Chronic hepatitis C virus (HCV) infection is a major global public health problem. HCV infection is supported by viral strategies to evade the innate antiviral response wherein the viral NS3-4A protease complex targets and cleaves the interferon promoter stimulator-1 (IPS-1) adaptor protein to ablate signaling of interferon α/β immune defenses. Here we examined the structural requirements of NS3-4A and the therapeutic potential of NS3-4A inhibitors to control the innate immune response against virus infection. The structural composition of NS3 includes an amino-terminal serine protease domain and a carboxyl-terminal RNA helicase domain. NS3 mutants lacking the helicase domain retained the ability to control virus signaling initiated by retinoic acid-inducible gene-1 (RIG-I) or melanoma differentiation antigen 5 and suppressed the downstream activation of interferon regulatory factor-3 (IRF-3) and nuclear factor κB (NF-κB) through the targeted proteolysis of IPS-1. This regulation was abrogated by truncation of the NS3 protease domain or by point mutations that ablated protease activity. NS3-4A protease control of antiviral immune signaling was due to targeted proteolysis of IPS-1 by the NS3 protease domain and minimal NS4A cofactor. Treatment of HCV-infected cells with an NS3 protease inhibitor prevented IPS-1 proteolysis by the HCV protease and restored RIG-I immune defense signaling during infection. Thus, the NS3-4A protease domain can target IPS-1 for cleavage and is essential for blocking RIG-I signaling to IRF-3 and NF-κB, whereas the helicase domain is dispensable for this action. Our results indicate that NS3-4A protease inhibitors have immunomodulatory potential to restore innate immune defenses to HCV infection.

Hepatitis C virus (HCV) establishes persistent infections in the majority of exposed individuals. There are nearly 200 million people worldwide with chronic HCV infection, and it is a major cause of hepatitis and liver disease. Moreover, HCV is associated with the development of liver cancer and is currently the leading indication for liver transplants (1, 2). HCV is a member of the Flaviviridae family of enveloped, single-stranded, positive-sense RNA viruses. The 9.6-kilobase genome of HCV encodes a single polyprotein that is post-translationally processed into at least 10 structural and nonstructural (NS) proteins by a combination of host and viral proteases (3). In addition to their primary role in HCV genome replication and virion maturation, various HCV proteins have been shown to antagonize host immune defenses. HCV control of the innate antiviral response and interferon (IFN) α/β antiviral defenses may provide a cellular foundation for viral persistence (4).

The innate antiviral response to RNA virus infection is triggered when intermediates of viral replication, including viral RNA or protein products, are recognized by specific cellular pathogen recognition receptors (5). Retinoic acid inducible gene-1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are cytosolic pathogen recognition receptors that bind to viral RNA and double-stranded RNA, albeit with variable efficiency (6, 7). Both are DEXD/H-box RNA helicases and encode tandem amino-terminal caspase activation and recruitment domains (CARDs) (6). RIG-I is an essential pathogen recognition receptor for various negative-strand viruses and Flaviviridae (8), including HCV (9, 10). RNA binding by RIG-I and MDA5 promotes a conformation change that potentiates signaling by the CARDs (9) to a CARD-containing adaptor protein termed IFN-β promoter stimulator 1 (IPS-1; for review, see Ref. 12), located on the mitochondrial membrane. IPS-1 confers downstream signaling to the latent cytoplasmic transcription factor IFN regulatory factor-3 (IRF-3) and nuclear factor-κB (NF-κB) (13–16). IRF-3 activation commences with its virus-induced phosphorylation, dimerization, and nuclear translocation, whereupon it binds to the promoter region of IRF-3-dependent genes, including IFN-β, IFN-stimulated gene (ISG) 15, and ISG56 (17–19). Parallel NF-κB activation induces a variety of proinflammatory genes and cooperates with IRF-3 to induce IFN-β expression (20). ISGs have antiviral and immunomodulatory activity that limit HCV infection (4, 21–23), and their expression marks the effector stage of the innate antiviral response (24).

The abbreviations used are: HCV, hepatitis C virus; NS, non-structural protein; IFN, interferon; RIG-I, retinoic acid-inducible gene I; MDA5, melanoma differentiation-associated gene 5; CARD, caspase activation and recruitment domain; IPS-1, interferon promoter stimulator 1; IRF-3, interferon regulatory factor-3; NF-κB, nuclear factor-κB; ISG, interferon-stimulated gene; aa, amino acid; Prot, NS3 protease domain; Hel, NS3 helicase domain; ΔArg, full-length NS3-4A lacking the arginine-rich region; S1165A, protease activate site mutation; SC, single-chain; WT, wild type; SenV, Sendai virus; RLU, relative luciferase units; CMV, cytomegalovirus; HEK cells, human embryonic kidney cells.

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HCV evades the innate antiviral response in part through the actions of the viral NS3/4A protease-helicase complex, which ablates RIG-I and MDA-5 signaling (6, 10, 25) through proteolytic cleavage and inactivation of IPS-1 (16, 26, 27). NS3/4A cleaves IPS-1 at Cys-508, thereby releasing it from the mitochondrial membrane and preventing its downstream activation of immune effectors (27). However, the NS3/4A structural motifs and features that direct IPS-1 cleavage and loss of RIG-I and MDA5 signaling have not been defined.

