Hepatitis C Virus NS5A Disrupts STAT1 Phosphorylation and Suppresses Type I Interferon Signaling

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Responses to alpha interferon (IFN-α)-based treatment are dependent on both host and viral factors and vary markedly among patients infected with different hepatitis C virus (HCV) genotypes (GTs). Patients infected with GT3 viruses consistently respond better to IFN treatment than do patients infected with GT1 viruses. The mechanisms underlying this difference are not well understood. In this study, we sought to determine the effects of HCV NS5A proteins from different genotypes on IFN signaling. We found that the overexpression of either GT1 or GT3 NS5A proteins significantly inhibited IFN-induced IFN-stimulated response element (ISRE) signaling, phosphorylated STAT1 (P-STAT1) levels, and IFN-stimulated gene (ISG) expression compared to controls. GT1 NS5A protein expression exhibited stronger inhibitory effects on IFN signaling than did GT3 NS5A protein expression. Furthermore, GT1 NS5A bound to STAT1 with a higher affinity than did GT3 NS5A. Domain mapping revealed that the C-terminal region of NS5A conferred these inhibitory effects on IFN signaling. The overexpression of HCV NS5A increased HCV replication levels in JFH1-infected cells through the further reduction of levels of P-STAT1, ISRE signaling, and downstream ISG responses. We demonstrated that the overexpression of GT1 NS5A proteins resulted in less IFN responsiveness than did the expression of GT3 NS5A proteins through stronger binding to STAT1. We confirmed that GT1 NS5A proteins exerted stronger IFN signaling inhibition than did GT3 NS5A proteins in an infectious recombinant JFH1 virus. The potent antiviral NS5A inhibitor BMS-790052 did not block NS5A-mediated IFN signaling suppression in an overexpression model, suggesting that NS5A’s contributions to replication are independent of its subversive action on IFN. We propose a model in which the binding of the C-terminal region of NS5A to STAT1 leads to decreased levels of P-STAT1, ISRE signaling, and ISG transcription and, ultimately, to preferential GT1 resistance to IFN treatment.

More than 170 million individuals are infected with hepatitis C virus (HCV) worldwide, and the disease often progresses to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1, 33). Alpha interferon (IFN-α) has been the backbone of therapy for hepatitis C virus infection. Unfortunately, a number of patients do not respond well to therapy (9, 15, 20, 40). The response rates to IFN therapy are also markedly different among patients infected with different HCV genotypes (GTs). Patients infected with HCV GT1 demonstrate sustained virological response (SVR) rates of 38 to 52%, whereas those infected with GT3 achieve higher SVR rates of 66 to 88% (5, 15, 34). However, the precise mechanisms for HCV GT1 hyporesponsiveness are still not fully understood (38, 40). Several HCV proteins, including core and NS5A, have been demonstrated to contribute to the lack of a response of patients with HCV infection to IFN treatment (35, 38, 40, 47). We have previously reported that HCV replication and core protein expression decrease phosphorylated STAT1 (P-STAT1) accumulation (28, 29). HCV NS5A was also shown to inhibit IFN signaling through the suppression of STAT1 phosphorylation (14, 24, 27). However, the molecular mechanisms by which HCV NS5A antagonizes IFN activity are not well characterized. Furthermore, HCV NS5A genotype-mediated IFN resistance is still controversial. In general, viral infection stimulates the type I IFN (IFN-α/β) pathway. The binding of IFNs to their cellular receptors activates an intracellular signaling cascade. The activated Jak1 and Tyk2 kinases further phosphorylate STAT1 and STAT2. The subsequent phosphorylation of the receptor-recruited STATs promotes the formation of heterodimers between STAT1 and STAT2, which further bind to a third protein, IFN-regulatory factor 9 (IRF9) or P48, to form the IFN-stimulated gene factor 3 (ISGF3) complex. This complex translocates into the nucleus and binds the IFN-stimulated response element (ISRE) in IFN-stimulated gene (ISG) promoters, leading to the upregulation of IFN effector proteins such as the double-stranded RNA-activated protein kinase (PKR), the 2′,5′-oligoadenylate synthetase (OAS), and the MxA (myxovirus resistance A) proteins (4, 13, 19, 44). The level of IFN-induced P-STAT1 has been shown to correlate with the global induction of ISGs and was significantly higher in sustained HCV virological responders than in nonresponders (3, 17). It was described previously that the NS5A protein plays a role in the inhibition of IFN activity via the interaction of its PKR binding domain (PKRBD) (amino acid [aa] positions 2209 to 2274) with the antiviral protein PKR, which blocks PKR activity (6, 11). This interaction is presumed to allow viral protein synthesis to occur during IFN treatment. In addition, a number of recent studies have demonstrated the inhibitory effects of the NS5A protein on...
the IFN-induced Jak-STAT signaling pathway (14, 24, 27, 43). However, the molecular mechanisms by which HCV NS5A interferes with P-STAT1 and IFN signaling have not been fully characterized. In order to characterize the mechanism of HCV resistance to IFN therapy among NS5A proteins from different genotypes, we investigated the effects of GT1 and GT3 HCV NS5A proteins on the IFN-α-induced Jak-STAT signaling pathway in overexpression and infectious recombinant virus (JFH1) models (39). NS5A is an essential component of the HCV replication complex (21, 41). The compound BMS-790052 is a potent NS5A inhibitor (8). We also tested the effects of BMS-790052 (12) on the IFN-α-mediated suppression of IFN signaling.

