Hepatitis C Virus Core Protein Blocks Interferon Signaling by Interaction with the STAT1 SH2 Domain

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Emerging data have indicated that hepatitis C virus (HCV) subverts the host antiviral response to ensure its persistence. We previously demonstrated that HCV protein expression suppresses type I interferon (IFN) signaling by leading to the reduction of phosphorylated STAT1 (P-STAT1). We also demonstrated that HCV core protein directly bound to STAT1. However, the detailed mechanisms by which HCV core protein impacts IFN signaling components have not been fully clarified. In this report, we show that the STAT1 interaction domain resides in the N-terminal portion of HCV core (amino acids [aa] 1 to 23). This domain is also required to produce P-STAT1 reduction and inhibit IFN signaling transduction. Conversely, the C-terminal region of STAT1, specifically the SH2 domain (aa 577 to 684), is required for the interaction of HCV core with STAT1. The STAT1 SH2 domain is critical for STAT1 hetero- or homodimerization. We propose a model by which the binding of HCV core to STAT1 results in decreased P-STAT, blocked STAT1 heterodimerization to STAT2, and, therefore, reduced IFN-stimulated gene factor-3 binding to DNA and disrupted IFN-stimulated gene transcription.

Hepatitis C virus (HCV) is an enveloped, single-stranded RNA virus member of the family *Flaviviridae*. Its 9.6-kb genome includes a 5' untranslated region that acts as an internal ribosome entry site (IRES). The IRES directs the translation of a single open reading frame encoding the 3,010-amino-acid polyprotein, which is posttranslationally cleaved by host and virus-encoded protease into mature structural and nonstructural proteins (1, 14). The structural proteins include core, which forms the HCV viral nucleocapsid, and two envelope glycoproteins, E1 and E2 (31). The HCV structural proteins are cleaved and further processed to generate the mature and stable form of p21 HCV core (amino acids 1 to 191) (43). The nonstructural proteins NS2 through NS5 support viral RNA replication. For example, the NS3/4A protease catalyzes the posttranslational processing of nonstructural proteins from the viral polyprotein. NS5B encodes the HCV-encoded RNA-dependent RNA polymerase that is critical for HCV RNA replication. The HCV core protein has been found to be primarily located within the membranes of cytoplasmic organelles. The C terminus of HCV core is hydrophobic, while the N-terminal domain of HCV core is basic (33).

HCV infection causes the development of chronic hepatitis and progression to liver cirrhosis and hepatocellular carcinoma. More than 170 million people are infected with HCV worldwide. Alpha interferon (IFN- α) alone or in combination with ribavirin is the only currently approved treatment for HCV infection (9). Unfortunately, in many genotype 1 HCV-infected patients, HCV is refractory to the action of interferon (15). The precise mechanisms for HCV persistence are still not

fully understood (21); thus, a more detailed understanding of virus-host interactions is critical.

Viral infection stimulates the host type I IFN pathway. Following binding of secreted IFN to the IFN receptor complex, Jak1 and Tyk2 kinases are activated and in turn phosphorylate STAT1 and STAT2. Jak1 association with the STAT1 Src-homology-2 (SH2) domain initiates STAT1 phosphorylation. Phosphorylated STAT1 (P-STAT1), together with phosphorylated STAT2, and interferon regulatory factor 9 form a heterotrimeric complex, IFN-stimulated gene (ISG) factor 3 (ISGF3). ISGF3 then translocates to the nucleus and binds the interferon-stimulated response element (ISRE) to upregulate a large number of ISGs (7, 19, 35). We have previously demonstrated that STAT1 is the critical component of innate antiviral immunity to HCV. For instance, full-length and subgenomic HCV expression were each associated with decreased P-STAT1 levels and blocked IFN signal transduction. HCV core, but not other viral proteins, also bound to STAT1 (10, 23). Therefore, HCV core protein's actions on the Jak-STAT pathway appear to be critical to ensuring the persistence of HCV in the human host (4). HCV core has been implicated in multiple functions. HCV core regulates innate immunity at several levels, including the pathogenesis and regulation of host innate antiviral immunity (39). For example, HCV core has been demonstrated to interact with several host cell signaling pathways, including the Jak-STAT pathway and other innate and adaptive defense pathways (16, 25, 27). The expression of HCV core protein has been shown to reduce STAT1 protein expression in IFN-treated mammalian cells (2). It has also been shown to inhibit interferon-induced nuclear import of P-STAT1 (26) and IFN- α -induced transcription of antiviral genes by decreasing the binding of ISGF3 to the IFN ISRE (12). HCV core protein expression can also induce the expres-

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sion of SOCS3 and inhibit IFN- α -mediated STAT1 activation (5, 17, 20, 29). However, the molecular basis by which HCV core mediates Jak-STAT signaling suppression by blocking P-STAT1 accumulation has yet to be elucidated (2). In order to better understand the role of HCV core in the subversion of the host IFN response, we used an HCV cell-based expression model to examine the specific interaction between HCV core protein expression and STAT1.