NS3/4A is a complex, bifunctional molecule (Fig. 1) that is essential for NS protein processing and viral RNA replication (28, 29). The amino-terminal region of NS3 contains a serine protease that non-covalently binds its cofactor, NS4A. NS4A is a small peptide necessary for efficient processing of the HCV polyprotein by NS3 and is thought to be involved in tethering the complex to intracellular membranes (30, 31). The carboxyl terminus of NS3 possesses a DEAD/H box helicase with nucleoside triphosphatase activity believed to participate in RNA unwinding during viral RNA replication (32). Its helicase activity was found to be positively modulated by the protease domain and NS4A (33–35). Conversely, conserved motif VI of the helicase domain has been shown to affect protease activity (33, 36–38). Moreover, structural studies of full-length NS3 indicate that the carboxyl terminus of the helicase domain may interact with the protease active site (39), suggesting a structural and functional interconnectivity of the two domains. NS3 also encodes an arginine-rich region shown to bind other host proteins (40, 41). NS3/4A interaction with its cellular targets could, therefore, occur through a variety of processes mediated by its helicase or protease domains.

Several peptidomimetic NS3/4A protease active site inhibitors are in preclinical development as HCV antiviral drugs (42). Future use of these compounds could represent an alternative or supplement to the current treatment regimen for HCV infection, IFN-α and ribavirin combination therapy, which is only effective in about 50% of patients (43). NS3/4A protease inhibitors may also exhibit immunomodulatory properties by removing the protease-dependent blockade to the innate antiviral response during HCV replication (23, 25), although the impact of IPS-1 in this process is not known. The current study was undertaken to define the domain structure and function of NS3/4A that regulates the host cell innate antiviral response.

**EXPERIMENTAL PROCEDURES**

Expression Cloning and Site-directed Mutagenesis—pFLAG NS3/4A and pFLAG NS3 were generated as described (23). Constructs (Fig. 1) containing NS3 helicase domain truncations at amino acids (aa) 1206 (protease domain alone, Prot), 1238, 1486, and 1501 as well as a protease domain deletion (NS3 aa 1207–1658, Hel) were made as follows. NS3 mutants were cloned by PCR (with primers containing a 5′ HindIII site and a 3′ XbaI site) using BD Advantage-HF 2 PCR kit (BD Biosciences) into pCR2.1 vector (TA cloning kit, Invitrogen). These constructs were then subcloned into the HindIII and XbaI sites of pFLAG-CMV-2 vector (Sigma). A protease active site mutant (NS3/4A S1165A) and deletion of an arginine-rich region (aa 1487–1501; NS3/4A Δ1487–1501) were constructed using QuickChange XL site-directed mutagenesis kit (Stratagene). Because NS4A forms part of the protease active site and is indispensable for full protease activity (31), a scheme was devised allowing expression of NS4A with the NS3 mutants. Expressing NS4A from a separate plasmid was not sufficient to result in efficient protease activity (data not shown). To circumvent this problem we created a primer containing an amino-terminal KpnI site, 12 amino acids of NS4A (aa 21–32; which intercalate into the NS3 protease active site), and a flexible linker (GSGS) region and subcloned this into the HindIII sites of the pFLAG NS3 1026–1206, creating a fully functional, single-chain (SC) NS3/4A protease domain (SC Protease). This strategy was similar to that described by Taremi et al. (44). The flexible linker was also subcloned into pFLAG NS3 (SC NS3), pFLAG NS3 1026–1238 (SC NS3 1026–1238), pFLAG NS3 1026–1486 (SC NS3 1026–1486), and pFLAG NS3 1026–1501 (SC NS3 1026–1501) to generate the SC constructs indicated in parentheses. NS3/4A, NS3/4A S1165A, and the SC Protease were subcloned into pEFl/Myc-His B (Invitrogen) to allow high efficiency expression of each. NS3/4A and NS3/4A S1165A were cut with HindIII, blunt-ended with Klenow fragment, digested with XbaI, and subcloned into the EcoRV and XbaI site of pEFl/Myc-HisB. The SC Protease was subcloned into the SacI and XbaI sites of pEFl/Myc-HisB. Primer sequences for all constructs are available upon request.

Plasmids and Transfection—pFLAG NS4A, pFLAG NS5A, and pFLAG NS5A-B were previously described (23, 25, 45). pEFBos-RIG-I, pEFBos-N-RIG, and pEFBos-C-RIG expression plasmids (7) as well as pEFBos-MDA5, pEFBos-N-MDA5, and pEFBos-C-MDA5 (6) are amino-terminal FLAG-tagged. pEFlBos, pEFlBos-IPS-1, pMyc-IPS-1, pIRF3-ΔN, pIFN-β-luc, pCMV-Renilla-luc, and pPRDII-luc were described elsewhere (23, 25, 27, 46). GenElute endotoxin-free plasmid midiprep kit (Sigma) was used to prepare plasmid DNA for transfections. Transfections were performed using FuGENE 6 transfection reagent (Roche Applied Science) or Lipofectamine 2000 (Invitrogen) according to manufacturer’s protocol.

Cells, Viruses, and Protease Inhibitors—Huh7 human hepatoma cells (47), Huh7.5, and HEK 293 cells were propagated in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% fetal bovine serum (HyClone), 1× nonessential amino acids, 2 mm L-glutamine, antibiotic-antimycotic solution, and 1 mm sodium pyruvate as described previously (25). Huh7.5 cells, a subline of Huh7 cells kindly provided by C. Rice, contain a defect in RIG-I and do not signal through the RIG-I pathway (10). UNS3/4A and UNS3 cells (a gift from D. Moradpour) are osteosarcoma cells conditionally expressing HCV 1a NS3/4A or HCV 1a NS3, respectively, and were described previously (30, 48). HP and K2040 cells are Huh7 cells harboring cell culture-adapted subgenomic HCV 1b replicons (11, 22). Cell culture methods for UNS3/4A, HP, and K2040 cells have been described elsewhere (11, 22, 23). For Sendai virus infection (Cantell strain, Charles River Laboratories), 1×10⁵ or 2×10⁴ cells/well were seeded into 12- or 48-well dishes respectively, and where indicated, transfected with expression constructs. Twenty-four hours after seeding or transfection, cells were washed in phosphate-buffered saline and mock-infected or infected with 100 hemagglutinin units of Sendai virus per ml of serum-free media for 1 h. Three volumes of culture media were
then added to wells, and cells were incubated another 17 or 19 h as described (23). HCV 2a stock was produced as described previously (27). For HCV infection ~4 × 10^4 cells seeded in a 12-well plate were infected at a multiplicity of infection of 1 with HCV 2a in culture media for 3 h and washed in phosphate-buffered saline, and culture media was added. Cells were then incubated until collection at the indicated time points. SCH6 (kindly provided by Schering-Plough) (23) and ITMN-C (a gift from Dr. S. Seiwert at Intermune, Brisbane, CA) protease inhibitor treatments were conducted by replacing culture medium with medium containing 20 μM SCH6 or the indicated amount of ITMN-C.