**MATERIALS AND METHODS**

**Cell cultures and infectious viruses.** The human hepatocellular carcinoma cell line Huh7.5.1 (50) and HCV JFH1-infected Huh7.5.1 cells were grown at 37°C in a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (0.1 mg/ml streptomycin and 100 U/ml penicillin). The Huh7.5.1 cells were infected with HCV JFH1 as previously described (30, 31, 45). The infectious GT1 and GT3 NS5A recombinant viruses were produced from recombinant DNA constructs, as previously reported (39). The infectious GT1 and GT3 NS5A recombinant viruses were developed by replacing the complete NS5A of the J6/JFH1 recombinant virus with NS5A from HCV GT1 isolate H77C (GT1a) or GT3 isolate S52 (GT3a) (31, 32, 39). An NS5A-specific inhibitor, BMS-790052 (12) (Selleck Chemicals LLC, Houston, TX), was tested for its effects on HCV JFH1 replication, ISRE signaling, and NS5A protein expression.

**Plasmid constructs and transfection.** Genotype 1 and 3 HCV strains are the most prevalent forms of infection in Thailand (16). Our project was initiated in Thailand several years ago, before the JFH1 virus was available. The GT1 and GT3 NS5A constructs used in this study were generated from HCV-infected patients in Thailand (26). Full-length cDNAs encoding HCV NS5A were prepared based on the consensus genotypes 1a, 1b, 3a, and 3b isolated from patient sera by reverse transcription (RT)–PCR. Primer pairs used for the amplification of the NS5A gene nototypes 1a, 1b, 3a, and 3b isolated from patient sera by reverse transcrip-

**Quantitative real-time PCR.** Total cellular RNAs from Huh7.5.1 cells transfected with a plasmid encoding the HCV NS5A protein, and JFH1-infected cells were harvested by using the QIA Shredder and RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Total cDNA was synthesized by reverse transcription using Applied Biosystems high-capacity cDNA reverse transcription kits (Invitrogen, Carlsbad, CA) with random primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HCV JFH1, PKR, OAS, and MxA mRNA levels were quantified by real-time PCR using the Bio-Rad IQ5 system (Bio-Rad Laboratories, Hercules, CA). GAPDH was used as a control for basal RNA levels. The primer sequences used are listed in Table 1. The reaction mixture was first heated at 95°C for 3 min, and 45 cycles of PCR amplification were then performed, as follows: 94°C for 20 s, 60°C for 30 s, and 72°C for 20 s. The mRNA level of each gene was normalized to GAPDH levels to obtain mRNA arbitrary units (folds).

**Protein sample preparation.** At the time of harvesting, cells were washed with phosphate-buffered saline (PBS) and lysed by using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA) containing a protease inhibitor cocktail (Sigma Life Science and Biochemicals, St. Louis, MO). Whole-cell protein lysates were subsequently sonicated and boiled at 95°C for 5 min in SDS-PAGE sample buffer and chilled on ice for another 5 min before being subjected to a Western blot (WB) assay.

**Western blotting.** While WB may not be precisely quantitative, it does provide semiquantitative information about protein levels. We also loaded equal quantities of protein (20 μg) into each lane. Protein samples were separated by SDS-PAGE with NuPAGE Novex precast 4-12%
incubated with 10 μl/well of NS5A constructs in a 6-well plate. Forty-eight hours after transfection, cells were lysed, and whole-cell lysates were collected. We added 20 μg of protein to each well to ensure equal protein loading in each lane. Furthermore, 100 μg (1 μg/μl) of protein lysate was incubated with 10 μg (1 μg/μl) of antibody in each coimmunoprecipitation (co-IP) experiment. Twenty microliters of each immunoprecipitation (IP) lysate was loaded onto the WB. STAT1 immunoprecipitation was performed by using the Roche immunoprecipitation kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. The STAT1-NS5A protein complex (20 μl IP lysate) was then analyzed by Western blotting. Anti-V5 antibody (Invitrogen Life Technologies, Carlsbad, CA) was used as the primary antibody, and HRP-conjugated ECL sheep anti-mouse IgG (Amersham Biosciences, Piscataway, NJ) was used as the secondary antibody.