MATERIALS AND METHODS

Constructs and antibodies. Full-length HCV core constructs pC191 and pF1-191 (amino acids [aa] 1 to 191); C-terminally truncated HCV core constructs pC152 (aa 1 to 152), pC123 (aa 1 to 123), and pC111 (aa 1 to 111); and the N-terminally truncated core constructs pF24-191 (aa 24 to 191), pF38-191 (aa 38 to 191), and pF58-191 (aa 58 to 191) have been described previously (34) and were the gifts of Tetsuro Suzuki (National Institute of Infectious Diseases, Japan). Plasmid pF1-23 (aa 1 to 23) was constructed in our laboratory in an analogous manner (34). For this construct, plasmid pF1-191 was used as a template for PCR amplification. The PCR fragment was BglII digested and then cloned into the BglII site of the digested pF1-191 pCAGGS vector. The nucleotide sequences of cloned HCV core constructs (pF1-23) were verified by bidirectional sequencing.

The full-length STAT1 construct pSTAT1-Full (aa 1 to 750) and STAT1 point mutant constructs at 428-9 M-Glu, a putative DNA binding site (pST1-428/9 M); 701 M-Tyr, a JAK kinase phosphorylation site (pST1-701 M); 713 M-His, a splice site resulting in STAT1 β formation (pST1-713 M); and 727 M-Ser, a mitogen-activated protein kinase (MAPK) phosphorylation site (pST1-727 M), have been described previously (38). The plasmids encoding full-length STAT1 construct pST1-Full and C-terminally truncated STAT1 constructs pST1-C577 (Flag STAT1, aa 1 to 577) and pST1-C684 (Flag STAT1, aa 1 to 684) were previously described (40) and were gifts of Nobuhiro Fujii (Sapporo Medical University, Japan).

The antibodies used in this study included mouse monoclonal anti-HCV core immunoglobulin G1 (IgG1) (which recognizes an epitope between amino acid residues 21 and 40 of HCV core protein, clone C7-50) (Affinity BioReagents, Inc., Golden, CO), mouse anti-HCV core antigen (aa 70 to 90 HCV) (U.S. Biological, Swampscott, MA), rabbit anti-STAT1 IgG, rabbit anti-phospho-STAT1 (Tyr 701) IgG, rabbit anti-phospho-STAT1 (Ser 727) IgG (Cell Signaling Technology, Inc., Beverly, MA), mouse anti-STAT1 (N terminus) (BD Biosciences Pharmingen, San Diego, CA), and mouse anti-Flag M2 IgG1 (Sigma, St. Louis, MO).

Cell cultures and transfection. Huh7 cells (human hepatocellular carcinoma cells) and U3A cells (STAT1-deficient 2fTGH human fibrosarcoma cells) (11, 23, 32) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum medium.

Expression constructs corresponding to full-length, C-terminally truncated, or N-terminally truncated HCV core, together with the parental empty vector pCAG, were also transiently transfected into Huh7 cells or U3A cells. The full-length STAT1 construct, point mutant STAT1 constructs, and C-terminal-truncated STAT1 constructs were also cotransfected into U3A cells. All transient transfections of expression plasmids were performed in six-well plates using Lipofectamine reagent (Invitrogen, Carlsbad, CA) as described previously (23). For IFN treatment, IFN- α (interferon alpha-2b; Schering Co., Kenilworth, NJ) was added to a final concentration of 1,000 IU/ml immediately after the transfection. ISRE-mediated IFN signaling was monitored by a dual-luciferase reporter assay system after HCV core constructs were cotransfected with plasmids pISRE-luc (expressing firefly luciferase) and pRL-TK (expressing *Renilla* luciferase). Samples for the luciferase assay, protein lysates, and RNA quantification were harvested separately at 14 h posttransfection. Protein sample preparation, luciferase reporter gene assay, enzyme-linked immunosorbent assay, and Western blotting were performed as described previously (23). STAT1, P-STAT1, and HCV protein expression were investigated with immunoprecipitation and Western blotting.

RT-PCR and real-time PCR. STAT1 mRNA levels were monitored by reverse transcription-PCR (RT-PCR) and real-time PCR. Total cellular RNA was harvested using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Human STAT1 (GenBank accession no. BC002704) was amplified using the following primers: sense primer GAG AGC TGT CTA GGT TAA CGT TCG C and antisense primer AGT CAA GCT GCT GAA GTT CGT ACC. STAT1 mRNA was semiquantified by RT-PCR by using the GeneAmp

RNA PCR kit (Applied Biosystems, Branchburg, NJ) to amplify a 184-bp fragment PCR product. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank accession no. BC026907; GAPDH sense primer ACA GTC CAT GCC ATC ACT GCC and GAPDH antisense primer GCC TGC TTC ACC ACC TTC TTG) was used to amplify a 266-bp fragment PCR product as a control for basal RNA levels. STAT1 mRNA was also quantified by real-time PCR using the LightCycler instrument and technology (Roche Applied Science, Indianapolis, IN) and SYBR green I dye for detection as described previously by Castet et al. (6) with modifications.