**Confocal Microscopy**—2 × 10^4 cells were plated in 4-chamber slides, 200 ng of the indicated expression plasmids were transfected, and cells were collected 12 or 24 h later. Cells were fixed in 4% paraformaldehyde and blocked in phosphate-buffered saline containing 10% fetal bovine serum and 1% Triton X-100. Antibodies against the FLAG epitope (Sigma), IPS-1 (a gift from Z. Chen), IRF-3 (kindly provided by M. David), and HCV NS3 NCL (Novacawa laboratories) were used in combination with Alexa 488- or Alexa 594-conjugated secondary antibodies (Invitrogen). Slides were mounted with Prolong Gold (Invitrogen) and examined using a Zeiss Pascal laser scanning confocal microscope and Zeiss LSM software. The images shown are ~7 μm optical slices.

**Immunoblotting and Immunoprecipitation Analysis**—Immunoblots were performed as described (23). Blots were probed with antibodies against HCV NS3 A6 (kindly provided by J. Ye), ISG56 (a gift from G. Sen), ISG15 (kindly provided by A. Haas), Sendai virus (a gift from I. Julkunen), actin (Santa Cruz Biotechnology), NS3 goat polyclonal antibody (U. S. Biologicals), Phospho-IκBα (Cell Signaling), IκBα (Santa Cruz Biotechnology), glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology), HCV (a gift from W. Lee), ISG48 (Santa Cruz Biotechnology), Myc epitope (Bethyl Laboratories), or those described above. Anti-human and anti-goat horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson Laboratories; anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were obtained from PerkinElmer Life Sciences. Coimmunoprecipitation was performed using FLAG M2-agarose beads (Sigma) as described (45), and an immunoblot analysis was conducted.

**Promoter Luciferase Reporter Assays**—2 × 10^4 cells/well were seeded into 48-well dishes and cotransfected with 25 ng of pIFN-β-luc or pPRDII-luc and 5 ng of pCMV-Renilla along with the indicated amounts of expression constructs. Cells were then cultured alone or subjected to virus infection and harvested 20 h later for luciferase assay as described (23). Results were calculated relative to Renilla luciferase values.

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assay analysis of NF-κB DNA binding activity was conducted on UNS3-4A and UNS3 nuclear extracts as described (46). Briefly, cells were grown in the presence (−NS3-4A) or absence (+NS3-4A, +NS3) of 1 mg/ml tetracycline, and protease inhibitor (20 μM SCH6 or 10 μM ITMN-C) Me2SO (mock treatment) was added to the media for 24 h. Cells were then mock-infected or infected with 100 hemagglutinin units/ml of Sendai virus, and infection was allowed to proceed 18 h. As a positive control for NF-κB activation, some wells were washed twice in phosphate-buffered saline and treated for 30 min with 20 μg/ml tumor necrosis factor-α in serum-free media. Antibody supershifts were performed using antibodies against NF-κB p50 or p65 subunits (Santa Cruz Biotechnology). NF-κB consensus and mutant oligonucleotides were obtained from Santa Cruz Biotechnology. Non-radiolabeled, cold competitor was used as a negative control.

**Statistical Analysis**—Differences among treatments were examined using unpaired t-tests and were considered statistically significant when p < 0.02 and where marked with an asterisk. Statistical differences in which p ≤ 0.001 were marked with two asterisks.

