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The Linker Region of NS3 Plays a Critical Role in the Replication and Infectivity of Hepatitis C Virus

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Hepatitis C virus (HCV) NS3-4A is required for viral replication and assembly. We establish that virus assembly is sensitive to mutations in the linker region between the helicase and protease domains of NS3-4A. However, we find that the protease cleavage, RNA binding, and unwinding rates of NS3 are minimally affected *in vitro*. Thus, we conclude that the NS3 linker is critical for mediating protein-protein interactions and dynamic control rather than for modulating the enzymatic functions of NS3-4A.

Hepatitis C virus (HCV) nonstructural protein 3 (NS3) is a bifunctional protein that is required for viral replication and virion assembly (1, 2). NS3 consists of an N-terminal serine protease domain (residues 1 to 181) and a C-terminal helicase domain (residues 196 to 632). The serine protease domain (NS3pro) forms a complex with HCV nonstructural protein 4A (NS4A) (3, 4), and the NS3-4A complex catalyzes co- and posttranslational cleavage of the HCV polyprotein (1, 5). The helicase domain (NS3hel) is a superfamily 2 DExH/D helicase with DNA/RNA binding, NTPase, and unwinding activities (6, 7). Although helicase activity is required for HCV replication (8), it is unknown if enzymatic activities of NS3 are required for virion assembly.

Through allosteric interactions between the protease and helicase domains, NS3 can modulate the efficiency of functional RNA binding, unwinding, translocation stepping (9, 10), and peptide cleavage (11). Disruption of a β-sheet interaction between the domains induces formation of an extended NS3 conformation that forms functional complexes with RNA substrates and facilitates rapid unwinding (12). This conformation is hypothesized to promote replication (12); however, the mechanism by which NS3 toggles between open and closed states is not well understood. Notably, a direct-acting antiviral has been developed that disrupts HCV replication by locking NS3 into a closed conformation (13).

A short "linker" (residues 182 to 195) physically connects the NS3pro and NS3hel domains (14). The linker is highly conserved and includes multiple invariant prolines (Fig. 1) (15, 16). Interestingly, a cell culture replication-enhancing mutation, E1202G, in the serine protease domain adjacent to the linker region was found to increase HCV replication and abrogate viral infectivity in the genotype 1b Con1 isolate (17, 18). Although the linker has no obvious enzymatic function, we hypothesized that this region may play a functional role in the observed biochemical coupling between the NS3pro and NS3hel domains. Additionally, the invariant PxxP motif (P191/194) within the linker hints at interactions between NS3 and host factors containing SH3 domains (19). This hypothesis is supported by an HCV interaction database that lists multiple interactions between the HCV replication complex and SH3 domain-containing proteins (20).

To examine the role of the linker, we assessed the HCV replication and infectivity levels of NS3 linker mutants by using a JFH1 (genotype 2a) *Gaussia princeps* luciferase bicistronic replicon system in a *trans*-packaging Huh-7.5[core-NS2] cell line (Fig. 2A) (21–23). This system yields high viral titers ($\geq 4 \times 10^5$ 50% tissue culture infective doses [$TCID_{50}$]/ml at 72 h; unpublished data), simplifies the selection of genetic revertants and suppressors (21), and reduces biohazard risk. We first measured the secreted luciferase expressed from the replicon to monitor HCV replication. To determine the level of released virus, we infected naive Huh-7.5[core-NS2] cells with media collected at each replication time point and measured newly secreted luciferase at 48 h postinfection.

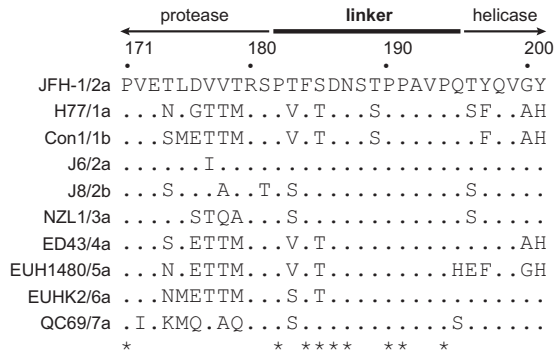

FIG 1 Amino acid alignment of the linker regions from several HCV isolates. Based on the structure of NS3, we define the linker region as the residues between NS3 P182 and P194 that connect the HCV NS3 N-terminal protease and C-terminal helicase domains (see PDB 3O8C) (14). Positions targeted for mutagenesis are indicated with stars. Conserved residues are represented as dots.