Luciferase reporter gene assay. Gene expression was monitored by the Promega dual-luciferase reporter assay system. To monitor IFN signaling directed by the ISRE, the plasmids pISRE-luc (500 ng/well), expressing firefly luciferase, and pRL-TK (50 ng/well), expressing *Renilla* luciferase, were cotransfected with appropriate plasmids (2 μ g/well) and relative luciferase activity was assessed. Relative luciferase activity was calculated by dividing the firefly luciferase value by the *Renilla* luciferase value.

Protein sample preparation. At the time of harvest, cells were washed twice with phosphate-buffered saline, and whole-cell protein samples were extracted with radioimmunoprecipitation assay buffer (0.5% Nonidet P-40, 10 mM Tris, pH 7.4, 150 mM NaCl, 1% sodium dodecyl sulfate [SDS]). Whole-cell lysates were sonicated, boiled at 95°C for 5 min, and chilled on ice for 10 min.

Western blotting. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) with NuPAGE Novex Bis-Tris precast 4 to 12% gradient gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride (PVDF) membranes. The primary antibodies used for Western blots were as follows: rabbit anti-STAT1 (1:2,000), rabbit anti-P-STAT1 (Tyr701) (1:2,000) (Cell Signaling Technology, Inc., Beverly, MA), mouse anti-STAT1 (N terminus) (1:1,000) (BD Biosciences Pharmingen, San Diego, CA), mouse anti-HCV core (1:2,000) (Affinity BioReagents, Inc., Golden, CO), mouse anti-HCV core (aa 70 to 90 HCV) (1:2,000) (U. S. Biological, Swampscott, MA), and mouse anti-Flag M2 IgG1 (1:2,000) (Sigma, St. Louis, MO). The secondary antibody was horseradish peroxidase-conjugated ECL donkey anti-rabbit IgG (1:2,000) or horseradish peroxidase-conjugated ECL sheep anti-mouse IgG (1:4,000) (Amersham Biosciences, Piscataway, NJ). Chemiluminescent signals were detected by using the ECL Western blotting detection kit (Amersham Biosciences, Piscataway, NJ).

Immunoprecipitation analysis. Immunoprecipitation analysis with anti-STAT1 monoclonal antibody (MAb) (Cell Signaling Technology, Inc., Beverly, MA), anti-HCV core IgG1 antibody (Affinity BioReagents, Inc., Golden, CO), or mouse anti-Flag M2 IgG1 antibody (Sigma, St. Louis, MO) was performed using the Roche immunoprecipitation kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Anti-HCV core antibodies, anti-STAT1 antibodies, and mouse anti-Flag M2 antibodies were used as primary antibodies for Western blots, while the previously described secondary antibodies were used for detection.

RESULTS

Wild-type HCV core produces a loss of P-STAT1 and suppresses ISRE-mediated IFN signaling. We previously observed that the expression of full-length HCV polyprotein resulted in a reduction of P-STAT1. To address whether HCV core alone produces the inhibition of IFN signaling and reduction of P-STAT1, we cotransfected wild-type HCV core (pC191) into Huh7 cells (Fig. 1A to C). The transfection of full-length HCV core inhibited IFN signaling to a level comparable to that of full-length HCV polyprotein (a 2.5-fold reduction) (Fig. 1B, lane 2). Similarly, full-length HCV core was associated with significantly decreased P-STAT1 accumulation (Fig. 1C, lanes 2 and 7). These data confirm that HCV core alone suffices to inhibit IFN signal transduction and reduce P-STAT1 levels. The finding of significant inhibition of P-STAT1 raises the possibility of paracrine effects of core on P-STAT1 levels in untransfected cells.

STAT1 mRNA levels are not affected by HCV transfection. To address whether the observed effect of HCV core on the reduction of STAT1 levels is transcriptional, we monitored STAT1 mRNA levels by RT-PCR and real-time PCR. HCV core expression had no effect on STAT1 mRNA levels (Fig. 2),