**RESULTS**

**Characterization of NS3-4A Constructs**—To determine the minimal region of NS3-4A that could modulate the innate antiviral response, a series of FLAG epitope-tagged NS3 truncation and deletion mutants were constructed (Fig. 1). To assess indi-
FIGURE 2. Comparison of HCV NS3-4A construct regulation of innate immune response signaling. A, expression and cleavage of a NS5A/B substrate by NS3-4A constructs. Huh7 cells were co-transfected with vector or a NS5A/B polyprotein construct and the NS3 expression construct indicated above each lane. Twenty-four hours later cells were collected, and immunoblot analysis was performed. Unless indicated, constructs were expressed from the pFLAG-CMV2 vector. Blots were probed with antibodies against NS3 (upper panel), HCV (immune patient serum; middle panel), FLAG (M2) (far right lower panel), or actin as indicated. Because NS3 ΔArg, Protease (Prot), SC 1238, SC 1486, and SC 1501 were expressed at low levels from the corresponding constructs, the far right panel showing a blot with higher input protein was included to demonstrate protein expression (lanes 14–18). Arrows to the left mark the positions of NS3 proteins, NS5A/B polyprotein, or the NS5A cleavage product. NS3, S1165A, and helicase (Hel) proteins are shown in both upper and middle panels. The identical positions of the Protease and the SC Protease (SC Prot) are marked by a single arrow. Arrows at the right indicate the nearly identical positions of SC 1486 and SC 1501 (upper) or mark the position of the SC 1238 construct (lower). These abbreviations for the NS3 constructs are similarly used in all subsequent figures. B, -fold activation of the IFN-β-luc reporter in response to Sendai virus infection. Huh7 cells were co-transfected with the indicated NS3/4A mutant, CMV-Renilla, and IFNβ-luc (containing an IRF-3 dependent promoter element) plasmids for 24 h. Cells were then mock-treated (empty bars) or infected with Sendai virus (black bars), and infection was allowed to proceed for 18 h. Luciferase assay was performed, and results were normalized to Renilla values and mock infection. Bars show the mean relative luciferase units and S.D. Differences between Sendai virus-infected treatments were subjected to unpaired t test. Single asterisks indicate p < 0.02; double asterisks indicate p < 0.001. C, effect of NS3-4A mutants on virus-induced ISG expression. Huh7 cells were transfected with 500 ng of the indicated plasmid. Twenty-four hours later cells were infected with Sendai virus (+) or mock-infected (−) for 18 h, and samples were collected for immunoblot analysis. Arrows mark the positions of the respective NS3 proteins. Results represent at least two independent experiments.
idual domains of NS3 and NS4A in innate immune regulation, we produced constructs encoding NS4A alone (23), the NS3 protease domain (Protease), helicase domain (Helicase), or full-length NS3 lacking the arginine-rich region (ΔArg) as well as full-length NS3/4A harboring a protease active site mutation (S1165A) as a negative control. These were placed under the control of the CMV-Immediate-Early promoter. Previous studies have identified a 12-amino acid region of NS4A (aa 21–32) that intercalates into the NS3 protease active site and is indispensable for full protease activity (31). To express full NS3/4A protease activity from a single plasmid, we attached these 12 aa of NS4A to each NS3 mutant via a flexible linker, creating a fully functional, SC NS3/4A protease encoding full-length NS3 (SC NS3), progressive deletions of the NS3 helicase domain (SC 1501, SC 1486, SC 1238), or the protease domain alone (SC Protease).

Use of similar SC constructs as a tool for examining the NS3/4A protease structure and function has been well established in crystallization studies (39) and studies identifying small molecule protease inhibitors (49). Moreover, Taremi et al. (44) found that such SC Protease constructs displayed protease activity comparable with wild type (WT) NS3/4A. As shown in Fig. 2A, when coexpressed in Huh7 cells, a N55AB polypeptide substrate was efficiently cleaved by WT NS3/4A, SC Protease, SC NS3, ΔArg, SC 1238, SC 1486, and SC 1501 constructs (middle panel, lanes 4, 7, 8, 14, 16–18, respectively). The N55AB polypeptide was not cleaved by NS4A, NS3/4A S1165A, Helicase, SC NS3 S1165A, or protease (lanes 3, 5, 6, 9, 15, respectively) and was only partially cleaved by NS3 alone (Fig. 2A, lane 2). When expressed from the elongation factor-1 promoter expression plasmid (pEF), pEF WT NS3/4A, and pEF SC Protease but not pEF NS3/4A S1165A also mediated NS5AB cleavage (compare lanes 11–13). These results validated the expression and cleavage activity of the NS3 constructs and show that efficient NS3/4A proteolytic activity requires the protease domain and its NS4A cofactor but does not require the NS3 helicase domain.

The Helicase Domain Is Dispensable for Inhibition of Innate Defense Signaling—To determine the NS3 structural requirements for inhibition of host antiviral signaling, we evaluated each construct for its ability to regulate IFN-β promoter induction in response to Sendai virus infection. It should be noted that Sendai virus and HCV trigger the innate antiviral response through similar mechanisms that are blocked by NS3/4A (10, 23, 25). WT NS3/4A significantly suppressed Sendai virus induction of IFN-β promoter activity comparable with a dominant-negative IRF-3 control construct (Fig. 2B, IRF3–ΔN). However, constructs lacking NS4A and/or the intact protease domain of NS3 failed to block signaling to the IFN-β promoter. Of note, the SC NS3 construct, similar to that used in crystallization studies (39), mediated a weaker albeit significant suppression of virus signaling to the IFN-β promoter. Further studies revealed that the SC NS3 protein did not localize to membrane-bound compartments (data not shown), suggesting its decreased ability to block viral activation of the IFN-β promoter could be due to aberrant localization. We also assessed Sendai virus-induced ISG expression in cells transfected with constructs encoding WT NS3/4A, NS3/4A S1165A, or the SC Protease. As shown in Fig. 2C, WT NS3/4A and the SC Protease suppressed ISG production, but ISGs were induced in cells expressing the protease-deficient NS3/4A S1165A mutant. Together these results indicate that the fully functional NS3 protease domain with its minimal NS4A cofactor is necessary for suppressing the host cell signaling of IFN-β promoter expression.

The Functional NS3/4A Protease Domain Inhibits Signaling by RIG-1 and MDA5—NS3/4A suppresses RIG-1 signaling of the innate antiviral response (25). We verified that our WT NS3/4A construct could suppress RIG-1-enhanced virus signaling as well as constitutive signaling by ectopic N-RIG (encoding the CARD domains alone) (Fig. 3A). We then assessed regulation of RIG-1 signaling to the IFN-β promoter by a subset of our NS3 constructs. As shown in Fig. 3B, the SC Protease and ΔArg constructs suppressed N-RIG signaling of the IFN-β promoter to an extent similar to WT NS3/4A. Expression of NS3 alone, NS4A, NS3/4A S1165A, or the NS3 helicase domain had no significant effect on promoter activation. Moreover, when expressed in Huh7 cells, WT NS3/4A and the SC Protease suppressed the expression of the IRF-3 target genes, ISG56 and ISG15, but NS3/4A S1165A and the NS3 protease domain failed to suppress IRF-3 target gene expression (Fig. 3C).