**Statistical analysis.** Data analysis was performed by using a 2-tailed Student t test. Data are expressed as means ± standard deviations (SD) of data from at least three sample replicates, unless stated otherwise.

**RESULTS**

Both HCV JFH1 infection and NS5A expression inhibit IFN-α-induced ISRE signaling. We first observed that IFN-α could induce ISRE signaling in Huh7.5.1 cells in a dose-dependent manner (from 1 to 10,000 IU/ml), as expected (Fig. 1A). We confirmed that HCV strongly decreased IFN-induced ISRE signaling in JFH1-infected Huh7.5.1 cells (Fig. 1A). Interestingly, we found that the GT1a, GT1b, GT3a, and GT3b NS5A proteins each significantly inhibited IFN-induced ISRE signaling by 52.9% (P < 0.001), 55.0% (P < 0.001), 31.5% (P < 0.001), and 6.3% (P = 0.016), respectively, compared to the empty vector control (Fig. 1B). In contrast, NS4B (GT1a) did not block ISRE signaling. Between genotypes, we noted that GT1a, GT1b, GT3a, and GT3b NS5A proteins each significantly inhibited ISRE signaling by 52.9% (P < 0.001), 55.0% (P < 0.001), 31.5% (P < 0.001), and 6.3% (P = 0.016), respectively, compared to the empty vector control (Fig. 1B). In contrast, NS4B (GT1a) did not block ISRE signaling. Between genotypes, we noted that GT1a, GT1b, GT3a, and GT3b NS5A proteins each significantly inhibited ISRE signaling by 52.9% (P < 0.001), 55.0% (P < 0.001), 31.5% (P < 0.001), and 6.3% (P = 0.016), respectively, compared to the empty vector control (Fig. 1B).
NS5A proteins inhibited IFN signaling significantly more strongly than did GT3 NS5A proteins (P < 0.05) (Fig. 1B). Western blot results confirmed the expression of each of the NS5A, NS4B, and core viral proteins in transfected and JFH1-infected Huh7.5.1 cells (Fig. 1C). Western blotting and densitometry verified equivalent levels of NS5A protein expression corresponding to HCV JFH1, GT1a, GT1b, GT3a, and GT3b (Fig. 1D).

Both HCV JFH1 infection and NS5A expression block IFN-α-induced STAT1 phosphorylation. To elucidate the effects of the NS5A protein on IFN-α-induced STAT1 phosphorylation (P-STAT1), we performed a P-STAT1 Western blot analysis with Huh7.5.1 cells expressing the NS5A protein. Huh7.5.1 cells were transfected with NS5A constructs for 48 h. Huh7.5.1, JFH1-infected Huh7.5.1, or construct-transfected Huh7.5.1 cells were incubated with IFN-α (100 IU/ml) for 15 min, 1 h, or 4 h. Whole-cell lysates were analyzed by Western blot analysis for P-STAT1, P-STAT2, STAT1, STAT2, NS5A, NS4B, and actin protein levels. IFN-α activated P-STAT1 and P-STAT2 levels in Huh7.5.1 cells (lane 3). JFH1-HCV replication (lane 4) as well as the overexpression of the GT1a, GT1b, GT3a, and GT3b NS5A proteins (lanes 6, 7, 8, and 9, respectively) strongly decreased P-STAT1 levels. The empty vector and NS4B overexpression had no effect on P-STAT1 levels (lanes 5 and 10, respectively). JFH1 infection or NS5A overexpression had no effect on P-STAT2 levels. GT1a and GT1b NS5A (lanes 6 and 7, respectively) expression exhibited a stronger suppression of P-STAT1 levels than did GT3a and GT3b NS5A (lanes 8 and 9, respectively) expression. Lanes: 1 and 3, Huh7.5.1 cells; 2 and 4, JFH1-infected Huh7.5.1 cells; 5, pCAGGS-V5/His; 6, GT1a NS5A; 7, GT1b NS5A; 8, GT3a NS5A; 9, GT3b NS5A; 10, NS4B. IFN-α treatment is shown in lanes 3 to 10.