FIG. 1. Full-length HCV core and N-terminal HCV core constructs produce similar levels of P-STAT1 loss and IFN signaling inhibition in Huh7 cells. (A) Map of full-length HCV core and C-terminal truncated or N-terminally truncated HCV core constructs. pC191 is a full-length HCV core construct (aa 1 to 191). pC152 (aa 1 to 152), pC123 (aa 1 to 123), and pC111 (aa 1 to 111) are C-terminally truncated HCV core constructs. pF24-191 (aa 24 to 191), pF38-191 (aa 58 to 191), and pF58-191 (aa 58 to 191) are N-terminally truncated HCV core constructs. (B) Expression of full-length and C-terminally truncated HCV core suppresses IFN signaling in Huh7 cells. The full-length HCV core construct pC191; the C-terminally truncated HCV core construct pC152, pC123, or pC111; the N-terminally truncated HCV core construct pF24-191, pF38-191, or pF58-191; or the empty vector pCAG was cotransfected with the plasmids pISRE-luc (expressing firefly luciferase) and pRL-TK (expressing *Renilla* luciferase) into Huh7 cells. ISRE-mediated IFN signaling was monitored by a dual-luciferase reporter assay system at 12 h posttransfection. Compared to values for empty vector (pCAG)-transfected cells, transfection of full-length HCV core construct pC191 decreased relative luciferase activity by about 67%, while transfections of the C-terminal truncated mutants also produced the inhibition of IFN signaling to comparable degrees (transfection of pC152, pC123, and pC111 reduced relative luciferase activity by about 57.2, 57, and 47%, respectively). In contrast, after transfection with N-terminally truncated HCV core constructs (pF24-191, pF38-191, and pF58-191), mutants lost the ability to suppress IFN signaling. Error bars indicate standard deviations. (C) Anti-STAT1 and anti-P-STAT1 Western blots. The full-length HCV core construct

FIG. 2. HCV core expression does not downregulate STAT1 gene expression. (A) Reverse transcription-PCR. (B) Real-time PCR. To monitor whether HCV core has an effect on STAT1 gene expression, STAT1 mRNA levels were measured in Huh7 cells or Huh7 cells transfected with the full-length HCV core construct pC191 or the parental empty vector pCAG. GAPDH gene levels were measured as a relative quantitative control. HCV expression had no effect on STAT1 mRNA levels. Error bars indicate standard deviations.

pC191; the C-terminally truncated HCV core construct pC152, pC123, or pC111; the N-terminally truncated HCV core construct pF24-191, pF38-191, or pF58-191; or empty vector pCAG was transfected into Huh7 cells treated with or without IFN- α (1,000 IU/ml). The protein lysates were harvested 12 h posttransfection for anti-STAT1 or Western blotting of anti-P-STAT1. Blotting of anti-actin was done as a protein quantitative control. Transfections with full-length HCV core and C-terminally truncated HCV core protein constructs reduced P-STAT1. In contrast, transfection with N-terminal truncated HCV core protein failed to reduce P-STAT1 accumulation. Panel i shows the C-terminally truncated HCV core. Lanes: 1, pCAG (empty) vector; 2, pC191; 3, pC152; 4, pC123; 5, pC111. Panel ii shows the N-terminally truncated HCV core. Lanes: 6, pCAG (empty) vector; 7, pC191; 8, pF24-191; 9, pF38-191; 10, pF58-191.

indicating that HCV's effect on STAT1 protein levels occurs posttranscriptionally.

N-terminal HCV core sequences are essential for its effects on IFN signaling inhibition and P-STAT1 reduction. To determine which domain of HCV core is responsible for the inhibition of IFN signaling and reduction of P-STAT1 levels, full-length HCV core (pC191) and C-terminally truncated or N-terminally truncated HCV core constructs were transfected into Huh7 cells (Fig. 1A to C). Transfections of full-length HCV core and C-terminally truncated HCV core produced comparable degrees of IFN signaling inhibition. In contrast, the transfection of N-terminally truncated HCV core did not inhibit IFN signaling (Fig. 1A to C). By Western blotting of P-STAT1, we found that transfections of full-length HCV core and the C-terminal deletion mutant were each associated with an impaired accumulation of P-STAT1 (Fig. 1C, panel i) but that the transfection of N-terminally truncated HCV core constructs had no effect on P-STAT1 levels (Fig. 1C, panel ii). These observations indicate that N-terminal HCV core sequences are critical for the inhibition of IFN signaling and reduction of P-STAT1.

STAT1 interacts with full-length and C-terminally truncated HCV. To determine which domain of HCV core is responsible for physical association with STAT1, we performed the immunoprecipitation of STAT1, followed by Western blotting of HCV core in cells transfected with full-length, C-terminal-truncated, or N-terminally truncated HCV core constructs. STAT1 binds to full-length HCV core and C-terminally truncated core in the presence of the proteasome inhibitor MG132 (Fig. 3A). In contrast, STAT1 did not bind to N-terminal-truncated HCV core in Huh7 cells treated with MG132 (Fig. 3B). The demonstration of a physical interaction between STAT1 and N-terminal HCV core implies that the HCV core-STAT1 interaction domain resides in the N-terminal portion of HCV core (amino acids 1 to 23). To further confirm whether the N terminus of HCV core interacts with STAT1, we performed HCV core reciprocal immunoprecipitation assays, followed by STAT1 Western blotting in Huh7 cells (with or without MG132) transfected with C- or N-terminally truncated HCV core. We found that C-terminally truncated HCV core bound to STAT1 in Huh7 cells (Fig. 4A). In contrast, N-terminally truncated HCV core did not bind to STAT1 (Fig. 4B). The interaction between HCV core and STAT1 is preserved in the absence of MG132, suggesting that this is a bona fide *in vivo* interaction. These results further suggest that the region comprising amino acid residues 1 to 23 of N-terminal HCV core is critical for STAT1 binding. To verify that the N-terminal HCV core domain (aa 1 to 23) inhibits IFN signaling and physically associates with STAT1, we transfected HCV core N-terminal construct pF1-23 into Huh7 cells. We found that full-length HCV core as well as N-terminal HCV core (amino acids 1 to 23) produced an inhibition of IFN signaling and reduction of P-STAT1 to comparable degrees (Fig. 5A, B, and C). The finding that HCV core protein directly interacts with STAT1, coupled with evidence that its expression is associated with the reduction of P-STAT1 (Fig. 5B and C), implies that the binding of the N-terminal HCV core domain (aa 1 to 23) to STAT1 is responsible for the inhibition of type I IFN signaling and reduction of P-STAT1.

