Control of Innate Immune Signaling and Membrane Targeting by the Hepatitis C Virus NS3/4A Protease Are Governed by the NS3 Helix α0

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Published Ahead of Print 11 January 2012.
Control of Innate Immune Signaling and Membrane Targeting by the Hepatitis C Virus NS3/4A Protease Are Governed by the NS3 Helix $\alpha_0$

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Hepatitis C virus (HCV) infection is sensed in the host cell by the cytosolic pathogen recognition receptor RIG-I. RIG-I signaling is propagated through its signaling adaptor protein MAVS to drive activation of innate immunity. However, HCV blocks RIG-I signaling through viral NS3/4A protease cleavage of MAVS on the mitochondrion-associated endoplasmic reticulum (ER) membrane (MAM). The multifunctional HCV NS3/4A serine protease is associated with intracellular membranes, including the MAM, through membrane-targeting domains within NS4A and also at the amphipathic helix $\alpha_0$ of NS3. The serine protease domain of NS3 is required for both cleavage of MAVS, a tail-anchored membrane protein, and processing the HCV polyprotein. Here, we show that hydrophobic amino acids in the NS3 helix $\alpha_0$ are required for selective cleavage of membrane-anchored portions of the HCV polyprotein and for cleavage of MAVS for control of RIG-I pathway signaling of innate immunity. Further, we found that the hydrophobic composition of NS3 helix $\alpha_0$ is essential to establish HCV replication and infection. Alanine substitution of individual hydrophobic amino acids in the NS3 helix $\alpha_0$ impaired HCV RNA replication in cells with a functional RIG-I pathway, but viral RNA replication was rescued in cells lacking RIG-I signaling. Therefore, the hydrophobic amphipathic helix $\alpha_0$ of NS3 is required for NS3/4A control of RIG-I signaling and HCV replication by directing the membrane targeting of both viral and cellular substrates.

Materials and Methods

Cell culture and viruses. Huh7 and Huh7.5 cells are human hepatoma cells that have been described previously (30). These cells, as well as HEK293 cells, were propagated in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% fetal bovine serum (HyClone), as described previously (30). Sendai virus strain Cantell was obtained from Charles River Laboratories.

Immunoblotting and immunoprecipitation. Cells were lysed in a modified radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 0.5% sodium deoxycholate, and 1% Triton X-100) supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail II (Calbiochem). After harvest, protein was resolved by SDS-PAGE, transferred to nitrocellulose membranes in a 25 mM Tris-192 mM glycine-0.01% SDS buffer and blocked in 5% milk.

Received 3 November 2011 Accepted 3 January 2012
Published ahead of print 11 January 2012
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doi:10.1128/JVI.06727-11
phosphate-buffered saline with 0.1% Tween 20 (PBS-T) buffer. For NS4A immuno blotting (IB), transfer onto Immobilon-P membranes (Millipore) was done in buffer without SDS. After washing, membranes were incubated with species-specific horseradish peroxidase-conjugated anti bodies (Jackson ImmunoResearch) followed by treatment of the membrane with ECL+ (GE Healthcare) and imaging on X-ray film. For immuno precipitation (IP), cells were lysed in IP buffer (10 mM Tris [pH 7.5], 150 mM NaCl, and 1% Triton X-100, in some cases supplemented with 10% glycerol). Protein was immunoprecipitated and captured on anti-Flag affinity gel (Sigma). Bound protein complexes were washed in IP buffer and then eluted using SDS loading buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 0.004% bromophenol blue, 10% β-mercaptoethanol). For NS3/NS4A immunoprecipitations, protein was immunoprecipitated with either anti-NS3 or anti-NS4A overnight and bound to protein G plus agarose (Calbiochem).

The following antibodies were used for immunoblot analysis: anti-HCV NS3 1B6 (gift from D. Moradpour), anti-HCV-NS4A (Virogen), anti-HCV polycyclonal sera (gift from W. Lee), anti-Flag M2 (Sigma), anti-My c (Abcam), anti-tubulin (Sigma), and anti-Cardif (for MAVS; Axorra).

