater than a threefold increase in HCVcc binding over background (Fig. 5B).

**Blocking TIR1 Endocytosis Inhibits HCVcc and HCVpp.** Finally, Tosoni et al. identified TTP as an endocytic protein uniquely required for TIR1 endocytosis (9). Therefore, we tested whether inhibiting TIR1 internalization via TTP knockdown affects HCVcc entry. Huh7 cells were transfected with an irrelevant control siRNA targeting GFP or siRNAs specific for TIR1 or TTP. We observed greater than 90% TTP and TIR1 knockdown in cells transfected with the specific siRNAs compared with control (Fig. S5A). Four days posttransfection, cells were inoculated with HCVcc. When intracellular HCV RNA levels were measured by RT-qPCR 24 and 48 h p.i., we observed that HCV levels were reduced in TTP knockdown cells to the same extent as in the TIR1 knockdown cells (Fig. 5C).

Likewise, we tested the effect of TTP knockdown on HCVpp entry. Four days after transfecting cells with control, CD81-, TIR1-, or TTP-specific siRNA target gene expression was reduced (Fig. S5B) and cells were inoculated with HCV JFHpp, MACVpp, or VSVpp. As expected, CD81 and TIR1 knockdown inhibited HCVpp entry but had no effect on VSVpp (Fig. 5D). A decrease in HCVpp also occurred in TTP knockdown cells, suggesting that disrupting TIR1 internalization affected HCVpp entry. Notably, although TIR1 knockdown resulted in a decrease in MACVpp entry, TTP knockdown did not affect MACVpp entry.

**Discussion**

Several cellular proteins have been demonstrated to be involved in HCV entry, suggesting it is a complex, multistep process. In this study, we observed down-regulation of cellular TIR1 mRNA and protein during HCV infection (Fig. 1). After siRNA knockdown or blocking of TIR1, we observed inhibition of HCVcc and HCVpp infection, demonstrating that TIR1 plays a role in E1/E2-dependent HCV entry (Figs. 2 and 3). However, although TIR1 mediates cell-free HCV entry, it does not appear to be absolutely required for HCV cell-to-cell viral spread (Fig. 4). Kinetic analysis indicated that TIR1 acts as a postbinding step after the requirement for CD81 (Fig. 5A). Mechanistic studies suggest that TIR1 may exert its effects via binding to the viral particle (Fig. 5B), possibly mediating TIR1/TTP-dependent endocytosis (Fig. 5C and D).

**Possible Interaction Between HCV and TIR1.** SRB1 and CD81 have both been shown to interact with soluble (s)E2, whereas a direct interaction between the HCV glycoproteins and CLDN1 and OCLN has not been observed (23, 24). Although CD81 has been shown to bind sE2, Evans et al. (23) observed enhanced HCVcc
binding to CHO cells expressing cell surface SRBI compared with both normal CHO cells and CHO cells expressing cell surface CD81, a result consistent with the hypothesis that a previous engagement between the E1/E2 glycoprotein complex and SRBI may be necessary for efficient binding of the viral particle to CD81 (33). In our study, HCVcc binding to CHO cells expressing surface human TfR1 was enhanced consistently with a direct interaction between the viral particle and TfR1. Notably, the relative degree of binding detected suggests that the viral particle can bind TfR1 effectively without the need for previous engagement with another receptor to physically prime the interaction. However, additional studies are needed to confirm and define the nature of the interaction between HCV and TfR1.

Regardless of the viral components involved in the interaction, future mapping studies to determine the functional domain within TfR1 required for HCV entry would be informative and perhaps identify a specific HCV antiviral target. The anti-human TfR1 antibody used in our blocking experiments has been shown to recognize a mouse–human TfR1 chimera containing human residues 187–383, but not a mouse–human TfR1 chimera containing human residues 187–207 or 213–383 (34), suggesting the epitope recognized may be contained within residues 208–212. Hence this apical domain would be a promising place to begin initial mapping studies.

Role of TfR1 in HCV Entry. Several lines of evidence suggest TfR1 may play a late role in HCV entry, perhaps in endocytosis. Similar to HCV, TfR1 is internalized via clathrin-mediated endocytosis. However, the protein TTP has been identified as a cargo-specific protein required for TfR1 internalization. TTP is thought to be TfR1-specific, as it directly binds TfR1 via its SH3 domain and functionally has not been found to be involved in clathrin-mediated uptake of any other cellular receptors tested (e.g., epidermal growth factor receptor and LDL-R) (9). This, combined with the fact that the magnitude and kinetics of HCV inhibition seen after TTP knockdown was virtually identical to the inhibition seen after TfR1 knockdown, suggests that TfR1 endocytosis in particular may be required for HCV infection. Interestingly, although MACV uses hTfR1 for viral entry, it was relatively insensitive to TTP knockdown. This is similar to what has been observed with MMTV, which uses mouse TfR1 to enter cells but has been reported to be TTP-independent (12). Thus, dependence on TTP does seem to be functionally relevant and, to some extent, defines TTP as yet another cellular factor required for HCV entry.