To further determine the effects of NS5A on the IFN-α-induced downstream ISG response, we monitored PKR, OAS, and MxA mRNA levels in JFH1-infected and NS5A-overexpressing Huh7.5.1 cells. We confirmed that IFN-α activated PKR, OAS, and MxA mRNA expressions in Huh7.5.1 cells transfected with the empty vector (Fig. 3). On the other hand, we found that HCV infection strongly decreased PKR, OAS, and MxA mRNA levels in JFH1-infected Huh7.5.1 cells transfected with the empty vector (Fig. 3). The overexpression of the GT1a, GT1b, GT3a, and GT3b NS5A proteins significantly inhibited PKR mRNA levels by 59.1% ± 6.7% (P < 0.001), 69.9% ± 6.6% (P < 0.001), 37% ± 10.8% (P = 0.004), and 43.6% ± 9.7% (P = 0.002), respectively, compared to the empty vector control. In contrast, NS4B had no effect on PKR expression (Fig. 3A). Moreover, the overexpression of the GT1a, GT1b, GT3a, and GT3b NS5A proteins also significantly decreased OAS mRNA levels by 56.6% ± 8.3% (P = 0.005), 63% ± 2.2% (P = 0.002), 36.7% ± 14.1% (P = 0.03), and 46.3% ± 12.8% (P = 0.01), respectively, and MxA mRNA levels by 64.7% ± 1.2% (P = 0.001), 66.6% ± 4.1% (P = 0.001), 42.2% ± 3.5% (P = 0.007), and 47.3% ± 2.7% (P = 0.004), respectively, compared to the empty vector control (Fig. 3B and C). We found that the GT1 NS5A proteins (GT1a and GT1b) induced a stronger reduction of the ISG expression level than did the GT3 NS5A proteins (GT3a

![FIG 2](jvi.asm.org) HCV NS5A reduces STAT1 phosphorylation. The inhibitory effect of NS5A on IFN-α-induced STAT1 phosphorylation was assessed in cells expressing the NS5A protein. Huh7.5.1 cells were transfected with NS5A constructs for 48 h. Huh7.5.1, JFH1-infected Huh7.5.1, or construct-transfected Huh7.5.1 cells were incubated with IFN-α (100 IU/ml) for 15 min, 1 h, or 4 h. Whole-cell lysates were analyzed by Western blot analysis for P-STAT1, P-STAT2, STAT1, STAT2, NS5A, NS4B, and actin protein levels. IFN-α activated P-STAT1 and P-STAT2 levels in Huh7.5.1 cells (lane 3). JFH1-HCV replication (lane 4) as well as the overexpression of the GT1a, GT1b, GT3a, and GT3b NS5A proteins (lanes 6, 7, 8, and 9, respectively) strongly decreased P-STAT1 levels. The empty vector and NS4B overexpression had no effect on P-STAT1 levels (lanes 5 and 10, respectively). JFH1 infection or NS5A overexpression had no effect on P-STAT2 levels. GT1a and GT1b NS5A (lanes 6 and 7, respectively) expression exhibited a stronger suppression of P-STAT1 levels than did GT3a and GT3b NS5A (lanes 8 and 9, respectively) expression. Lanes: 1 and 3, Huh7.5.1 cells; 2 and 4, JFH1-infected Huh7.5.1 cells; 5, pCAGGS-V5/His; 6, GT1a NS5A; 7, GT1b NS5A; 8, GT3a NS5A; 9, GT3b NS5A; 10, NS4B. IFN-α treatment is shown in lanes 3 to 10.

Both HCV JFH1 infection and NS5A expression block IFN-α-induced STAT1 phosphorylation. In contrast, NS4B had no effect on PKR expression (Fig. 3A). Moreover, the overexpression of the GT1a, GT1b, GT3a, and GT3b NS5A proteins also significantly decreased OAS mRNA levels by 56.6% ± 8.3% (P = 0.005), 63% ± 2.2% (P = 0.002), 36.7% ± 14.1% (P = 0.03), and 46.3% ± 12.8% (P = 0.01), respectively, and MxA mRNA levels by 64.7% ± 1.2% (P = 0.001), 66.6% ± 4.1% (P = 0.001), 42.2% ± 3.5% (P = 0.007), and 47.3% ± 2.7% (P = 0.004), respectively, compared to the empty vector control (Fig. 3B and C). We found that the GT1 NS5A proteins (GT1a and GT1b) induced a stronger reduction of the ISG expression level than did the GT3 NS5A proteins (GT3a
and GT3b). These data imply that HCV replication or NS5A overexpression impairs ISG expression through an inhibition of STAT1 phosphorylation.

