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Arginine 112 is involved in HCV translation modulation by NS5A domain I

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ABSTRACT

HCV NS5A has three domains, which have multiple roles in the viral life cycle. We previously found that NS5A is able to down-regulate HCV RNA translation through a mechanism requiring the polyU/UC region within the viral 3′UTR to which NS5A binds. In this study, we further investigated the role of domain I in modulating viral translation. Using a series of deletion and substitution mutants, we identified a number of positively charged residues that played a role in this modulatory effect, most prominently R112. The R112A mutation negated the ability of domain I and full-length NS5A to modulate viral translation. Additionally, the R112A mutation impeded domain I binding to the polyU/UC RNA, suggesting a mechanism for this down-regulatory effect. Finally, the R112A mutation rendered HCV replication deficient. These results collectively point to a crucial role for the R112 residue of NS5A in the modulation of HCV life cycle by NS5A.

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1. Introduction

Hepatitis C virus (HCV), as member of the *Flaviviridae* family, is a small positive sense single-stranded RNA virus [10]. The 9.6 Kb viral genome contains a single open reading frame (ORF) which is flanked by highly conserved 5′ and 3′ untranslated regions (UTRs), which are involved in the modulation of viral translation and replication [10,29]. Translation of this single ORF, mediated by an internal ribosomal entry site (IRES) located within the 5′UTR, results in the production of a polyprotein [29]. The polyprotein is cleaved co- and post-translationally by both cellular and viral proteases into at least 10 viral proteins: three structural proteins (core, E1, and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The IRES, located in the 5′UTR, directly recruits the 40S ribosomal subunit to initiate viral translation in a cap-independent manner [10]. Viral polyprotein translation is also modulated by viral cis-acting factors and both viral and cellular trans-acting factors [10,24]. One of the viral cis-acting factors that modulates translation is the viral 3′UTR [10,24]. While the 3′UTR serves as the initiation site for negative strand synthesis and as such is essential for viral replication, it also functions to stimulate viral translation [3,5,30]. The 3′UTR is composed of three regions: a variable region, a polyU/UC tract and an X-tail region which is composed of three stem loops: SL1, SL2 and SL3 [24]. The variable region, polyU/UC region and SL1 have been found to significantly contribute to the enhancement of viral translation mediated by the 3′UTR [30]. The mechanism of this enhancement is not clearly defined but proposed mechanisms include the recruitment of viral and/or cellular factors which may mediate 5′ and 3′UTR interaction [10]. Additionally, it has been observed that the 3′UTR may function in ribosome recycling between the two UTRs as the 3′UTR is able to bind the 40S ribosomal subunit and enhance successive rounds of translation [2].

HCV non-structural 5A protein (NS5A) is essential for viral replication and assembly although its exact role in the viral life cycle remains unclear [1,8,13,14,23,27,28,31]. NS5A is composed of an N-terminal amphipathic α-helix (AH) and three domains which are separated by two regions of low complexity sequences (LCSs) [22]. The AH is necessary and sufficient for membrane localization of NS5A, at the site of the viral replication complex, and is essential for replication [4,6,25]. NS5A domains I and II have been found to be essential for viral replication [26,27,32,33]. Domain III, while largely

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dispensable for viral replication, plays a role in modulating viral assembly [1,14,32]. NS5A is an RNA binding protein that binds with high affinity to the polyU/UC region in the viral 3′UTR [11]. All three NS5A domains are capable of specifically interacting with the 3′UTR independently [7]. Additional studies have suggested that domain I is the key mediator of NS5A RNA binding [15].

In a previous study, we have shown that the viral NS5A protein functions to down-regulate viral translation through a mechanism that requires the polyU/UC region of the 3′UTR, where the protein has been shown to bind [9]. We have also shown that domain I is able to modulate viral translation as effectively as the full NS5A protein [9]. Given the role of domain I in modulating viral replication and NS5A RNA binding, we further investigated the ability of domain I to modulate viral translation in this study. Using a series of deletion and substitution mutants, we identified that R112 plays a prominent role in this modulatory effect. R112A mutation impedes the ability of domain I to bind the polyU/UC RNA, suggesting a mechanism for this down-regulatory effect. In addition, the R112A mutation is lethal for HCV replication.