We also examined NS3 construct regulation of MDA5 signaling. To determine the effect of MDA5 regulation in the absence of RIG-1, we utilized Huh7.5 cells lacking RIG-1 function (10). As shown in Fig. 4A, Huh7.5 cells failed to activate the IFN-β promoter in response to Sendai virus infection, but ectopic MDA5 complemented this defect. Moreover, we verified that our WT NS3/4A construct could suppress WT MDA5 or N-MDA5 (encoding the CARD domains) signaling to the IFN-β promoter in Huh7.5 cells. To determine the minimal domain of NS3/4A necessary to block MDA5 signaling, we evaluated the ability of constructs encoding WT NS3/4A (positive control), NS3/4A S1165A (negative control), the SC Protease, and Protease to regulate signaling to the IFN-β promoter by N-MDA5 (Fig. 4B). The SC Protease inhibited promoter activation to the same extent as WT NS3/4A and the IRF3–ΔN control, but neither the NS3 protease domain alone nor NS3/4A S1165A blocked promoter signaling. Both WT NS3/4A and the SC Protease suppressed ISG15 expression in response to ectopic N-MDA5 (Fig. 4C). Taken together, these results demonstrate that the SC Protease is sufficient to block signaling by both RIG-1 (Fig. 3) and MDA5 (Fig. 4) in a process that requires the NS3 protease domain and its NS4A cofactor. The NS3 helicase domain, however, is dispensable for innate immune response regulation.

The SC Protease Blocks Activation of IRF-3 and Exhibits a Subcellular Localization Pattern Similar to WT NS3/4A—To verify that the SC Protease was sufficient for inhibition of IRF-3 activation, we examined IRF-3 and SC Protease distribution in transfected Huh7 cells. Sendai virus infection of cells caused the redistribution of IRF-3 from the cytoplasm to the nucleus (23), consistent with its virus-induced activation, but this response was blocked in control cells expressing WT NS3/4A (Fig. 5). Expression of the SC Protease also blocked IRF-3 activation, maintaining it in a cytoplasmic context during Sendai virus infection. Notably, despite the lack of the putative amino-terminal membrane-anchoring motif of NS4A (30), the SC Protea-
ase localized to a subcellular compartment similar to WT NS3-4A. Thus, the NS3 protease domain and a minimal NS4A cofactor are sufficient to confer proper localization and inhibition of IRF-3 activation.

Regulation of NF-κB by NS3-4A—RIG-I and MDA5 signal the parallel activation of IRF-3 and NF-κB (6), and we sought to

**FIGURE 3. Effect of the NS3-4A protease on RIG-I signaling.** A, effect of NS3-4A on RIG-I, N-RIG, and C-RIG induction of the IFN-β promoter. Huh7 cells were co-transfected with 20 ng of the indicated plasmid, 10 ng of CMV-RepH1, 35 ng of IFNβ-luc, and 80 ng of WT NS3-4A or vector plasmid. Twenty-four hours later cells were mock (empty bars)- or Sendai virus (black bars)-infected, harvested 20 h later, and luciferase assay was conducted. Luciferase results were normalized to Renilla values. Bars show the mean relative luciferase units and S.D. B, effect of NS3-4A mutant constructs on N-RIG-mediated stimulation of the IFN-β promoter. Huh7.5 cells were transfected with CMV-RepH1, IFNβ-luc, the NS3-4A constructs shown, and vector (empty bars) or N-RIG (black bars), collected, and analyzed as in A. S.D. is indicated by error bars. Unpaired t test was used to determine statistical significance of results. Asterisks indicate p < 0.001. C, immunoblot analysis of ISG production in response to N-RIG stimulation. Huh7 cells were co-transfected with 400 ng of NS3-4A mutants and 100 ng of vector (−) or N-RIG (+), and production of ISGs was examined. Arrows mark the positions of NS3 proteins. Results are representative of at least three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**FIGURE 4. NS3-4A regulation of MDA5 signaling.** A, effect of NS3-4A on MDA5, N-MDA5, and C-MDA5 induction of the IFN-β promoter. Huh7.5 cells, lacking a functional RIG-I, were co-transfected with 20 ng of the indicated MDA5 variant, 10 ng of CMV-RepH1, 35 ng of IFNβ-luc, and 80 ng of WT NS3-4A or vector plasmid. Twenty-four hours later cells were mock (empty bars)- or Sendai virus (black bars)-infected and harvested 20 h later, and luciferase assay was conducted. Luciferase results were normalized to Renilla values. Bars show the mean relative luciferase units and S.D. B, effect of NS3-4A mutant constructs on N-MDA5-mediated stimulation of the IFN-β promoter. Huh7.5 cells were transfected with CMV-RepH1, IFNβ-luc, the NS3-4A constructs shown, and vector (empty bars) or N-MDA5 (black bars), collected, and analyzed as in A. S.D. is indicated by error bars. Unpaired t test was used to determine statistical significance of results. Asterisks indicate p < 0.001. C, immunoblot analysis of ISG production in response to N-MDA5 stimulation. Huh7 cells were co-transfected with 400 ng of NS3-4A mutants and 100 ng of vector (−) or N-RIG (+), and production of ISGs was examined. Arrows mark the positions of NS3 proteins. Results are representative of at least three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
examine the effect of NS3-4A and its requirement for protease activity on regulation of NF-κB function. We performed an electrophoretic mobility shift assay to measure NF-κB binding to a target probe in nuclear extracts of cells treated with tumor necrosis factor-α (TNF-α; control) or infected with Sendai virus. Tumor necrosis factor-α or Sendai virus infection triggered NF-κB binding (Fig. 6A, lanes 1 and 5, respectively). Expression of NS3-4A (lane 11) but not NS3 alone (lane 17) blocked Sendai virus–induced NF-κB binding. The NS3-4A blockade of NF-κB binding was relieved when cells were treated with SCH6 or ITMN-C peptide mimetic active site inhibitors of the NS3-4A protease (lanes 13 and 15, respectively). These results were verified by luciferase reporter assay, in which WT NS3-4A suppressed Sendai virus induction of an NF-κB–dependent PRDII–luciferase promoter and its enhancement by ectopic RIG-I or MDA5 (Fig. 6B). To determine the domain of NS3-4A necessary for this effect, HEK 293 cells were transiently transfected with the indicated NS3 construct followed by Sendai virus infection (Fig. 6C, left panel) or were cotransfected with a construct encoding constitutively active N-RIG to trigger PRDII promoter signaling (Fig. 6C, right panel). The SC Protease inhibited activation of the PRDII promoter element in response to both Sendai virus infection and ectopic N-RIG. Similar results were observed when the PRDII promoter element was stimulated by ectopic N-MDA5 (data not shown). We also examined the influence of NS3-4A upon virus–induced phosphorylation of the NF-κB inhibitor, IκBo. As shown in Fig. 6D, Sendai virus infection of HEK 293 cells induced the phosphorylation of IκBo within 20 h post-infection, but expression of either WT NS3-4A or the SC Protease but not NS3-4A S1165A prevented virus–induced IκBo phosphorylation (compare lanes 2, 4, 6, and 8). These results link HCV control of NF-κB function with NS3-4A protease domain regulation of RIG-I and MDA5.