**GT1 NS5A binds to STAT1 with a higher affinity than GT3 NS5A.** To further investigate whether the HCV NS5A protein physically associates with STAT1, we performed STAT1 immunoprecipitation followed by anti-V5 Western blotting. We found that the GT1a, GT1b, GT3a, and GT3b NS5A proteins each bind to the STAT1 protein (Fig. 4). Interestingly, we observed that the GT1 NS5A proteins (GT1a and GT1b) bound to STAT1 with a higher affinity than the GT3 NS5A proteins (GT3a and GT3b). Western blotting confirmed NS5A, STAT1, and actin protein expressions (Fig. 4). These data suggest a direct physical interaction between the NS5A and STAT1 proteins. Taken together, we speculate that the binding of NS5A to the STAT1 protein impairs STAT1 phosphorylation, ISRE signaling, and downstream ISG responses. Furthermore, we found that the GT1a and GT1b NS5A proteins suppress IFN responsiveness more strongly than the GT3a and GT3b NS5A proteins in conjunction with stronger binding to STAT1. Moreover, the WB-IP assay and densitometry revealed that the STAT1-NS5A interaction differences were more pronounced between HCV GT1 and GT3 (Fig. 4) than were differences in ISRE signaling (Fig. 1B) or downstream ISG mRNA expression (Fig. 3) between the two genotypes. This disparity suggests the possibility that GT1 and GT3 exert other differential effects at points downstream of STAT1 binding (10, 18, 23).

**Overexpression of NS5A in JFH1-infected Huh7.5.1 cells is permissive for HCV replication.** GT1 and GT3 NS5A proteins may exhibit different degrees of suppression of the Jak-STAT antiviral pathway and the downstream ISG response, which may result in a reduction of the IFN-induced antiviral response. We next examined the functional effects of the overexpression of the GT1a, GT1b, GT3a, and GT3b NS5A proteins on IFN signaling, ISG expression, and JFH1 replication. Huh7.5.1 cells were first transfected with NS5A constructs, followed by HCV JFH1 infection. The cells were then treated with or without IFN-α (100 IU/ml). We again confirmed that IFN-induced ISRE signaling was strongly inhibited in JFH1-infected Huh7.5.1 cells. Interestingly, the overexpression of the GT1a, GT1b, GT3a, and GT3b NS5A proteins in JFH1-infected Huh7.5.1 cells produced a stronger reduction in the level of ISRE signaling than did JFH1 infection alone. The overexpression of the GT1a and GT1b NS5A proteins exhibited a stronger reduction of the level of ISRE signaling than did the overexpression of the GT3a and GT3b NS5A proteins (Fig. 5A). In contrast, NS4B overexpression led to no further decrease in the level of signaling. Furthermore, NS5A overexpression in combination with JFH1 infection further decreased P-STAT1 levels compared to levels found with JFH1 infection alone (Fig. 5B). We found that the overexpression of NS5A further decreased mRNA levels of PKR, OAS, and MxA in JFH1-infected Huh7.5.1 cells compared to cells infected with JFH1 alone. The overexpression of the GT1a and GT1b NS5A proteins displayed a more substantial reduction of ISG mRNA levels than did the overexpression of the GT3a and GT3b NS5A proteins (Fig. 5C to E). In contrast, the overexpression of NS4B led to no further decrease in the level of ISG expression. We also observed that the overexpression of NS5A in JFH1-infected Huh7.5.1 cells further increased HCV replication levels compared to those in cells infected with...
JFH1 alone, whereas NS4B had no effect on HCV replication in JFH1-infected Huh7.5.1 cells. The overexpression of GT1a or GT1b NS5A proteins had greater permissive effects on HCV replication level than did the overexpression of GT3a or GT3b NS5A proteins (top). Total cell lysates were used for Western blotting to monitor the expressions of the NS5A (anti-V5), STAT1, and actin proteins. (B) NAPAU for STAT1 immunoprecipitation (IP) lysates of GT1a, GT1b, GT3a, and GT3b from the corresponding densitometry. The NAPAU (from three independent WB images) were 0.84 ± 0.06 (GT1a), 0.81 ± 0.08 (GT1b), 0.29 ± 0.08 (GT3a), and 0.26 ± 0.23 (GT3b). These densitometry data confirmed that the GT1 (GT1a and GT1b) NS5A proteins had stronger binding to STAT1 than did the GT3 (GT3a and GT3b) NS5A proteins. (C) NAPAU for whole-cell lysates of GT1a, GT1b, GT3a, and GT3b from the corresponding densitometry. The NAPAU (from three independent WB images) were 0.90 ± 0.09 (GT1a), 0.94 ± 0.12 (GT1b), 0.88 ± 0.11 (GT3a), and 0.96 ± 0.18 (GT3b). These densitometry data verified the relative equal NS5A protein expression levels between GT1 (GT1a and GT1b) and GT3 (GT3a and GT3b).