FIG. 3. STAT1 binds to full-length and C-terminally truncated HCV core in Huh7 cells treated with MG132 in an immunoprecipitation (IP) analysis of STAT1 and by Western blotting of anti-HCV core. Protein lysates from Huh7 cells transfected with the full-length HCV core construct pC191, the C-terminally truncated core constructs (pC152, pC123, and pC111), or N-terminally truncated HCV core constructs (pF24-191, pF38-191, and pF58-191) (with the introduction of proteasome inhibitor MG132 [10 μ M for 12 h]) and STAT1-immunoprecipitated lysates were separated by SDS-PAGE and transferred to PVDF membranes. Anti-HCV core MAb was used for Western blotting. (A) Transfections of C-terminally truncated HCV core constructs. Full-length HCV core protein and C-terminal truncated HCV core bound to STAT1. Lanes: 1, pCAG; 2, pC191; 3, pC152; 4, pC123; 5, pC111; 6, pCAG; 7, pC191; 8, pC152; 9, pC123; 10, pC111. (B) Transfections of N-terminally truncated HCV core constructs. N-terminally truncated HCV core protein did not bind to STAT1. Lanes: 1, pCAG; 2, pF1-191; 3, pF24-191; 4, pF38-191; 5, pF58-191; 6, pCAG; 7, pF1-191; 8, pF24-191; 9, pF38-191; 10, pF58-191. +, with; -, without.

HCV core interacts with the STAT1 SH2 domain. In a previous study, we demonstrated that transfected STAT1 in STAT1-deficient U3A cells restored ISRE-mediated IFN signaling to the level of wild-type 2fTGH cells (23). To determine the HCV core interaction domain of STAT1, we performed the immunoprecipitation of HCV core, followed by Western blotting of STAT1 in U3A cells cotransfected with HCV core pC191 and either full-length STAT1 (pST1-Full [aa 1 to 750]) or C-terminally truncated STAT1 construct pST1-C684 (aa 1 to 684) or pST1-C577 (aa 1 to 577) (Fig. 6A) (40). HCV core bound to pST1-C684 but not to pST1-C577 (Fig. 6C). As expected, the transfection of wild-type STAT1 rescues IFN signaling in IFN-treated U3A cells (Fig. 6B). In contrast, the transfection of C-terminally truncated STAT1 (pST1-C684 and pST1-C577) provides only partial or no restoration, respectively, of IFN signaling in U3A cells (Fig. 6B). The cotransfection of HCV full-length core specifically reduced STAT1-res-

FIG. 4. N-terminal HCV core is essential for interaction with STAT1, as shown by the immunoprecipitation of HCV core and Western blotting of anti-STAT1. Protein lysates from Huh7 cells transfected with the full-length HCV core construct pC191, the C-terminally truncated core constructs (pC152, pC123, and pC111), or the N-terminally truncated HCV core construct (pF24-191, pF38-191, and pF58-191) (with or without the introduction of proteasome inhibitor MG132 [10 μ M for 12 h]) and HCV core-immunoprecipitated lysates were separated by SDS-PAGE and transferred to PVDF membranes. Anti-STAT1 MAb was used for Western blotting. (A) C-terminally truncated HCV core protein bound to STAT1. Lanes: 1, pCAG; 2, pC191; 3, pC152; 4, pC123; 5, pC111; 6, pCAG; 7, pC191; 8, pC152; 9, pC123; 10, pC111. (B) The N-terminally truncated HCV core lost the ability to interact with STAT1. Lanes: 1, pCAG; 2, pF1-191; 3, pF24-191; 4, pF38-191; 5, pF58-191; 6, pCAG; 7, pF1-191; 8, pF24-191; 9, pF38-191; 10, pF58-191. +, with; -, without.

cued IFN signaling in wild-type STAT1-transfected U3A cells and ablated the partial signaling conferred by pST1-C684. These observations further confirm that HCV core suppresses ISRE-mediated IFN signaling (Fig. 6B). These results indicated that STAT1 binding to HCV core mapped to the C-terminal domain of STAT1, specifically to the SH2 domain. In reciprocal experiments, in which the immunoprecipitation of STAT1 was followed by Western blotting of HCV core (Fig. 6D), we confirmed the dependence of STAT1 binding to HCV core on STAT1 amino acids 577 to 684. In IFN signaling assays, transfections of C-terminally truncated STAT1 failed to rescue IFN signaling in U3A cells (Fig. 6B), consistent with the previously reported importance of STAT1 701-Tyr (Fig. 7A), the STAT1 transactivation region (aa 683 to 750), and the SH2 domain in activating IFN- α -induced ISRE-mediated signaling.