Consistent with the expectation that a molecule involved in virion internalization would act later in the entry process, our data demonstrate that TfR1 does act later than CD81 with kinetics similar to the late-entry factor Niemann-Pick C1-Like 1 (25). Recently, Farquhar et al. (35) reported that CD81 and CLDN1 endocytosis is induced by HCV, with a significant increase in intracellular CD81 and CLDN1 in the presence of infectious HCV particles but not heat-inactivated HCV. Although this was interpreted to suggest that a CD81/CLDN1 complex might be involved in HCV endocytosis, down-regulation of the surface expression of these viral receptors may be independent of viral uptake and, instead, be related to the fact that many viruses down-regulate expression of their receptors postinfection. A role of CD81 in HCV endocytosis would also be inconsistent with the many reports indicating that CD81 is an early HCV entry factor (31). Hence, further mechanistic studies are needed to clarify the roles of all of the different cellular factors involved in HCV entry. In this regard, we plan to use virion labeling techniques such as those used by the Randall group (36) to investigate which host cell entry factors are internalized with the viral particle.

Interplay Between Iron Homeostasis and HCV Infection. In terms of whether changes in cellular iron homeostasis can directly affect cellular permissiveness to HCV infection, it has been shown that intracellular iron levels regulate TfR1 expression; however, because TfR1 does not appear to be essential for HCV cell-to-cell spread, it is likely that changes in TfR1 expression do not influence the spread of previously established HCV infections. Equally intriguing is whether the effects of HCV on TfR1 are responsible for the increased propensity for hepatic iron overload observed in chronically infected patients. Although the characterization of TfR1 as an HCV entry factor reported here does not address this question, it certainly provides a unique line of investigation regarding the link between chronic HCV infection and iron overload.

Meanwhile, we demonstrate here that TfR1 and TTP are required for cell-free HCV entry, expanding our understanding of the complex HCV entry process. Our data suggest that TfR1 binds the HCV virion and may contribute specifically to virion endocytosis. Interestingly, however, TfR1 is not absolutely required for HCV cell-to-cell spread, perhaps providing some insight into the different mechanisms involved in HCV cell-free entry vs. cell-to-cell spread. As such, more extensive studies on the role of TfR1 in HCV entry may contribute to better understanding of the mechanisms involved in virion internalization, cell-to-cell spread, and/or alternative therapeutic strategies.

Materials and Methods

Cells and Reagents. The plasmid containing the HCV genotype 2a JFH-1 genome (pJFH1) and the construct containing the sg JFH-1 replicon clone (pSRG-JFH1) was provided by T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan) (28, 37). Huh7 human hepatoma cells were obtained from F. V. Chisari (The Scripps Research Institute, CA) and grown in Dulbecco modified Eagle’s medium (HyClone) supplemented with 10% (vol/vol) FBS, 10 mM Heps, 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine (DMEM) at 5% CO2. Nondividing Huh7 cells were established in DMEM supplemented with 1% (vol/vol) dimethyl sulfoxide, as previously described (27, 38). Huh7 cells stably transfected and constitutively replicating the sgJFH-1 replicon were maintained in DMEM supplemented with 500 μg/mL geneticin. CHO cells were cultured in Ham’s F-12 medium supplemented with 10% (vol/vol) FBS (HyClone), 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine. CHO-hCD81 and hSRBI clones were maintained in 600 μg/mL geneticin. Anti-human TfR1 monoclonal antibody (clone M-A712) was used for blocking and Western blot and HRP-conjugated rat anti-mouse IgG monoclonal antibody (clone 356) were purchased from BD Pharmingen, and mouse anti-human TfR1 monoclonal antibody (clone 66G10) used for flow cytometry was purchased from Hycult Biotech. Mouse anti-human CD81 monoclonal antibody (clone 1D6) was purchased from AbD Serotec, monoclonal anti-human NS5B (clone 9-2G) was obtained from ViroGen, rabbit anti-human CLDN1 monoclonal antibody was obtained from AbCam, mouse anti-HCV NS5A E910 monoclonal antibody was a gift from Charles Rice (Rockefeller University, New York, NY), HRP-conjugated anti-mouse secondary antibodies were purchased from Pierce, and HRP-conjugated monoclonal anti–p-actin (clone AC-15) was obtained from Sigma. The TfR1 inhibitor, NSC306071, also known as ferritatin, was obtained from the National Cancer Institute.