**The NS5A C terminus physically interacts with STAT1 and inhibits IFN signaling.** To determine the physical domain of HCV NS5A responsible for the disruption of IFN signaling and the reduction of P-STAT1, ISRE signaling, and downstream ISG responses, we constructed and transfected plasmids expressing full-length NS5A (aa residues 1 to 447), the N-terminal region of NS5A (N-terminal NS5A) (aa residues 1 to 236), and C-terminal NS5A (aa residues 237 to 447) into Huh7.5.1 cells. We found that the overexpression of C-terminal NS5A produced a degree of ISRE signaling inhibition comparable to that of full-length NS5A. In contrast, the overexpression of N-terminal NS5A or NS4B did not block ISRE signaling. A similar result was seen for both GT1 and GT3 (Fig. 6A). In addition, the overexpression of full-length NS5A as well as C-terminal NS5A (in both GT1b and GT3a) was associated with a reduction of P-STAT1 levels, whereas N-terminal NS5A had no effect on P-STAT1 levels (Fig. 6B). We also found that the overexpression of C-terminal NS5A (in both GT1b and GT3a) decreased ISG (including PKR, OAS, and MxA) mRNA levels similarly to full-length NS5A, whereas N-terminal NS5A had no effect on ISG expression (Fig. 6C to E). To investigate whether C-terminal NS5A is responsible for the physical association with STAT1, we performed STAT1 immunoprecipitation followed by NS5A-V5 immunoblotting with cells transfected with the full-length, N-terminal, or C-terminal NS5A construct. We found that both full-length NS5A and the C terminus of NS5A (both GT1b and GT3a) bind to STAT1 in Huh7.5.1 cells (Fig. 6F). In contrast, N-terminal NS5A did not bind to STAT1. These data indicate that the STAT1 interaction domain is located in the C terminus of NS5A (aa residues 237 to 447) and that this domain is responsible for the inhibition of type I IFN signaling and the reduction in levels of P-STAT1 and the downstream ISG response.

**GT1 NS5A exhibits higher resistance to IFN than GT3 NS5A in an infectious replication model.** To test the effects of the GT1 and GT3 NS5A proteins on IFN resistance in the context of an infectious HCV replication model, we examined the influence of an alteration of the NS5A genotype on sensitivity to IFN-α by using H77 (GT1a NS5A)/JFH1 (H77) and S52 (GT3a NS5A)/JFH1 (S52) recombinant viruses (39). In these constructs, full-length NS5A in J6/JFH1 was replaced with GT1a or GT3a NS5A to generate H77 or S52 infectious recombinant virus, as previously reported (39). It was reported previously that NS5A has distinct cis- and trans-acting functions in HCV RNA replication. HCV NS5A’s cis-acting function may occur as part of the HCV replication complex through basally phosphorylated NS5A, while the

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**FIG 4** GT1 NS5A has stronger binding to STAT1 than GT3 NS5A. The plasmid DNA constructs encoding HCV NS5A of GT1a, GT1b, GT3a, or GT3b were transfected into Huh7.5.1 cells. Cell lysates were harvested at 48 h after transfection. STAT1 immunoprecipitation was performed by using the Roche immunoprecipitation (IP) kit. IP cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes, followed by anti-V5 immunoblotting to determine interactions between NS5A and STAT1. (A) GT1a and GT1b NS5A proteins have stronger binding affinity for STAT1 than GT3a and GT3b NS5A proteins (top). Total cell lysates were used for Western blotting to monitor the expressions of the NS5A (anti-V5), STAT1, and actin proteins. (B) NAPAU for STAT1-IP lysates of GT1a, GT1b, GT3a, and GT3b from the corresponding densitometry. The NAPAU (from three independent WB images) were 0.84 ± 0.06 (GT1a), 0.81 ± 0.08 (GT1b), 0.29 ± 0.08 (GT3a), and 0.26 ± 0.23 (GT3b). These densitometry data confirmed that the GT1 (GT1a and GT1b) NS5A proteins had stronger binding to STAT1 than did the GT3 (GT3a and GT3b) NS5A proteins. (C) NAPAU for whole-cell lysates of GT1a, GT1b, GT3a, and GT3b from the corresponding densitometry. The NAPAU (from three independent WB images) were 0.90 ± 0.09 (GT1a), 0.94 ± 0.12 (GT1b), 0.88 ± 0.11 (GT3a), and 0.96 ± 0.18 (GT3b). These densitometry data verified the relative equal NS5A protein expression levels between GT1 (GT1a and GT1b) and GT3 (GT3a and GT3b).
-acting function may occur outside the replication complex and requires hyperphosphorylation (7). We found that both the H77 and S52 viruses replicated equivalently by analyzing both RNA and protein levels (NS5A and core) without IFN treatment (Fig. 7A and C). Although we did not monitor NS5A adaptive mutations in the H77 and S52 viruses, our findings and our previously reported description (39) indicate that H77, S52, and J6/JFH1 are comparably replicative viruses. In addition, HCV RNA was markedly inhibited by IFN treatment in a time-dependent manner (Fig. 7A). Interestingly, HCV H77 (GT1a NS5A) replication demonstrated a weaker response to IFN than did HCV S52 (GT3a NS5A) replication (Fig. 7A). We also found that the H77 (GT1a NS5A) recombinant virus exhibited a more substantial reduction in IFN-induced ISRE signaling (Fig. 7B) and P-STAT1 levels (Fig. 7C) than did the S52 (GT3a NS5A) virus. We also observed that infection with the H77 (GT1a NS5A) recombinant virus led to a stronger reduction of the ISG expression level, including PKR, OAS, and MxA mRNA levels, than did infection with the S52 (GT3a NS5A) virus (Fig. 7D to F). These results confirm that GT1 NS5A has greater inhibitory effects on IFN signaling than does GT3 NS5A when inserted into an infectious replication model. It would be of interest to study NS5A sequences corresponding to the selection of GT3 NS5A-harboring clones (S52) that have acquired the phenotype associated with GT1 NS5A-harboring clones (H77).