We designed mutants to modulate linker flexibility and to disrupt any potential SH3 domain interactions (Table 1). Each of the invariant prolines in the NS3 linker—P182G, P190G, P191G, P194G, and P190G/P191G (referred to as "Double")—was mutated to glycine. We also mutated an invariant proline in the pro-

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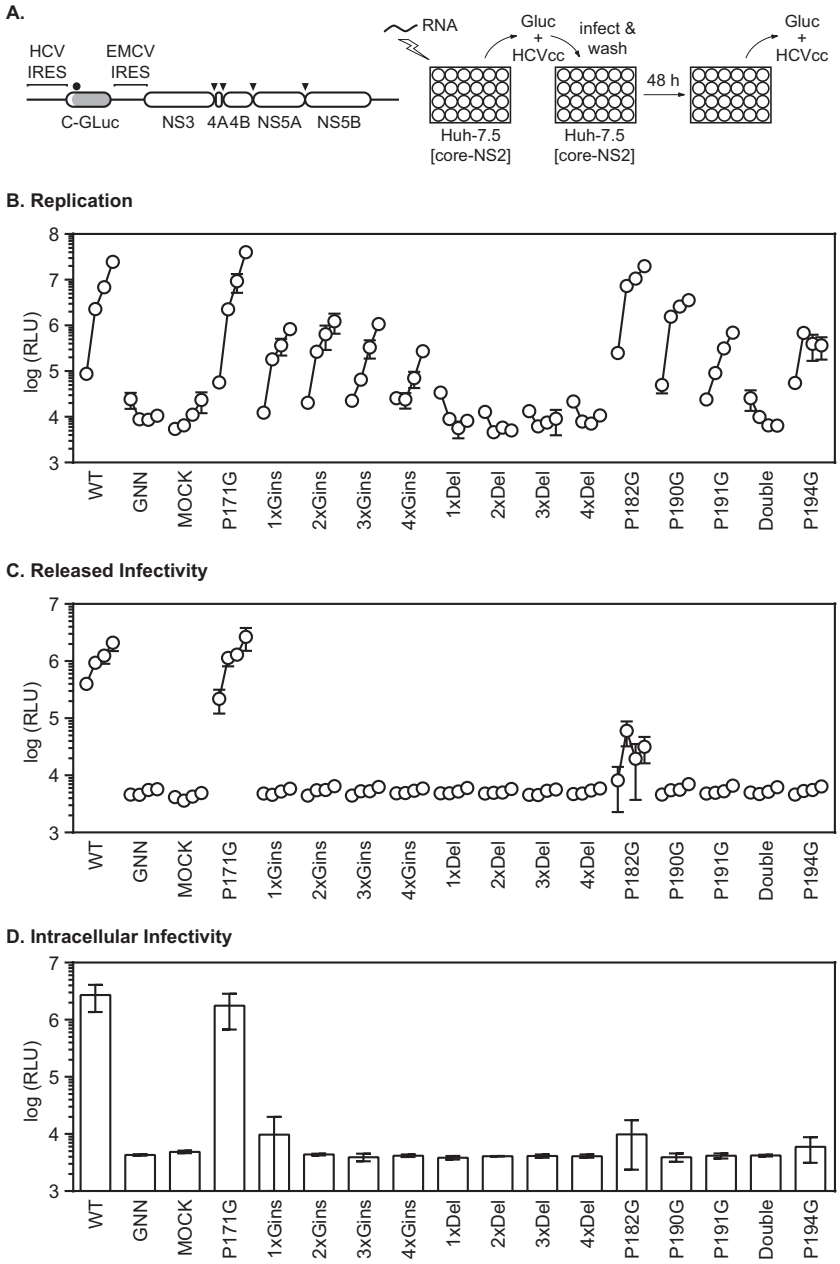

FIG 2 Replication and infectivity of HCV NS3 linker mutants in Huh-7.5 cells expressing core-NS2. (A) Diagram of the GLuc replicon construct for monitoring HCV replication and design of the replication and released-infectivity experiments. Triangles indicate sites cleaved by NS3-4A, and the circle indicates a signal peptidase cleavage site at the N terminus of the GLuc gene. IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus; HCVcc, cell culture–derived HCV. (B) Replication of NS3 linker mutants in the GLuc-JFH1 replicon context in Huh-7.5[core-NS2] cells, monitored as previously described (25). GNN is a mutation that disrupts the active site of the NS5B RNA-dependent RNA polymerase. (C) Released infectivity measurements of the media collected at each time point. The media tested for replication as described for panel B were used to infect naive cells, and then fresh media were assayed for secreted luciferase 48 h postinfection. For panels B and C, the luciferase activity data (*y* axis) at each of the time points—24, 48, 72, and 96 h—are plotted in succession from left to right (*x* axis) for the NS3 mutants. RLU, relative light units. (D) Intracellular infectivity of lysate from infected cells. Cell lysates were used to infect naive cells, and then fresh media were assayed for secreted luciferase 48 h postinfection. "Double" refers to P190G/P191G, "1xGins" to "4xGins" refer to 1 to 4 glycines inserted after P182, and "1xDel" to "4xDel" refer to deletion of 1 to 4 residues between F184 and N187 (starting with F184). WT, wild type. Each data point represents the average of the results of three experiments, and the error bars represent the standard deviations.

tease domain outside the linker region (P171G) for comparison. To modulate linker length, we inserted up to four glycines after P182 and deleted up to four residues between F184 and N187. Residues were inserted and deleted adjacent to P182 because the reduced conservation at this location suggests that it is amenable to substitution (Fig. 1).