Immunofluorescence analysis and confocal microscopy. Cells were stained with 2 nm of Drage5 (Axorra) according to the manufacturer’s protocol before fixation. Cells were fixed in 4% paraformaldehyde, permeabilized, and immunostained as described previously (16). For immunofluorescence, we used anti-HCV NS3 (Novocastra) and anti-calmexin (Enzo Life Sciences). Samples were imaged on a Zeiss 510 Meta confocal microscope with a 63× oil immersion lens with a numerical aperture of 1.4 at the Nyquist limit. Images were collected as z stacks with a focal step size of 0.43 µm and processed with ImageJ. z stacks were deconvolved using AutoDeblur (Media Cybernetics).

Plasmids and transfections. The plasmids used in this study have been described previously and are as follows: pEFS-N3, pEFS-NS3/4A, and pEFS-Flag-SC-pro tease (wild type [wt] and S139A) (10); pEFS-BoS-N-RIG-I (38); and pMyc-MAVS (wt and C508Y) and pEFTak-Flag-MAVS (17). DNA transfections were done using Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche). IFN-β promoter luciferase assays were conducted as described previously (30). The NS3/4A helix α0 mutant containing five mutations, namely, L13A, L14A, I17A, I18A, and L21A, was created using recombinant PCR with overlapping mutagenic primers (5'-GGccGactagtcGcGcGacggggcgtgacagaa-3'; 5'-GGccGactagtcGcGcGacggggcgtgacagaa-3'; 5'-GGccGactagtcGcGcGacggggcgtgacagaa-3'; 5'-GGccGactagtcGcGcGacggggcgtgacagaa-3'; 5'-GGccGactagtcGcGcGacggggcgtgacagaa-3'); uppercase represents nucleotide mutations) and subcloned into the EcoRI and XbaI restriction sites in the pEF expression vector. pEFS-NS3/4A R24/26A and pEFS-Flag-SC-pro teease R24/26A, to substitute arginine at positions 24 and 26 of NS3 with alanine, as well as pEFS-NS3 L14A, pCDNA-Myc-MAVS C283Y, and pCDNA-Myc MAVS C452Y, were created using site-directed mutagenesis of membrane targeting domain that are essential for the preservation of membrane association (26). Therefore, we mutated the two amino acids located downstream of helix α0 of NS3 (R24, R26)

Crystal violet staining was performed after 3 weeks of G418 selection. Colonies from replicate plates were counted to determine the relative transduction efficiency of the corresponding HCV replicon RNA. Relative transduction efficiency is expressed as the percentage of colonies that were stably transduced in Huh7 cells relative to wt as compared to the percentage of colonies that were stably transduced in Huh7.5 cells relative to wt. For the α0 mutant, all remaining electroporated cells were plated in a 150-mm dish for G418 selection. The sole resulting colony was picked and expanded. RNA extracted (RNeasy kit; Qiagen) from this clonal cell popu lation was reverse transcribed into cDNA (Advantage cDNA polymerase; Clontech), and the NS3 region was cloned and sequenced to deduce the nucleotide sequence of NS3 in this replicon RNA.

HCV infection. HCV was produced from a molecular clone of HCV genotype 2a corresponding to the sequence of strain JFH1 and synthesized by Blue Heron, Inc. The corresponding cDNA was cloned into PENTR, allowing production of the full-length HCV RNA from the T7 promoter to make pSfi (for synthetic JFH1). pSfi/NS3 α0 containing alanine substitution of the hydrophobic amino acids in the NS3 helix α0 mutations in JFH1 (L13A, L14A, I17A, V18A, V19A, M21A) was created using recombinant PCR with overlapping mutagenic primers (5’-GCCGacggggcgtgacagaa-3’; 5’-GCCGacggggcgtgacagaa-3’; 5’-GCCGacggggcgtgacagaa-3’; 5’-GCCGacggggcgtgacagaa-3’; 5’-GCCGacggggcgtgacagaa-3’). The pSJ and pSJ/NS3 α0 plasmids were linearized at the 3’ end of the HCV cDNA by XbaI digestion. The linearized DNA was then purified and used as a template for in vitro transcription. RNA was purified free of DNA and transfected into Huh7.5 cells via electroporation, as above. HCV infection was assessed by focus forming assay, immunostaining, and immunoblotting for HCV proteins and by quantitative real-time (qRT)-PCR for intracellular RNA.