HCV core can bind STAT1 signaling point mutants. To investigate whether HCV core binds STAT1 at sites previously demonstrated to be essential for STAT1 signaling, we examined the effect of HCV core transfection in STAT1-deficient U3A cells cotransfected with wild-type STAT1 construct pST1-Full (aa 1 to 750) or point mutant STAT1 constructs pST1-428/9 M (putative DNA binding site), pST1-701 M (JAK kinase phosphorylation site), pST1-713 M (splice site resulting in STAT1 β formation), and pST1-727 M (MAPK phosphorylation site) (Fig. 7A) (11, 38). As expected, point mutant constructs pST1-701 M and pST1-713 M significantly lost their abilities to rescue IFN- α -induced ISRE-mediated signaling in U3A cells (Fig. 7A). HCV core cotransfection further sup-

pressed IFN- α signaling (Fig. 7A) in U3A cells cotransfected with either full-length STAT1 or each of the point mutant STAT1 constructs (Fig. 7A). We confirmed by Western blotting that full-length and point mutant STAT1 constructs were successfully expressed in U3A cells. HCV core expression reduced both wild-type and mutant STAT1 levels (Fig. 7B). To further investigate whether HCV core physically associates with point mutant STAT1, we performed STAT1 immunoprecipitation experiments using lysates from cells cotransfected with HCV core and mutant STAT1 in U3A cells in the presence of MG132, followed by Western blotting with anti-HCV core. We found that HCV core bound to full-length STAT1 and each of the STAT1 point mutants (Fig. 7C), suggesting that the STAT1-HCV core interaction was not disrupted by these point mutations. These data imply that HCV core does not impair signaling by binding to key STAT1 regulatory sites.

DISCUSSION

In this study, we demonstrated that HCV core protein plays an important role in subverting host innate immunity by blocking type I IFN signaling. We further showed that HCV core blocks Jak-STAT signaling by direct physical interaction with STAT1. The HCV core-STAT1 interaction maps to the N-terminal 23 amino acids of HCV core and the SH2 region of STAT1 (aa 578 to 684). These interactions are required for the observed loss of P-STAT1 and impairment of IFN signaling.

FIG. 5. N-terminal HCV core (aa 1 to 23) inhibits IFN signaling and reduces P-STAT1. (A) Full-length HCV core and N-terminal HCV core (aa 1 to 23) expression suppresses IFN signaling in Huh7 cells. The full-length HCV core construct pF1-191, the N-terminal HCV core construct pF1-23, the N-terminally truncated HCV core construct pF24-191, or the empty vector pCAG was cotransfected with plasmids pISRE-luc (expressing firefly luciferase) and pRL-TK (expressing *Renilla* luciferase) into Huh7 cells. ISRE-mediated IFN signaling was monitored by a dual-luciferase reporter assay system at 12 h posttransfection. The transfection of the full-length HCV core construct pF1-191 and the N-terminal HCV core construct pF1-23 produced the inhibition of IFN signaling to comparable degrees. In contrast, the transfection of the N-terminally truncated HCV core construct pF24-191 did not suppress IFN signaling. Error bars indicate standard deviations. (B) N-terminal HCV core (aa 1 to 23) interacts with STAT1 in FLAG immunoprecipitation–anti-STAT1 Western blotting. Protein lysates from Huh7 cells transfected with the full-length HCV core construct pF1-191, the N-terminal HCV core construct pF1-23, the N-terminally truncated HCV core construct pF24-191, or empty vector pCAG and anti-FLAG-immunoprecipitated lysates were separated by SDS-PAGE and transferred to PVDF membranes. Anti-STAT1 Mab was used for Western blotting. Full-length HCV core and N-terminal HCV core (aa 1 to 23) protein bound to HCV core. Lanes: 1, pCAG; 2, pF1-191; 3, pF1-23; 4, pF24-191; 5, pCAG; 6, pF1-191; 7, pF1-23; 8, pF24-191. +, with; –, without. (C) N-terminal HCV core produces a reduction of P-STAT1. Panel i shows anti-FLAG Western blots to confirm the production of HCV core proteins. Panel ii shows anti-P-STAT1 Western blots. The full-length HCV core construct pF1-191 and the N-terminal HCV core construct pF1-23 reduced the accumulation of P-STAT1. Lanes: 1, pCAG; 2, pF1-191; 3, pF1-23; 4, pF24-191. +, with; –, without.