Several linker NS3 mutants exhibited defects in replication (Fig. 2B), but most notably, every mutation in the linker region disrupted released infectivity (Fig. 2C). Deletion of linker residues severely disrupted HCV replication, whereas an increasing linker length (up to two glycines) was tolerated. Notably, there were no discernible defects in the NS3 P171G mutant, which resides outside the linker region.

TABLE 1 HCV NS3 linker mutants and RNA unwinding rate constants

HCV strain	Codon(s)[a]	Linker Sequence[b]	k_{obs} rate constant (s^{-1})[c]	Fraction unwound
WT		PVETLDVVTRSPTFSDNSTPPAVPQ	0.060 ± 0.004	0.37 ± 0.01
P171G	1201	GVETLDVVTRSPTFSDNSTPPAVPQ		
1xDel	Δ1214	PVETLDVVTRSPT-SDNSTPPAVPQ	0.073 ± 0.005	0.37 ± 0.01
2xDel	Δ1214–216	PVETLDVVTRSPT--DNSTPPAVPQ		
3xDel	Δ1214–217	PVETLDVVTRSPT---NSTPPAVPQ		
4xDel	Δ1214–218	PVETLDVVTRSPT----STPPAVPQ		
1xGins	@1213	PVETLDVVTRSPGTFSDNSTPPAVPQ	0.078 ± 0.004	0.32 ± 0.01
2xGins	@1213	PVETLDVVTRSPGGTFSDNSTPPAVPQ		
3xGins	@1213	PVETLDVVTRSPGGGTFSDNSTPPAVPQ		
4xGins	@1213	PVETLDVVTRSPGGGGTFSDNSTPPAVPQ	0.062 ± 0.006	0.25 ± 0.01
P182G	1212	PVETLDVVTRSGTFSDNSTPPAVPQ	0.046 ± 0.007	0.22 ± 0.01
P190G	1220	PVETLDVVTRSPTFSDNSTGPAVPQ	0.063 ± 0.005	0.31 ± 0.01
P191G	1221	PVETLDVVTRSPTFSDNSTPGAVPQ	0.060 ± 0.006	0.31 ± 0.01
Double	1220–1221	PVETLDVVTRSPTFSDNSTGGAVPQ	0.075 ± 0.005	0.25 ± 0.01
P194G	1224	PVETLDVVTRSPTFSDNSTPPAVGQ	0.078 ± 0.005	0.32 ± 0.01

[a] Codons are numbered per the JFH-1 genome (GenBank accession no. AB047639). @, insertion sites.
[b] Linker sequences (NS3 residues 171 to 195; JFH-1 codons 1201 to 1225) of the mutants are listed. Mutations (indicated by boxed residues) were created by Quick Change site-directed mutagenesis with primers containing the indicated changes flanked by 15 complementary upstream and downstream nucleotides. Glycine residues were coded by using GGG, GGC, or GGA codons.
[c] NS3-4A unwinding activity was measured as previously described (21). Rate constants and amplitudes for unwinding were determined from single exponential fits to the time courses.