RESULTS
Helix α0 of NS3 is required for MAVS cleavage and RIG-I pathway control by HCV NS3/4A. The amphipathic helix α0 at the amino terminus of NS3 (Fig. 1A) has a surface-exposed hydrophobic patch (Fig. 1B) that is highly conserved among HCV genotypes and confers membrane localization to the NS3/4A protease (4). To define how the NS3 helix α0 directs proteolytic targeting of MAVS by NS3/4A on intracellular membranes, we eliminated the surface-exposed hydrophobicity of the helix α0 (Fig. 1B) through alanine mutation (α0 mutant) and examined the ability of the encoded protein to target and cleave endogenous MAVS in human hepatoma (Huh7) cells, a hepatocyte cell line that supports HCV infection (33). These mutations have been shown to eliminate the interaction of the NS3 helix α0 with intracellular membranes (4). Confocal microscopy analysis revealed that the loss of the hydrophobic membrane-targeting amino acids in the NS3 helix α0 altered NS3/4A from a punctate pattern to diffuse staining throughout the cell that does not overlap with the ER marker calnexin (Fig. 1C), confirming that the NS3 helix α0 is a membrane-targeting domain (4).

Coexpression analysis of NS3/4A constructs with Myc-tagged MAVS demonstrated that while wt NS3/4A could cleave MAVS, the α0 mutant was deficient in MAVS cleavage, although not to the same extent as NS3 containing a mutation that inactivated the protease active site (S139A; compare α0 to SA) (Fig. 1D). These analyses also demonstrate the requirement for coexpression of NS3 with NS4A for MAVS cleavage, as NS3 alone was unable to cleave MAVS (Fig. 1D). Proteins targeted near mitochondria often contain basic residues in proximity to a hydrophobic membrane-targeting domain that are essential for the preservation of membrane association (26). Therefore, we mutated the two arginine residues located downstream of helix α0 of NS3 (R24, R26)
To investigate if they contributed to the membrane-targeting and proteolytic activities of NS3. However, these mutations did not affect cleavage of MAVS by NS3/4A (RA mutant) (Fig. 1D).

To ascertain the impact of the helix of NS3 on the proteolytic efficiency of viral substrates, we examined the role of the NS3 helix in cleavage of the hydrophobic amino acids in the helix to alanine (α) that were immunostained with anti-NS3 (green) and anti-calnexin (red). Nuclei were stained with Draq5 (blue). Bar: 10 μm. (D to F) Immunoblot analysis of extracts from cells expressing the indicated NS3/4A constructs and Myc-MAVS (D), Flag-NS5A/B (E), or Flag-NS4B/5A (F). Arrows mark full-length (FL) and cleaved (C) MAVS in panel D and full-length and cleaved HCV polyprotein in panels E and F. The constructs used express either NS3, wt NS3/4A, NS3/4A S139A (SA), NS3/4A αo mutant (αo), or NS3/4A R24/26A (RA). (G) IFN-β promoter reporter luciferase expression of Huh7 cells transfected with increasing amounts of NS3/4A expression plasmids and then mock or SenV infected. Values are mean ± standard deviation (SD) (n = 3). *, P ≤ 0.003 by unpaired Student’s t test. (Inset) Immunoblot for NS3 protein expression from this assay. (H) Immunoblot analysis of anti-NS3 or anti-NS4A immunoprecipitated extracts, as well as input, from cells expressing wt NS3/4A, αo mutant NS3/4A, or empty vector (V).