The type I IFN system is the first line of host defense against virus infection. HCV, like other viruses, has evolved a unique strategy to disrupt host IFN-induced innate antiviral responses through the action of its core protein. HCV core has protean actions. It is capable of broadly signaling across multiple transduction pathways that result in pleiotropic effects in suppressing host immunity and enhancing HCV replication (22, 24, 33). Among the best characterized of these pathways in hepatocyte-derived lines are the MAPK/extracellular signal-regulated kinase (13, 36) and NF- κ B pathways (18, 41). The HCV core N terminus (aa 1 to 20) has been demonstrated to bind to HCV IRES RNA and inhibit HCV IRES-dependent translation (22). There is also evidence that HCV core acts to suppress type I IFN signaling at several levels (3, 30, 39). For instance,

HCV core mediates the disruption of STAT1 phosphorylation through the induction of SOCS3 (5, 17, 20, 24, 29). It can also inhibit P-STAT1 nuclear import (4, 26). In this study, we found that the expression of the major Jak-STAT functional component P-STAT1 was markedly reduced in HCV-core-transfected cells. C-terminally truncated but not N-terminally truncated HCV core constructs produced parallel reductions in STAT1 binding, P-STAT1 loss, and blockage of IFN signaling. The HCV core binding and functional activities specifically map to its 23 N-terminal amino acids.

We further demonstrated that the STAT1 SH2 domain appears to be the critical HCV core interaction domain. The STAT1 protein sequence can be divided into N-terminal and C-terminal regions. The STAT1 protein N-terminal region in-

FIG. 6. HCV core interacts with STAT1 at the SH2 domain. (A) Map of STAT1 constructs. (a) Full-length STAT1 (pST1-Full, aa 1 to 750). (b) C-terminally truncated STAT1 construct pST1-C684 (Flag STAT1, aa 1 to 684). (c) C-terminally truncated STAT1 construct pST1-C577 (Flag STAT1, aa 1 to 577). (d) STAT1 point mutant constructs at 428/9 M-Glu, a putative DNA binding site (pST1-428/9 M); 701 M-Tyr, a JAK kinase phosphorylation site (pST1-701 M); 713 M-His, a splice site resulting in STAT1b formation (pST1-713 M); and 727 M-Ser, a MAPK phosphorylation site (pST1-727 M). (B) Transfection of C-terminally truncated STAT1 failed to rescue IFN signaling in U3A cells. IFN treatment (12 h) markedly induced ISRE-directed luciferase activity in U3A cells cotransfected with full-length STA1 (aa 1 to 750). In contrast, cotransfection with C-terminally truncated STAT1 constructs pST1-C684 (aa 1 to 684) and pST1-C577 (aa 1 to 577) failed to rescue the IFN signaling. Error bars indicate standard deviations. (C) HCV core immunoprecipitation (IP) and anti-STAT1 Western blotting. Protein lysates from U3A cells cotransfected with the full-length HCV core construct pC191, full-length STAT1, or truncated STAT1 constructs and anti-HCV core immunoprecipitated lysates were separated by SDS-PAGE and transferred to PVDF membranes. Anti-STAT1 IgG was used for Western blotting. With the introduction of proteasome inhibitor MG132 (10 μ M for 12 h), the HCV core protein bound to full-length STAT1 and the truncated STAT1 construct pST1-C684 but not the truncated STA1 construct pST1-C577. Lanes: 1, pST1-Full; 2, pST1-C684; 3, pST1-C577; 4, pST1-Full; 5, pST1-C684; 6, pST1-C577. +, with; -, without. (D) Anti-Flag immunoprecipitation and HCV core Western blot. Protein lysates from U3A cells cotransfected with the full-length HCV core construct pC191, full-length STAT1, or truncated STAT1 constructs and anti-Flag immunoprecipitated lysates were separated by SDS-PAGE and transferred to PVDF membranes. Anti-HCV core IgG was used for Western blotting. With the introduction of proteasome inhibitor MG132 (10 μ M for 12 h), full-length HCV core protein bound to full-length STAT1 and truncated STAT1 construct pST1-C684 but not truncated STA1 construct pST1-C577. Lanes: 1, pST1-Full; 2, pST1-C684; 3, pST1-C577; 4, pST1-Full; 5, pST1-C684; 6, pST1-C577. +, with; -, without.

cludes the NH2 region (aa 1 to 35), the coiled-coil domain (aa 135 to 315), and the DNA binding domain (315 to 487), while the C-terminal region includes the SH3 domain (aa 487 to 576), SH2 domain (aa 576 to 683), and transactivation domain (aa 683 to 750) (8, 19, 37, 40) (Fig. 5A). The STAT1 SH2

domain functions as a recognition site for phosphorylation by JAK kinases (7, 8, 19). The STAT1 SH2 domain is critical for two functions: (i) the recruitment of STAT1 to JAK kinases and its subsequent phosphorylation by JAK kinase and (ii) the promotion of homo- or heterodimerization of STAT1. STAT1