The Protease Domain of NS3-4A Is Sufficient to Target and Cleave IPS-1—RIG-I and MDA5 signal IRF-3 and NF-κB activation through the IPS-1 adaptor protein (13), which is targeted and cleaved by NS3-4A to inactivate signaling (16, 27). To determine whether the functional NS3-4A protease domain was sufficient to physically target and cleave IPS-1, we examined complex formation between IPS-1 and the SC Protease or a protease–defective derivative (Fig. 7A, wt and S4, respectively) in transfected HepG2 cells. Immunoprecipitation studies revealed that the functional SC Protease and an SC Protease S1165A mutant each bound to IPS-1 (Fig. 7A, lanes 2 and 3). When compared with the SC Protease, we observed an apparent reduction in the amount of SC Protease S1165A mutant bound to IPS-1 in these experiments. Although this could be due to protein expression or stability differences between the SC Protease constructs, it could also be explained by potential changes in the active site conformation caused by the serine to alanine switch, possibly resulting in decreased affinity for cleavage substrates. Importantly, the interaction between IPS-1 and the SC Protease was not disrupted by ITMN-C protease inhibitor treatment, and the SC Protease maintained an interaction with the cleaved form of IPS-1 (Fig. 7A, lanes 3 and 4). Ectopic IPS-1 is a potent trigger of the innate antiviral response (13), and we found that it triggered endogenous ISG15 expression in HepG2 cells (Fig. 7B). This reaction was blocked by both the SC Protease and WT NS3-4A concomitant with IPS-1 cleavage. Thus, the SC Protease is sufficient to target and cleave IPS-1. We also found that the SC Protease suppressed IFN-β promoter induction by ectopic IPS-1 as well as WT NS3-4A (Fig. 7C). Furthermore, the SC Protease blocked NF-κB–dependent induction of the PRDII promoter element by ectopic IPS-1, but neither NS3 nor the protease or helicase domains affected promoter induction (Fig. 7D). Thus, the targeted cleavage of IPS-1 by the NS3-4A protease domain inhibits downstream signaling to IRF-3 and NF-κB.

NS3-4A Protease Inhibitor Prevents Cleavage and Restores Localization of IPS-1—To determine the influence of the NS3-4A protease and protease inhibitor treatment on IPS-1 localization and function, we examined the subcellular distribution of IPS-1 in the presence of NS3-4A alone and in the context of HCV RNA replication when cells were treated with ITMN-C. As shown in Fig. 8A, IPS-1 showed a characteristic thread–like appearance in the cell cytoplasm, consistent with its mitochondrial membrane localization (27). However, in the presence of NS3-4A, this localization was disrupted, and IPS-1 exhibited the diffuse cytoplasmic staining pattern previously described (26, 27). When the cells were exposed to the ITMN-C protease inhibitor for 24 h, IPS-1 was restored to its proper localization in the presence of NS3-4A (Fig. 8A). We further examined this regulation in the context of HCV RNA replication in two cell lines harboring genetically distinct HCV replicons that are differentially resistant (HP replicon) and sensitive (K2040 replicon) to the antiviral actions of IFN therapy (11, 22). In the presence of NS3, IPS-1 membrane localization was disrupted, whereas K2040 cells with no visible NS3-4A staining typically retained a normal pattern of IPS-1 localization (Fig. 8B, mock–treated). When the cells were treated with ITMN-C, IPS-1 returned to a native distribution within a 24–h time interval.
FIGURE 6. Characterization of NF-κB signaling regulation by the NS3-4A protease. A, effect of NS3-4A and protease inhibitor therapy on Sendai virus-induced activation of NF-κB. U87 and U83 cells were cultured to suppress (~WT NS3-4A) or induce NS3-4A (+ WT NS3-4A) or NS3 (+ NS3) expression alone or in the presence of 20 μM SCH6 or 10 μM ITMN-C. HCV NS3-4A peptidomimetic protease active site inhibitors. Twenty-four hours later cells were either mock (M) or Sendai virus (SV)-infected for 18 h, and nuclear fractions were collected and analyzed by electrophoretic mobility shift assay. As a positive control, cells were treated with 20 ng/ml tumor necrosis factor-α (TNF-α) for 30 min. Antibody supershifts using antibodies to the p50 and p65 subunit of NF-κB, incubation with cold competitor (lane 18), or mutated binding site control DNA probe (NF-κB site; lane 19) confirm the specificity of binding. B, effect of NS3-4A on activation of an NF-κB-dependent promoter element. HEK 293 cells were transfected with 25 ng of a PRDII-luciferase reporter construct, 10 ng of CMV-Renilla, 80 ng of vector or WT NS3-4A, and 20 ng of the constructs as shown. Twenty-four hours later cells were mock (empty bars) or Sendai virus-infected (black bars) for 20 h, then collected and analyzed by luciferase assay. Bars show the mean relative luciferase units and S.D. C, effect of the SC Protease on Sendai virus- and N-RIG-mediated activation of the NF-κB-dependent promoter. HEK 293 cells were transfected with vector or the indicated NS3-4A construct, PRDII-luciferase reporter, and CMV-Renilla for 24 h followed by mock (empty bars) or Sendai virus (black bars) infection for 20 h (left panel) or co-transfection with vector (empty bars) or N-RIG (black bars) for 16 h (right panel). Results were analyzed by luciferase assay. Bars show the mean relative luciferase units and S.D. Data shown represent three separate experiments. D, effect of NS3-4A constructs on phosphorylation of IκBα. The inhibitor of NF-κB, the inhibitor of NS3-4A. HEK 293 cells were transfected with 500 ng of the indicated constructs for 24 h, then infected with Sendai virus for 20 h. Cell lysates were collected and analyzed by immunoblotting. Arrows indicate the positions of the various NS3 proteins. Unless stated otherwise, experiments are representative of two independent trials. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Structure-Function Analysis of HCV NS3-4A