(Fig. 1A) to investigate if they contributed to the membrane-targeting and proteolytic activities of NS3. However, these mutations did not affect cleavage of MAVS by NS3/4A (RA mutant) (Fig. 1D).

To ascertain the impact of the helix αo motif on the proteolytic efficiency of viral substrates, we examined the role of the NS3 helix αo in cleavage of HCV polyprotein substrates. We expressed the NS3/4A constructs with Flag-tagged HCV polyprotein substrates, Flag-NS4B/5A and Flag-NS5A/5B, in Huh7 cells and assessed the processing of each by immunoblot analysis. The NS4B/5A junction is known to be membrane associated and requires NS3 interaction with NS4A for efficient processing, while the NS5A/5B junction is not membrane associated and can be cleaved by NS3 alone (2, 15). Both wt NS3/4A and the αo mutant efficiently cleaved the NS5A/B junction to liberate NS5A (Fig. 1E). Moreover, mutation of the NS3 helix αo did not affect processing of the cis cleavage site between NS3 and NS4A (compare SA to αo) (Fig. 1E, top). However, mutation of NS3 helix αo reduced the efficiency of processing of the NS4B/5A polyprotein, as compared to both the wt and RA mutants of NS3/4A (Fig. 1F). Sendai virus (SenV) is an RNA virus that triggers the RIG-I pathway in a similar fashion as HCV (28, 30). Both wt NS3/4A and the NS3/4A RA mutant were able to block this SenV-mediated signaling to the IFN-β promoter (Fig. 1G). Consistent with a reduced cleavage efficiency of MAVS, the NS3/4A helix αo mutant failed to block SenV-mediated signaling to the IFN-β promoter, similar to the NS3/4A SA active-site mutant (Fig. 1G). Thus, the hydrophobic composition of helix αo is important for proteolytic targeting of membrane-anchored substrates, including MAVS and the HCV NS4B/5A polyprotein, as well as for control of RIG-I pathway signaling by NS3/4A during virus infection.
NS4A interacts with specific residues in the amino terminus of NS3, but it is not known to specifically interact with the NS3 helix α₀ (18). However, because the NS3 helix α₀ mutant demonstrated reduced ability to process the NS4B/5A polypeptide junction (which requires NS4A interaction with NS3 for efficient processing [2, 15]), we hypothesized that this construct may have a reduced interaction with NS4A compared to wt NS3. Results from coimmunoprecipitation assays of NS3 and NS4A showed that the NS3 helix α₀ mutant did not stably interact with NS4A as compared to wt NS3 (Fig. 1H), demonstrating that the NS3 helix α₀ is required for efficient interaction of NS3 with NS4A. Although we did not detect a stable interaction between NS4A and the NS3 helix α₀ mutant, it is likely that some transient interaction occurs between these two proteins, as the NS3 helix α₀ mutant was able to partially cleave the NS4B/5A polypeptide junction, albeit with reduced efficiency, and this cleavage requires NS3 interaction with NS4A.

Helix α₀ of NS3 required for membrane-associated proteolysis by NS3/4A. The NS3 cofactor NS4A contains a transmembrane domain at its amino terminus that confers membrane targeting (4) and is known to stimulate NS3 protease activity, especially for membrane-anchored substrates (2, 15). Therefore, to isolate the function of the NS3 helix α₀ from that of the NS4A transmembrane domain in conferring membrane targeting and function of the NS3 protease, we utilized a single-chain protease construct of NS3/4A, which lacks the transmembrane domain of NS4A but contains the central 12 amino acids of NS4A fused to the NS3 protease domain (SC-protease) (10). Use of the SC-protease eliminates any contribution of the NS4A transmembrane domain to the targeting function of the protein while maintaining interaction of the NS4A amino acids essential for full protease activity with NS3 by directly tethering them onto the amino terminus xof NS3.