2. Materials and methods

2.1. Plasmid construction and RNA synthesis

The monocistronic HCV RNA translation reporter plasmid T7 HCV 5′UTR-Core^{aa1-16}-rLuc-NS5B^{5}-3′UTR was described previously [9]. This reporter construct contains T7 promoter, HCV 5′UTR, sequence encoding the first 16 amino acids of the Core protein, an internal *Renilla* luciferase (rLuc) gene, sequence encoding the last five amino acids of the NS5B protein and the 3′UTR. The 5′UTR contains the viral IRES which drives the expression of the internal rLuc gene. In another construct, the polyU/UC region in the 3′UTR was cloned downstream of the T7 promoter sequence. HCV subgenomic replicon (SgR) construct is composed of the HCV 5′UTR, which directs translation of a luciferase reporter gene, followed by the IRES of the encephalomyocarditis virus (EMCV), which directs translation of the HCV replicase genes NS3-NS5B, and the 3′UTR (Fig. 4A) [20]. The SgR reporter allows for transient replication assay as the amount of luciferase produced can be measured as an indicator of viral replication [36]. As a negative control, the GDD sequence within the NS5B viral polymerase was deleted which rendered replication deficient. Plasmids expressing HCV NS5A with or without the amphipathic α-helix (AH) were reported previously [9]. The coding sequence for NS5A domain I was amplified by PCR using HCV-1b N Neo C-5B [16] as template and cloned into the pEF/cyto/myc vector (Invitrogen). NS5A domain I truncations and substitution mutants were constructed by a PCR-based approach. A myc tag was present at the C-terminus of the NS5A coding sequence in the expression plasmids. All plasmids were confirmed by DNA sequencing. HCV RNA translation reporter and SgR RNAs were produced from linearized plasmids by in vitro transcription using the MEGAscript T7 In Vitro Transcription kit (Ambion).

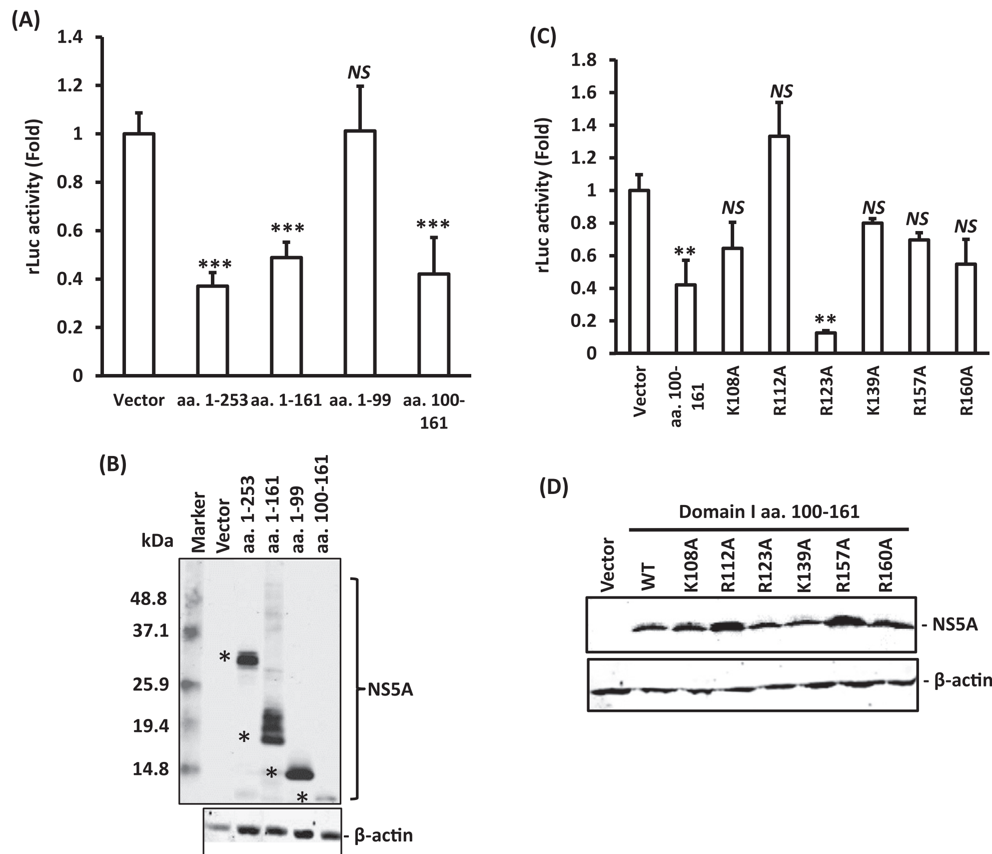

Fig. 1. Residues K108, R112, K139, R157 and R160 of NS5A domain I region aa. 100-161 are involved in the down-regulation of viral translation. (A and C). HuH-7 were co-transfected with vector, plasmids expressing myc-tagged NS5A or the domain I truncation mutants indicated (A), or with vector, or plasmids expressing myc-tagged NS5A aa. 100-161, wild-type or containing the amino acid substitutions indicated (C), and HCV RNA translation rLuc reporter RNA. Luciferase assay was performed 24 h after transfection. Statistical differences were indicated as * if $p \le 0.05$, ** if $p \le 0.01$, *** if $p \le 0.001$, or *NS* for not significant. (B and D). Expression of NS5A domain I truncation mutants was demonstrated by Western blotting using a myc-tag specific antibody. The location of truncated NS5A proteins in (B) was indicated by * in the blot. As a loading control, the level of β-actin was determined using a β-actin antibody.

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