**A**

Myc-IPS1: + + + +
ITMN-C: + wt wt wt
FLAG SC Prot: SA wt wt
Myc-IPS1: + + + +
NS3: (S1165A, reduced exposure)
Huh7 cells were cotransfected with Myc-IPS1 and vector, FLAG-tagged SC Protease S1165A (S1A), or WT SC Protease (WT) in the presence (+) or absence (−) of 1 μM ITMN-C protease inhibitor for 24 h. Cell lysates were collected and immunoprecipitated using FLAG-conjugated beads followed by immunoblot analysis of supernatant and pellet fractions. The cleaved form of IPS-1 is visible as a faster migrating species (lane 3). Reduced exposure of the NS3 panel demonstrates that a lower amount of the SC Protease S1165A mutant was expressed in the corresponding cells as compared with the SC Protease WT construct.

**B**

C-Vector
IPS-1

**C**

Huh7 cells

**D**

HEK 293 cells

**FIGURE 7. Targeting and cleavage of IPS-1 by the SC protease.** A, co-immunoprecipitation analysis of the SC Protease and IPS-1. Huh7 cells were cotransfected with Myc-IPS1 and vector, FLAG-tagged SC Protease S1165A (S1A), or WT SC Protease (WT) in the presence (+) or absence (−) of 1 μM ITMN-C protease inhibitor for 24 h. Cell lysates were collected and immunoprecipitated using FLAG-conjugated beads followed by immunoblot analysis of supernatant and pellet fractions. The cleaved form of IPS-1 is visible as a faster migrating species (lane 3). Reduced exposure of the NS3 panel demonstrates that a lower amount of the SC Protease S1165A mutant was expressed in the corresponding cells as compared with the SC Protease WT construct.

B, cleavage of overexpressed IPS-1 and inhibition of downstream signaling by the SC Protease. Huh7 cells were cotransfected with 125 ng of vector or Myc-IPS1 and 375 ng of the NS3-4A constructs indicated for 24 h, and cell lysates were collected and subjected to immunoblotting. The positions of full-length and cleaved forms of IPS-1 or S1165A and wild type forms of NS3 are indicated. Panels C and D, Huh7 (C) or HEK 293 (D) cells were transfected with IFN-β-luc or PRDII-luc, respectively, plus CMV-Renilla, 75 ng of the NS3-4A mutants shown, and 25 ng of vector (empty bars) or IPS-1 (black bars). Panel C includes cells transfected with dominant-negative IRF-3 (IRF3-DN) as a control. Cells were collected 20 h later, and luciferase reporter assays were performed. Error bars indicate S.D. Experiments were repeated at least two times.

We examined the effect of protease inhibitor treatment on IPS-1 and the innate antiviral response against HCV, we examined ITMN-C treatment of Huh7 cells harboring a HCV 2a replicon or infected with the JFH1 strain of HCV 2a. Treatment of HCV 2a replicon cells resulted in alteration of endogenous IPS-1 from a predominantly cleaved form to its native, full-length form concomitant with induction of ISG56 expression over a 48-h time course (Fig. 9, A and B). To verify these results in the context of an actual HCV infection, Huh7 cells were infected with JFH1 (50) at a multiplicity of infection of 1 for 48 h followed by 24, 36, or 48 h of ITMN-C treatment (Fig. 9C). After 48 h of HCV infection, endogenous IPS-1 levels were reduced with a large portion of the remaining protein present as the cleaved form, consistent with previously published data (27). Within 24 h of ITMN-C treatment and throughout the time course, we observed a recovery of full-length IPS-1 levels with corresponding induction of ISG56 expression and reduction in viral protein abundance (Fig. 9C).

**DISCUSSION**

Our results show that the NS3 protease domain and minimal NS4A cofactor are sufficient to inhibit antiviral signaling through RIG-I and MDA5 and block activation of the downstream transcription factors IRF-3 and NF-κB. This is accomplished by protease domain targeting and cleavage of IPS-1. Our data indicate that the NS3 helicase domain does not play a role in innate immune regulation and inactivation of IPS-1. Moreover, infection studies demonstrated for the first time that NS3-4A protease inhibitor therapy of HCV infection can effectively remove the NS3-4A blockade to the innate immune response, restoring RIG-I signaling to IPS-1 and activation of ISG expression in infected cells.