As shown in Fig. 2A, the NS3 helix α₀ mutations in the SC-protease altered its localization from a punctate pattern to diffuse staining throughout the cell that did not overlap with the ER marker calnexin (Fig. 2A). Immunoblot analysis of extracts from cells expressing the SC-protease and Myc-tagged MAVS found that the SC-protease α₀ mutant was unable to cleave MAVS at the canonical C508 cleavage site (Fig. 2B). Furthermore, while the loss of the NS3 helix α₀ in the SC-protease had no effect on proteolytic processing of NS5A/B (Fig. 2C), it ablated processing of the NS4B/5A polypeptide at the canonical cleavage site, which was instead processed at an alternate site to liberate a protein product of higher mass than the canonical NS4B product (Fig. 2D; indicated by the star). Examination of the NS4B/5A polypeptide sequence revealed an alternative consensus serine protease cleavage sequence within NS5A (DPSHIT↓AETA; P6, P-1, and P1 residues are in bold) that is likely the alternative site of cleavage of the SC-protease helix α₀ mutant. Taken together with the previous data, these data demonstrate that in the context of either the SC-protease or the NS3/4 protease complex, the NS3 helix α₀ is required for cleavage of MAVS and the NS4B/5A polypeptide substrate but not the NS5A/5B polypeptide substrate. Further loss of hydrophobicity in the NS3 helix α₀ prevents the cleavage of membrane-anchored substrates at membrane-proximal cleavage sites while redirecting proteolytic function to non-membrane-associated cleavage sites of specific substrates.

SC-protease cleaves MAVS at an alternate site to block RIG-I pathway signaling. Surprisingly, we found that even though the SC-protease helix α₀ mutant failed to cleave MAVS at the canonical C508 cleavage site (Fig. 2B), it blocked SenV-mediated signaling to the IFN-β promoter at similar levels as the wt SC-protease, while the SC-protease harboring the protease active-site mutation (S139A) did not block this signaling (Fig. 3A). This result was different than that seen with full-length NS3/A, where helix α₀ mutation blocked signaling regulation by NS3/4A (Fig. 1G). As NS3/4A has been reported to block MAVS oligimerization, which is essential for downstream signaling to IFN-β (1), we hypothesized that the SC-protease helix α₀ mutant, unlike the NS3/4A helix α₀ mutant, was forming a stable complex with MAVS that would fully block MAVS oligimerization and subsequent down-

![Image](http://jvi.asm.org/remote-access/doi/10.1128/JVI.01335-12/images/fig2a.png)
stream signaling to IFN-β. Indeed, we have shown previously that both SC-protease wt and S139A are able to form a stable complex with MAVS (10). Using a MAVS oligomerization assay that measures interaction between overexpressed Flag-tagged MAVS and Myc-tagged MAVS, we found that expression of the SC-protease with the helix α₀ mutation only partially blocked MAVS oligomerization, as compared to the complete block in MAVS oligomerization by the wt SC-protease (Fig. 3B). Therefore, the SC-protease helix α₀ mutant block of RIG-I pathway signaling is not solely due to the inhibition of MAVS oligomerization. Upon closer analysis of the immunoblots measuring cleavage of MAVS in these assays, we found that while the wt SC-protease cleaves MAVS at the canonical C508 cleavage site and the helix α₀ mutant of the SC-protease does not, it appeared that both of these constructs cleaved MAVS at a second site, releasing a MAVS cleavage product of a lower molecular weight (Fig. 3C and E). This secondary cleav-