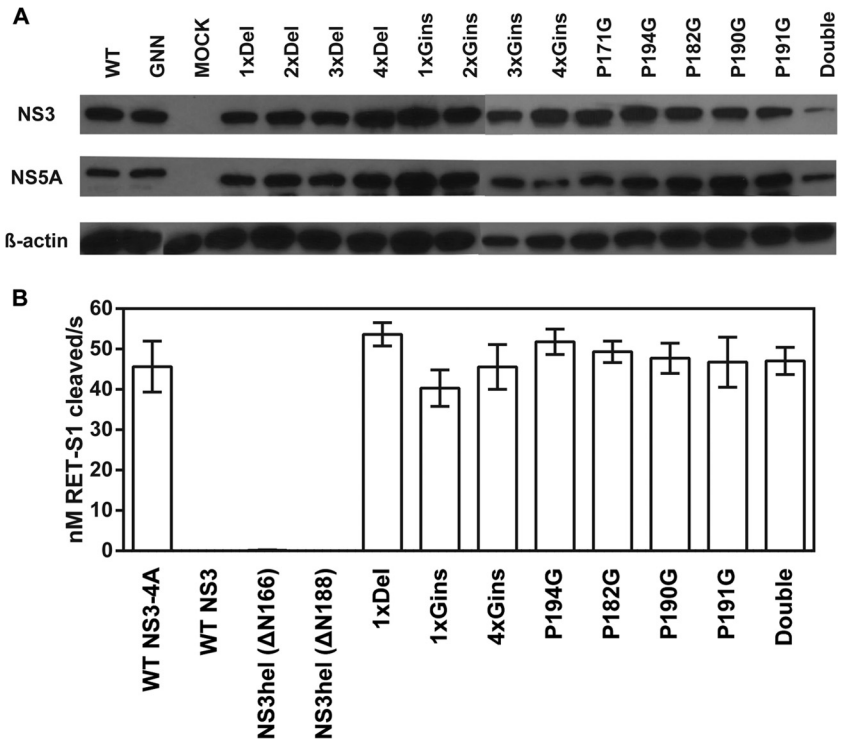

FIG 3 NS3 linker mutations do not disrupt polyprotein processing. (A) The vaccinia virus T7 RNA polymerase expression system described by Fuerst et al. (26) was used to drive HCV polyprotein expression to test for levels of NS3 expression and proper polyprotein processing of each NS3-4A linker mutant replicon construct. NS3 P190/P191 was the only replicon construct to display lower NS3 expression levels. The image is a composite of two gels. (B) The RET-S1 peptide cleavage assay described by Taliani et al. (27) was used to evaluate the serine protease activity of recombinantly expressed NS3-4A linker mutants and three NS3 controls—NS3, NS3hel[ΔN166] (amino acids [aa] 167 to 631), and NS3hel[ΔN188] (aa 189 to 631). Steady-state protease rates were calculated between the first 10 and 40 s of the reaction. Each column represents the average of the results of three experiments, and the error bars represent the standard deviations.

The lack of infectious particles for every linker mutant was striking, and we confirmed the defects in released infectious virus by measuring intracellular infectivity, using cell lysates to infect naive (core-NS2) Huh-7.5 cells. Intracellular infectivity was disrupted for every mutant, indicating that the infectivity defects were indeed caused by disruption of virus assembly (Fig. 2D).

To establish whether the cell culture phenotypes were due to defects in the proteolytic processing of the HCV polyprotein or in protein stability, we employed a vaccinia virus-T7 system to express constant levels of polyprotein for each construct independently of replication efficiency. At 24 h posttransfection, we collected cells, lysed them, and performed a Western blot analysis for NS3, NS5A, and β-actin. Comparable levels of NS3 and NS5A in the Western blots revealed that the NS3 linker mutations do not disrupt polyprotein processing (Fig. 3A). Additionally, none of the linker mutations decreased NS3 stability, with the exception of NS3 P190/P191.

To further characterize the enzymatic function of the linker mutants, we expressed recombinant NS3-4A and mutants 1xDel (ΔF184), 1xGins, 4xGins, P182G, P190G, P191G, P190G/P191G (Double), and P194G. By using a previously described RET-S1 cleavage assay (27), we measured the serine protease activity of each construct and confirmed that modifying the linker region did not disrupt peptide cleavage (Fig. 3B). These data suggest that the NS3 linker region influences replication and virus assembly independently of HCV polyprotein processing or differences in NS3 stability.

We next examined whether the NS3 linker mutations disrupted any of the measurable *in vitro* helicase activities of NS3-4A. By using a set of unwinding substrates with a constant 3′ overhang of 18 nucleotides and a variable-length duplex of 12 to 36 bp, we first measured RNA binding for each NS3-4A linker mutant (24). We observed that the linker mutations did not substantially affect RNA binding and calculated dissociation constants (K_d values) of approximately 1 to 10 nM for each protein construct (data not shown). Then, by using the 24/42mer (top-strand/bottom-strand) substrate under single-cycle unwinding conditions, we measured unwinding time courses as previously described (21). We similarly observed minimal changes in the unwinding rate constant and unwinding amplitude for each NS3-4A mutant (Table 1).

In this report, we establish that the conserved linker region of HCV NS3 is a distinct functional region of the protein that contributes to HCV replication and virus assembly. We demonstrate that the *in vitro* serine protease activity, RNA binding, and unwinding of NS3-4A are not disrupted by mutations to the linker. The lack of enzymatic defects in NS3-4A linker mutants supports the hypothesis that the linker region does not exert direct effects on NS3-4A catalysis but rather serves a critical role in the virus life cycle by modulating NS3-4A conformation and/or serving as a node for interactions with host or viral proteins.

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