**HCV NS5A ISDR variation may contribute to GT1 and GT3 sensitivity to IFN.** NS5A interferon sensitivity-determining-region (ISDR) substitutions have been associated with the IFN response and HCV viral load (25, 46). To assess the possible correlation between the NS5A ISDR and IFN efficacy, we compared ISDR sequences (amino acids 2209 to 2248) between the GT1a, GT1b, GT3a, and GT3b NS5A proteins. We found five amino acid differences between GT1 and GT3, located at positions 2216, 2220, 2221, 2239, and 2248 (Table 2). We speculate that these amino acid variations may be associated with GT3 viruses being more responsive to IFN than GT1 viruses, and we will perform these studies as a logical follow-up to the present study.

**BMS-790052 does not affect NS5A-STAT1 interactions.** To evaluate the effect of a pharmacological NS5A inhibitor on HCV replication, we monitored HCV RNA and core protein levels in JFH1-infected cells treated with the NS5A inhibitor BMS-790052. As expected, we found that BMS-790052 inhibited HCV RNA levels in JFH1-infected cells in a dose-dependent manner (Fig. 8A). Western blotting for the core protein confirmed the proportional reduction of HCV core protein and RNA levels (Fig. 8A, bottom). We also found that BMS-790052 rescued the HCV-me-
diation inhibition of ISRE signaling in the JFH1 replication model in a dose-dependent manner (Fig. 8B). However, BMS-790052 had no effect on the N55A overexpression-mediated inhibition of ISRE signaling (Fig. 8B). Western blotting demonstrated that BMS-790052 reduced the HCV core level in JFH1-infected cells. However, BMS-790052 had no effect on the overexpression of the N55A protein (Fig. 8C). We also found that BMS-790052 neither rescued N55A overexpression-mediated P-STAT1 reduction nor interrupted N55A-STAT1 interactions (data not shown). These results indicate that the N55A inhibitor BMS-790052 reduces HCV RNA and core levels by blocking HCV replication but does not disrupt the N55A-STAT1 interaction.

**DISCUSSION**

IFN-α has been the mainstay of therapy for chronic hepatitis C. Unfortunately, many patients do not respond well to therapy. The response rates to IFN treatment vary among patients infected with different HCV genotypes. Patients infected with genotype 3 viruses respond much more effectively to IFN treatment than patients infected with genotype 1 viruses (5, 15, 34, 48). However, the mechanisms underlying this difference are not well understood. In the present study, we used both HCV N55A overexpress-
previous study (39), it was reported that an NS5A (GT1 to GT7)/JFH1 recombinant virus had a similar sensitivity to IFN-treatment. We speculate that the discrepancy between our findings and those of the previous report is attributable to the HCV detection methods used. We monitored HCV replication in the presence of IFN-treatment by measuring both HCV RNA levels by quantitative PCR (qPCR) and HCV NS5A and core protein levels by Western blotting, compared to the previous report, in which HCV infectivity was measured by calculating the percentage of HCV NS3-positive cells by immunostaining. We also found that NS5A proteins bind to STAT1 and that GT1 NS5A proteins exhibit stronger binding to STAT1 than do GT3 NS5A proteins, suggesting a basis for these differential functional effects. It was reported previously that the C-terminal region of NS5A is required for IFN antagonism (43). Our results reveal, for the first time, that the C terminus of NS5A (both GT1 and GT3) but not the N terminus of NS5A exhibits inhibitory effects on IFN signaling through binding to the STAT1 protein.