![FIG 3](http://jvi.asm.org) SC-protease cleaves MAVS at an alternate site to block RIG-I pathway signaling. (A) IFN-β promoter reporter luciferase expression of 293 cells transfected with increasing amounts of SC-protease expression plasmids and then mock or SenV infected. Values are mean ± SD (n = 3) of one of four replicate experiments. (Inset) Immunoblot for Flag-SC-protease protein expression from this assay. (B) Immunoblot analysis of anti-Myc immunoprecipitations and input from 293 cells expressing Flag-MAVS, Myc-MAVS, and increasing amounts of indicated SC-protease constructs (wt, S139A, or α₀) or vector. (C) Entire panel of the Flag-MAVS immunoblot from panel B is shown. Arrows mark full-length (FL), cleaved (C, at C508), and alternate cleaved (C-2) forms of MAVS. The asterisk indicates an alternate form of MAVS not resulting from cleavage. (D) The sequence of the HCV polyprotein cleavage sites, as well as known and possible cleavage sites of MAVS, are shown, with the P1 and P1’ residues shown in boldface. (E) Immunoblot analysis of MAVS cleavage at alternate sites by the SC-protease. Blots were probed with anti-Myc (for MAVS) and anti-Flag (for SC-protease). Arrows mark full-length (FL) and cleaved (C, at C508; C-2, at C283) MAVS. (F) IFN-β promoter reporter luciferase expression of 293 cells following MAVS wt overexpression alone or with increasing doses of MAVS aa 284 to 540. Values are mean ± SD (n = 3).
age of MAVS occurs only upon coexpression of the SC-protease but not with the wt NS3/4A protein, which cleaves MAVS only at C508 (references 14 and 20 and data not shown). Analysis of the MAVS protein sequence revealed additional cysteine residues as candidate serine protease cleavage sites (Fig. 3D). To test if these cysteine residues were the alternate MAVS cleavage sites by the SC-protease, we inactivated them by tyrosine mutation, coexpressed the MAVS constructs with the SC-protease constructs, and monitored cleavage by immunoblot assay. The C508Y mutation in MAVS abolished SC-protease cleavage at the canonical NS3/4A cleavage site (C508) but not at the secondary cleavage site (Fig. 3E). The C452Y mutation in MAVS did not prevent cleavage at the secondary site (data not shown). However, the C283Y mutant prevented MAVS cleavage at the secondary site by both the wt and helix α0 mutant SC-protease, thereby identifying C283 as a secondary cleavage site in MAVS by the SC-protease (Fig. 3E). As MAVS lacking a transmembrane domain can function as a dominant negative factor in MAVS pathway signaling via oligomerization of MAVS CARD domains (Y. M. Loo and M. Gale, Jr., unpublished results), we hypothesized that the cleavage at C283 by the SC-protease helix α0 mutant liberated a fragment of MAVS that acted as a dominant negative to block RIG-I pathway signaling to IFN-β. To test this notion, we expressed wt MAVS, along with increasing doses of the truncated MAVS 284- to 540 fragment, and measured signaling to the IFN-β promoter in luciferase assays. Indeed, while wt MAVS overexpression induced signaling to the IFN-β promoter, coexpression with MAVS 284 to 540 abrogated this effect (Fig. 3F), demonstrating that it acts as a dominant negative to block MAVS signaling. Therefore, while the SC-protease helix α0 mutant does not cleave MAVS at C508, it does cleave MAVS at C283, and this resulting cleavage prevents RIG-I pathway signal transduction. Further, as the helix α0 mutant does not cleave MAVS at C508, which is positioned at intracellular membranes next to the transmembrane domain of MAVS (aa 514 to 535), our results identify that the NS3 helix α0 is required to direct the protease active site of NS3/4A for cleavage of the host immune protein MAVS at amino acids located near the membrane.