The protease domain of NS3 has been shown to mediate internal interactions with the carboxyl-terminal helicase domain, in which the latter has been suggested to potentiate protease activity (37–39). We found that the helicase domain was dispensable for NS3-4A control of the innate antiviral response and signaling by RIG-I and MDA5. That the helicase domain is not involved in innate immune control is further supported by the observations that the SC Protease, lacking the complete helicase domain of NS3, mediated binding and cleavage of IPS-1 similar to WT NS3-4A. Taken together, these results imply that the NS3 helicase domain does not play a role in directing NS3-4A interactions with IPS-1. Importantly, our results show that neither full-length NS3 nor the NS3 protease domain alone could block signaling by RIG-I or MDA5 when expressed in the absence of NS4A; however, NS4A alone does not regulate the innate immune response. In contrast, each NS3 construct efficiently suppressed signaling when expressed as a SC construct with aa 21–32 of NS4A, encoding the NS4A central core region integral to NS3 protease activity (31). Previous work has associated NS4A aa 2–19 with localization of NS3 to intracellular membranes (30). Despite lacking this domain of NS4A, our SC constructs effectively cleaved NS5AB and blocked innate immune signaling. Moreover, when expressed in Huh7 cells, the SC Protease construct demonstrated a subcellular distribution pattern equivalent to WT NS3-4A that similarly supported IPS-1 targeting. We conclude that whereas NS4A is required for full proteolytic action of NS3, localization and IPS-1 targeting by NS3-4A are controlled by residues located within the NS3 protease domain and/or the NS4A central core.
IPS-1 proteolysis (Fig. 7A). Under these conditions ITMN-C is expected to occupy the protease active site and catalytic residues (53). Thus, the SC Protease may bind IPS-1 independently of substrate occupancy in the protease active site. This suggests a model in which NS3-4A may first interact with IPS-1 through protease domain residues outside the catalytic pocket, thereby positioning it for subsequent proteolysis. NS3-4A exhibits a shallow substrate binding cleft that might accommodate other protein substrates (39, 54, 55). Indeed, the TIR domain-containing adaptor-inducing interferon (TRIF) adaptor protein has been identified as an in vitro substrate of NS3-4A proteolysis (56). In this case residues within the 3/10 helix of NS3 located adjacent to the protease active site bind to a region of TRIF near the cleavage site (57). This shared relationship between protein interaction and cleavage could indicate that NS3-4A may target and interact with TRIF and IPS-1 through similar processes. The mechanics of the NS3-4A-IPS-1 interaction are currently under investigation.

Our study demonstrates that NS3-4A proteolysis of IPS-1 blocks signaling by both RIG-1 and MDA5 and ablates virus activation of downstream IRF-3 and NF-κB, thus confirming that IPS-1 serves as the essential adaptor for RIG-1 and MDA5 signaling. In the case of NF-κB, we found that the NS3-4A or SC Protease blockade of NF-κB activation was concomitant with suppression of IκB-α phosphorylation and that NF-κB activation could be fully restored when cells were treated with a NS3-4A protease inhibitor. Thus, the RIG-1/MDA5 axis may signal NF-κB activation through a canonical process of kinase phosphorylation of IκB and unmasking of NF-κB DNA binding activity (58). Similar to the control of IRF-3 signaling (23, 25, 27), these processes of NF-κB signaling are disrupted by IPS-1 proteolysis. As a signaling adaptor protein, IPS-1 may serve to recruit factors of IRF-3 and/or NF-κB signaling. This idea is supported by studies that have demonstrated IPS-1 interactions with various signaling components, including the IKKε protein kinase (59), TRAF3 (60), and FADD (13) into a complex with IPS-1. In the context of HCV infection, an IPS-1 signaling complex would

The amino-terminal 21 aa of NS3 consist of several highly hydrophobic residues, and crystal structure analysis showed that this region extends away from the rest of the protease (51), suggesting it may be involved in membrane tethering. Additionally, a recent study demonstrated a mitochondrial membrane localization of NS4A in the context of HCV RNA replication (52), implicating NS4A in directing NS3 to the mitochondria and site of interaction with IPS-1. These possibilities are presently being examined.

We found that the SC Protease could bind IPS-1 and that this interaction was maintained in the presence of the peptidomimetic protease active site inhibitor ITMN-C, which abrogated
possibly be disrupted through the targeted proteolysis of IPS-1 by NS3-4A to ablate a wide range of immune signaling action.

NS3-4A protease inhibitors are being developed for use as future HCV therapeutics (42), and our studies define a novel role for these compounds as immunomodulatory agents. NS3-4A cleavage of IPS-1 during HCV RNA replication and infection results in IPS-1 release from the mitochondrial membrane and diffuse redistribution throughout the cytoplasm with associated loss of host immune signaling (27). We found that ITMN-C treatment of cells harboring distinct HCV 1b replicons mediated a potent inhibition of NS3-4A, restoring the native distribution of endogenous IPS-1 as early as 12 h after treatment regardless of the differential sensitivities of each HCV replicon to IFN-α therapy (11, 22). Moreover, protease inhibitor treatment of Huh7 cells harboring an HCV 2a replicon or infected with the HCV 2a JFH1 clone caused the accumulation of IPS-1 from a NS3-4A-cleaved form to a full-length form and related induction of ISG56 expression. These results indicate that 1) ITMN-C treatment and inhibition of NS3-4A protease function permits a rapid restoration of IPS-1 function, innate immune response signaling, and immune action directly within HCV-infected cells, 2) endogenous HCV RNA is a potent trigger of RIG-I signaling in infected cells (9), and 3) RIG-I-responsive genes, including ISG56 (22), may directly suppress HCV replication. We conclude that NS3-4A protease inhibitors possess immunomodulatory activity through prevention of IPS-1 proteolysis and restoration of RIG-I signaling.

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