RNA Interference. A transfection mix consisting of 1 μL RNAlMAX (Invitrogen) and 12 nM TR1 siRNA or TTP siRNAs (Silencer Select siRNAs s24313, s24314, and s24315) purchased from Ambion in OptiMem (Invitrogen) was mixed with suspended cells for 20 min at room temperature before seeding at 5,000 cells per well in 96-well plates. Medium was changed to DMEM after 24 h. At indicated times posttransfection, cultures were mock-inoculated with medium collected from uninfected cells or inoculated with JFH-1 HCVcc at an MOI of 0.05 FFU per cell. Total cellular RNA was extracted in 1x Nucleic Acid Purification Lysis Solution (Applied Biosystems) at the indicated times p.i. for RT-qPCR analysis.

Western Blot. Huh7 cells were infected with JFH-1 HCVcc at an MOI of 1. Cells were harvested in 1.25% (vol/vol) SDS/PAGE X-100 lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 2 mM EDTA) supplemented with protease inhibitor mixture (Roche Applied Science). Thirty-five micrograms of protein were run on 10% (vol/vol) SDS/PAGE and transferred to Hybond nitrocellulose membranes. Membranes were blocked with 5% (wt/vol) nonfat milk followed by...
incubation with primary antibodies to TR1 and/or N53 at a 1:1,000 dilution. Membranes were washed 3 times with 1× TBS containing 0.05% Tween20 (vol/vol) and incubated with HRP-conjugated goat anti-mouse for 30 min. Membranes were also probed with an HRP-conjugated β-actin monoclonal antibody. SuperSignal chemiluminescent substrate was used to detect bound antibody complexes.

**Indirect Immunofluorescence.** Cells in chamber well slides were fixed and stained as previously described (25). Primary antibodies against TR1 and E2 were incubated at a 1:1,000 dilution overnight at 4 °C. Conjugated secondary antibodies, anti-mouse Alexa-555 (TR1), and anti-rabbit Alexa-488 (HCV E2; Molecular Probes) were incubated at a 1:500 dilution for 1 h at room temperature. Nuclei were stained with Hoechst dye. Images were captured via confocal microscopy (63×, Zeiss LSM 510) and analyzed using Zeiss LSM Alpha Imager Browser v. 4.0 software.

**Antibody Inhibition of HCVcc Infection.** Cells were incubated with 25 µg/mL isotype control, CD81, or TR1 antibodies for 1 h before being inoculated with HCVcc at an MOI of 0.01 in the presence of antibodies. At 24 h post-infection, cellular RNA was harvested in 1× Nucleic Acid Purification Lysis Solution (Applied Biosystems), and HCV RNA copies were determined by RT-qPCR. For time-of-antibody addition experiments, antibodies were added 1 h before MOI 0.01 HCVcc inoculation for 1 h at 4 °C or added at the indicated times post-infection. Antibody interactions with cultures were shifted to 37 °C. Thirty hours post-virus binding, cells were lysed with 1× Nucleic Acid Purification Lysis Solution, and HCV RNA levels were measured by RT-qPCR.

**RNA Isolation and RT-qPCR Analysis.** RNA was purified using an ABI Prism 6100 Nucleic Acid PrepStation, as per manufacturer’s instructions. Purified RNA was used to generate cDNA using TaqMan reverse transcription reagents. Gene expression was measured by SYBR green RT-qPCR, using an Applied Biosystems 7300 real-time thermocycler, as previously described (25). HCV copy number was determined relative to a standard curve and normalized to GAPDH. See Table 51 for list of primers.

**HCV Pseudotype Particle Production.** Pseudotyped viruses were produced as previously described (25). Briefly, pseudotyped viruses were generated by cotransfection of DNA vectors encoding the HCV E1/E2, VSV, LCMV, or MACV envelope glycoproteins with an Env-deficient HIV vector carrying a luciferase reporter gene (pNL4-3-Luc–R–E−) into human embryonic kidney cells transformed with the SV40 large T antigen gene producer cells, HEK 293T. Supernatants were collected 48 h posttransfection and filtered through a 0.45-µm pore-size filter (BD Biosciences). Infectivity levels were determined 72 h p.i. by lysing infected cultures in 20 µL lysis reagent to measure luciferase activity (Promega), using a FLUOstar Optima microplate reader (BMG Labtechnologies).

**Binding Assay.** CHO cells and CHO cell lines stably transfected with human 5R1B, human CD81, or human TR1 expression plasmids were seeded in 12-well plates. Cultures were inoculated with 400 µL HCVcc at 4 °C for 1 h and then washed 3 times with 1× PBS to remove nonbound virus. Cell-associated RNA was extracted for RT-qPCR analysis.

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