Helix α0 of NS3 is required for HCV replication. To directly test the role of the NS3 helix α0 in HCV replication, we engineered the mutant helix α0 motif into an HCV genotype 1B subgenomic replicon encoding a G418-selectable marker (the HP replicon) (31) and assessed its ability to mediate persistent replication in Huh7 or Huh7.5 cells transfected with in vitro-transcribed replicon RNA and subjected to G418 selection. While Huh7 cells have an intact RIG-I pathway, Huh7.5 cells lack RIG-I function and are highly permissive for HCV replication (30). Neither Huh7 nor Huh7.5 cells express functional TLR3 (12). While G418-resistant colonies arose in both Huh7 and Huh7.5 cells transfected with the parental replicon RNA, no G418-resistant colonies arose in either Huh7 or Huh7.5 cells transfected with the NS3 helix α0 mutant replicon RNA, demonstrating that the hydrophobic amino acids in this domain are essential for HCV replication (Fig. 4A). Moreover, an HCV genotype 2A strain JFH1 engineered with the mutant NS3 helix α0 motif also failed to replicate to a level that supported productive infection in Huh7.5 cells after direct transfection of cells with viral RNA (data not shown). HCV replicon RNA harboring NS3 mutations at R24 and R26 displayed reduced transduction efficiency in both Huh7 and Huh7.5 cells (Fig. 4A). To test if any of the hydrophobic amino acids in the NS3 helix α0 domain provided a more dominant contribution to either HCV replication or HCV control of innate immunity, we mutated each individual hydrophobic amino acid in this domain to alanine (L13A, L14A, I17A, I18A, L21A), transduced the resulting replicon RNA into Huh7 or Huh7.5 cells, and examined the number of G418-resistant colonies that arose following selection. Remarkably, mutation of the individual hydrophobic amino acids in the NS3 helix α0 to alanine did not affect HCV RNA replication in Huh7.5 cells, as these constructs displayed a similar number of G418-resistant colonies as the parental HCV replicon RNA (Fig. 4A, bottom). However, in Huh7 cells, mutation of any of the individual hydrophobic amino acids in the NS3 helix α0 resulted in fewer G418-resistant colonies than seen for the parental replicon RNA (Fig. 4A, top). These results demonstrate that in the absence of efficient substrate targeting by the NS3/4A protease, the host cell can impart RIG-I-dependent suppression of viral RNA replication, and reveal that the hydrophobic amino acids in the NS3 helix α0 play a role in RIG-I pathway targeting and evasion during HCV replication.

Whereas we observed no G418-resistant colonies after 3 weeks of selection of Huh7 cells transfected with the NS3 helix α0 mutant replicon RNA in two separate experiments, a sole colony of G418-resistant Huh7.5 cells supporting HCV RNA replication eventually grew. This colony was expanded and characterized. We found that both MAVS cleavage and HCV polyprotein processing occurred efficiently in these cells and paralleled levels observed in cells harboring the parental HCV replicon (Fig. 4B). We determined the viral RNA sequence from these cells and found that the predominant HCV RNA species was retained at only one of the originally engineered mutations (L14A) in the NS3 helix α0 motif, while the other four mutations reverted back to the parental NS3 sequence (Fig. 4C). Introduction of this mutation (L14A) into the NS3/4A expression construct maintained HCV polyprotein processing by NS3/4A at the NS3/4A, NS5A/B, and NS4B/5A junctions (Fig. 4D and E). Taken together, our data demonstrate that the overall hydrophobic nature of NS3 helix α0 is required for efficient HCV RNA replication and contributes to viral RNA replication fitness under pressure from RIG-I signaling of MAVS-dependent immune defenses imposed by the host cell.

DISCUSSION

Our results demonstrate that the hydrophobic amino acids in the amphipathic NS3 helix α0 are required for cleavage of membrane-anchored substrates, including the viral HCV NS4B/5A polyprotein and the host cell immune adaptor protein MAVS. Loss of hydrophobicity in the NS3 helix α0 results in an inability to establish persistence by HCV RNA replicons and infection by HCV. Further, loss of this domain prevents NS3/4A from controlling RIG-I pathway signaling during RNA virus infection. Therefore, the NS3 helix α0 governs control of both viral and cellular processes through targeting of membrane-anchored substrates.