Table 2 Comparison of amino acid sequences of NS5A ISDRs between GT1a, GT1b, GT3a, and GT3b

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino acid sequence at residues 2209–2248*</th>
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<tbody>
<tr>
<td>GT1a NS5A</td>
<td>PSLKATCpngpHgDpaBqKieKwLqQeMgqGmNtrBveSn</td>
</tr>
<tr>
<td>GT1b NS5A</td>
<td>PSLRATGpngpHgDpaBqKieKwLqQeMgqGmNtrBveSn</td>
</tr>
<tr>
<td>GT3a NS5A</td>
<td>PSLKATCpngpHgDpaBqKieKwLqQeMgqGmNtrBveSn</td>
</tr>
<tr>
<td>GT3b NS5A</td>
<td>PSLKATCpngpHgDpaBqKieKwLqQeMgqGmNtrBveSn</td>
</tr>
</tbody>
</table>

*The underlined sequences indicate variant amino acids between GT1 and GT3 NS5A which are at positions 2216, 2220, 2221, 2239, and 2248.

FIG 7 GT1 NS5A proteins exhibit higher levels of resistance to IFN than GT3 NS5A proteins in NS5A/JFH1 recombinant virus. To determine GT1 and 3 NS5A resistances to IFN in the replication model, we used the H77 (GT1a NS5A)/JFH1 (H77) and S52 (GT3a NS5A)/JFH1 (S52) recombinant viruses. Full-length NS5A in JFH1 was replaced with GT1a or GT3a NS5A to generate the infectious recombinant virus H77 or S52, as previously reported (39). Huh7.5.1 cells were infected with the H77 or S52 recombinant virus. (A) HCV H77 and S52 respond to IFN treatment in a time-dependent manner. Twenty-four hours after infection, cells were treated with IFN-α (100 IU/ml) or without IFN for another 24, 48, and 72 h. We confirmed the relative equal HCV RNA levels between the H77 and S52 viruses without IFN treatment. We found that IFN treatments reduced HCV RNA levels in a time-dependent manner. S52 (GT3 NS5A) was more sensitive to IFN treatment than H77 (GT1 NS5A). (B) HCV H77 exhibited a stronger inhibition of ISRE signaling than HCV S52. Plasmids pISRE-luc and pRL-TK were cotransfected into H77- or S52-infected Huh7.5.1 cells for 24 h. Cells were treated with IFN-α (100 IU/ml) for another 24 h. The relative luciferase activity was finally assessed. We found that the H77 (GT1 NS5A) recombinant virus exhibited a greater reduction of IFN-induced ISRE signaling did HCV S52 (GT3 NS5A). #, P < 0.01 (for comparisons between H77 and S52). (C) HCV H77 exhibited a greater reduction of the P-STAT1 level. Huh7.5.1 cells were infected with HCV H77 or S52 for 48 h. The cells were treated with IFN-α (100 IU/ml) for 1 h. IFN-α induced P-STAT1 accumulation in Huh7.5.1 cells. HCV H77 reduced the P-STAT1 protein level more than did HCV S52. The NAPAU for H77 and S52 were 0.87 ± 0.05 and 0.84 ± 0.10, respectively. HCV core and NS5A Western blotting confirmed the relative equal HCV replication levels between H77 and S52. (D to F) HCV H77 inhibited ISG expression more than HCV S52. Huh7.5.1 cells were infected with HCV H77 or S52 for 24 h. The cells were treated with IFN-α (100 IU/ml) for 24 h. Cell lysates were harvested to measure mRNA levels. H77 showed a stronger suppression of ISG expression levels, including PKR (D), OAS (E), and MxA (F) mRNA levels, than S52. #, P < 0.05 (for comparisons between H77 and S52).
790052 exhibited no significant effect on the activity of the overexpressed NS5A protein or on the NS5A-STAT1 interaction, implying a functional dissociation between NS5A’s replication and IFN subversion functions.

It was identified previously that the inhibitory activity of BMS-790052 maps to the first 100 amino acids of HCV NS5A (N-terminal NS5A) (12). In this study, we demonstrated that C-terminal NS5A confers a blockage of IFN signaling pathway through binding to STAT1. It is likely that the existence of distinct binding sites on NS5A for BMS-790052 and STAT1 explains the finding that BMS-790052 does not affect the overexpression of NS5A-mediated IFN antagonism. We therefore conclude that NS5A impairs type I IFN signaling in both HCV replication and NS5A expression models. GT1 NS5A suppresses IFN responsiveness more strongly than GT3 NS5A in conjunction with stronger binding to STAT1. We propose a model in which HCV mediates its antagonistic effects on IFN treatment through C-terminal NS5A binding to STAT1, which results in reduced P-STAT1, ISRE signaling, and ISG expression levels. These observations provide new insights into the mechanisms by which HCV NS5A proteins from different genotypes influence IFN signaling and may account for genotype-related differences in IFN treatment responses. The results further suggest a potential target for the development of novel antiviral strategies. Further analyses of NS5A’s qualitative and quantitative effects at more physiologic levels will take steps toward an understanding of HCV evasion of host control in a native context.

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REFERENCES