FIG. 7. HCV core associates with point mutant STAT1. (A) Full-length STAT1 (pST1-Full) transfection restored IFN-induced ISRE-mediated signaling in U3A cells (STAT1 deficiency). IFN treatment (12 h) markedly induced ISRE-directed luciferase activity in U3A cells cotransfected with pSTAT1-Full and pCAG (empty vector). However, in the presence of HCV core the construct pC191, this IFN-stimulated activity was blocked more than threefold. Transfected STAT1 mutants (pST1-701 M and pST1-713 M) have no abilities to rescue IFN signaling in U3A cells cotransfected with pCAG or the HCV core construct pC191. Error bars indicate standard deviations. (B) STAT1 Western blotting. Point mutant STAT1 constructs were cotransfected with the empty vector pCAG (panel i) or the HCV core construct pC191 (panel ii) into U3A cells. The transfection of HCV core reduced expressions of full-length and point mutant STAT1 in U3A cells. Proteasome inhibitor MG132 preserved the expression of STAT1. Lanes: 1, pST1-Full; 2, pST1-428/9 M; 3, pST1-701 M; 4, pST1-713 M; 5, pST1-727 M; 6, U3A (STAT1 deficient); 7 pST1-Full. (C) STAT1 immunoprecipitation-anti-HCV core Western blot. Protein lysates from U3A cells cotransfected with the full-length HCV core construct pC191 and STAT1 point mutant constructs and STAT1-immunoprecipitated lysates were separated by SDS-PAGE and transferred to PVDF membranes. Anti-HCV core Mab was used for Western blotting. With the introduction of the proteasome inhibitor MG132 (10 μ M for 12 h), full-length HCV core protein bound to full-length STAT1 and point mutant STAT1. Lanes: 1, pST1-Full; 2, pST1-428/9 M; 3, pST1-701 M; 4, pST1-713 M; 5, pST1-727 M; 6, U3A (STAT1 deficient); 7, pST1-WT; 8, pST1-428/9 M; 9, pST1-701 M; 10, pST1-713 M; 11, pST1-727 M; 12, U3A (STAT1 deficient).

SH2 heterodimerization with STAT2 and binding to interferon regulatory factor 9 are essential for the formation of a heterodimerization DNA binding complex that induces ISRE-mediated transcription of ISGs (8, 19, 42). In this study, we have shown that the interaction between HCV core and STAT1 localizes to the STAT1 SH2 region (aa 576 to 683) and that N-terminal HCV core mutants that fail to bind STAT1 do not block P-STAT1 formation. A plausible model explaining the observed events may proceed as follows: the binding of HCV core to the STAT1 SH2 domain inhibits the recruitment of STAT1 to JAK kinases, in turn inhibiting JAK-induced phosphorylation of STAT1. In addition, STAT1-STAT2 heterodimerization may be inhibited by both blockade of the SH2 domain and inhibition of STAT1 phosphorylation. The net result of these events is decreased DNA binding of ISGs. Our previous finding demonstrating that HCV expression is associated with reduced ISGF3 formation is consistent with this model (23). Our data also show that point mutations at other key STAT1 regulatory sites do not disrupt the HCV core-STAT1 interaction, implying that these regulatory sites are not blocked by HCV core. STAT1 deletion studies have demonstrated that its N ter-

minus is required for its proteasome-mediated degradation by mumps protein V (40). Rabies virus P protein has been reported to interact with STAT1 and blocks the IFN signaling pathway (37). The carboxy-terminal portion of rabies virus P protein has been demonstrated to inhibit the IFN signal transduction pathway by interacting with a region containing the STAT1 DNA binding domain (aa 315 to 487) and the coiled-coil domain (aa 135 to 315) (37). On the other hand, C-terminal STAT1 (aa 577 to 750) was reported to be unnecessary for mumps virus V protein-induced STAT1 degradation (40). There are several reports of blocked IFN signaling caused by the degradation of STAT1 induced by V proteins encoded by human parainfluenza virus type 2 and rubulaviruses simian virus 5, simian virus 41, mumps virus, and Newcastle disease virus (28, 40). These viruses have each evolved different mechanisms to counteract STAT1-driven type I IFN signaling. For HCV, we propose a unique model in which the interaction of the HCV core N-terminal domain (aa 1 to 23) with the STAT1 SH2 domain (aa 576 to 683) results in decreased STAT1 phosphorylation, leading to decreased STAT1-STAT2 heterodimerization, reduced ISGF3 formation, decreased DNA binding to the ISRE of IFN-stimulated genes, and, ultimately, decreased IFN-stim-

ulated gene transcription. These findings have important implications for the understanding of complex interactions between HCV proteins and the host cell antiviral defenses and may lead to novel strategies to interrupt the HCV core-STAT1 interaction and its adverse downstream effects on IFN signal transduction. We speculate that HCV core, in addition to blocking the formation of P-STAT1, also promotes ubiquitin-mediated proteasome-dependent degradation of STAT1. Further studies to address whether HCV core directly promotes STAT1 degradation are warranted.

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