The NS3 helix α0 is required to position the protease active site to properly cleave membrane-anchored viral and host substrates. Indeed, SC-protease constructs lacking the hydrophobic amino acids in the NS3 helix α0 cleave the membrane-anchored substrates NS4B/5A and MAVS at alternate, membrane-distal sites. This selectivity in the cleavage of membrane-anchored substrates could be due to the ability of the NS3 helix α0 to sense or induce membrane curvature (19). Our data indicate that the NS4A tar-
geting subunit of the NS3/4A protease complex also contributes to this process, as even the wt SC-protease, which lacks the NS4A transmembrane anchor, cleaves MAVS at an alternate site (C283) distal from the MAVS transmembrane anchor. Therefore, amino acids within both the NS4A transmembrane anchor and the NS3 interacting domain (aa 21 to 32), as well as the hydrophobic amino acids in the NS3 helix αα, contribute to canonical MAVS cleavage and disruption of RIG-I pathway signaling. These results support the model by Brass et al. (4), who performed structural analysis of the NS3 helix αα and the NS4A transmembrane α-helix and proposed that both of these domains are required to properly position the protease active site at the membrane surface for the cleavage of substrates. It is also possible that the NS3 helicase domain, which is not present in the SC-protease, could contribute to this process, as functional communication between the NS3 helicase and protease domains has been reported (3).

Our data indicate that the membrane association of the NS3 helix αα is essential to establish HCV RNA replication, even in...
Huh7.5 cells which do not have functional RIG-I signaling. This is not surprising, considering that the NS3 helix α6 mutant has a reduced capacity for processing of the HCV NS4B/5A polyprotein and reduced interaction with NS4A. Further, the fact that the only HCV RNA replicon we obtained after transduction of Huh7.5 cells with HCV RNA containing the five mutations in the NS3 helix α6 domain reverted four of these five amino acids back to their exact wt amino acid sequence highlights the essential nature of the hydrophobicity in this domain for NS3 function. By inactivating single hydrophobic amino acids, we were also able to show that the entire hydrophobic nature of the NS3 helix α6 is also required for the control of RIG-I pathway signaling in the context of HCV RNA replication. These mutant RNA replicons (L13A, L14A, I17A, I18A, and L21A) were established at equal frequency in the Huh7.5 cells; however, they displayed reduced transduction efficiency in the Huh7 cells, in which the HCV RNA activates RIG-I pathway signaling. Interestingly, we were unable to demonstrate an effect on MAVS cleavage or RIG-I pathway control by overexpressed NS3/4A proteins containing these mutations in cell culture assays (data not shown). Therefore, we hypothesize that to establish RNA replication, there is likely a race between HCV and the host, wherein any small defects in MAVS cleavage or in viral RNA replication/polyprotein processing by NS3/4A will compromise the ability of the viral RNA to establish productive replication during innate immune pressure.

The multifunctional HCV NS3/4A protease has emerged as the target of the first set of direct-acting antiviral drugs approved for HCV therapy. In addition to MAVS, NS3/4A has also been reported to cleave the host cell proteins TRIF (13) and T-cell protein phosphatase (5). While it remains to be seen exactly how the cleavage of these molecules impacts viral persistence in vivo, it is clear that the HCV protease has the ability to cleave multiple cellular targets, likely to promote its replication and viral pathogenesis in the host, and these antiviral agents, which act directly against NS3/4A, will also inhibit these processes. Examination of the spatial-temporal regulation of NS3/4A-mediated cleavage of viral polyprotein versus that of host cell proteins, as well as the selectivity of these processes, will likely reveal the roles of the host cell protein cleavages in the virus life cycle. Taking these findings together with our previous studies and those of others (4, 9, 36), we propose a model wherein MAVS cleavage by NS3/4A is governed through a bipartite process. In this model, NS4A serves as the primary membrane subcellular targeting subunit to bring NS3/4A to the MAM for the cleavage of MAVS on the MAM, which is directed by the amphiphilic helix α6 of NS3. The diverse roles of NS3 helix α6 in HCV replication and in regulating RIG-I signaling through MAVS cleavage highlight the importance of this domain for productive HCV infection.

ACKNOWLEDGMENTS

We thank the indicated colleagues for antibodies, C. L. Johnson and K. Chang for technical assistance, C. Wilkins for discussion and critical reading of the manuscript, and the W. M. Keck Center for Advanced Studies in Neural Signaling for microcopy assistance.

This work was supported by NIH grants AI060389, AI00035, and DA024563, the Burroughs Welcome Fund (M.G.), and the Irvington Institute Fellowship Program of the Cancer Research Institute (S.M.H.).

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