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Structural Studies of Peptide Binding Interaction of HCV IRES Domain IV

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Abstract The hepatitis C virus (HCV) internal ribosome entry site (IRES) is an RNA structure located in the 5'-UTR of the HCV RNA genome. The HCV IRES consists of four domains I, II, III, and IV, where domains II - IV are recognized by 40S ribosomal subunit and the domain III is bound to eukaryotic initiation factor 3 (eIF3) for translation initiation. Here, we have characterized the tertiary interaction between an L-/K- rich peptide and the HCV IRES domain IV. To probe the peptide binding interface in RNA, we synthesized ^{13}C- and ^{15}N-double labeled RNA and the binding site was identified by using the chemical shift perturbation (CSP) NMR methods. Our results showed that the peptide binds to the upper stem of the IRES domain IV, indicating that the tertiary interaction between the IRES domain IV and the peptide would disrupt the initiation of translation of HCV mRNA by blocking the start codon exposure. This study will provide an insight into the new peptide-based anti-viral drug design targeting HCV IRES RNA.

Keywords HCV IRES, HCV IRES domain IV, RNA structure, RNA binding peptide, NMR

Introduction

Hepatitis C virus (HCV) is a human pathogen that causes a liver disease infecting an estimated 71 million People worldwide (www.who.int/mediacentre/factsh-eets/fs164/en). Although the treatment of interferon with ribavirin is an effective therapy for Hepatitis C, patients could still face further risk in liver and kidney diseases. Therefore, more effective drugs with less side-effects need to be developed.[1]

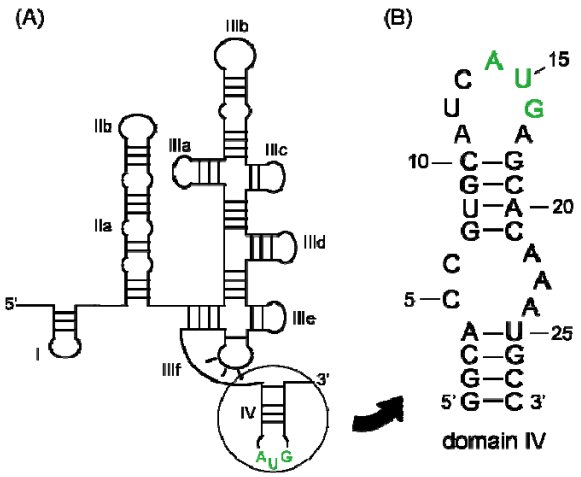

Figure 1. Secondary structure of the HCV IRES RNA. (A) Schematic representation of the HCV IRES RNA. The domain names are indicated. (B) Secondary structure of domain IV of HCV IRES RNA. The initiation codon (AUG) for translation is highlighted in green.

HCV contains a positive single-stranded RNA genome that includes a large open reading frame (ORF) encoding for a polyprotein. In the 5'-untranslated region (UTR) of RNA genome, there is a highly conserved structured region called 'the

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internal ribosome entry site' (IRES). Translations of proteins are initiated by interaction of IRES with 40S small ribosomal subunit and eukaryotic initiation factor 3 (eIF3).[2]

The HCV IRES consists of ~ 300 nucleotides, and it is composed of four structural domains (Figure 1A).[2] Among these four domains, the domains II and III are essential to recruit host ribosome and eIF3 for translation of viral mRNA. In particular the four-way junctional region that is composed of stem-loop IIId, IIIe, IIIf, and IV is the binding site for the 40S ribosomal subunit. The domain IV is predicted to be folded into stem-loop hairpin in the protein free-form[3], where the start codon (AUG) sequence is in the apical loop of the RNA structure. When HCV IRES is bound to the 40S ribosomal subunit, this hairpin no longer exists and the start codon is exposed for initiation of translation.[4]

Previously, an L- and K- rich peptide that strongly binds to the HCV IRES domain IV hairpin was reported.[5] Here, we report further structural characterization of the tertiary interaction between the peptide and HCV IRES domain IV RNA using NMR methods. The chemical shift perturbation (CSP) studies indicate that the peptide preferably binds to the upper stem of the IRES RNA, suggesting that this peptide would inhibit the denaturation of the domain IV hairpin structure. Since it is essential to expose the AUG start codon for translation, the tertiary interaction between the peptide and RNA would inhibit of the translation mediated by HCV IRES.

Experimental Methods

The 13- mer peptide 1' (LKKLLKLLKKLLK) was kindly supplied by Professor Injae Shin at Yonsei University, Seoul, Korea. For NMR studies, unlabeled and fully or base specifically ^{15}N-, ^{13}C-labeled HCV IRES RNA (5'- GGCAC CGUGC AUCAU GAGCA CAAAU GCC -3') were synthesized *in vitro* by using T7 RNA polymerase.[6] After 6 hour transcription at 37 °C, RNA was ethanol precipitated, purified by 15 % denaturing PAGE, electro-eluted, and further purified by anion-exchange column (5 mL Hi-Trap Q, GE Healthcare). The purified RNAs were desalted and exchanged into water (Amicon, Millipore). The RNA samples were heated to 95°C under dilute concentration (~ 10 μM) for 5 minutes, cooled on ice for 30min, and concentrated to ~ 1 mM. NMR samples were prepared in 10 mM sodium phosphate buffer (pH 6.5) with 10 % D_2O for assignments of exchangeable protons, and 100 % D_2O for assignments of non-exchangeable protons.

NMR spectra were recorded on Bruker Avance III HD 800MHz spectrometer (KIST) equipped with a HCN cryoprobe. Exchangeable proton spectra were taken in 90 % H_2O/10 % D_2O at 283 K and non-exchangeable proton spectra in 100 % D_2O (Sigma) at 293 K. With unlabeled RNA samples, the H1' and H8/H6/H5/H2 protons were sequentially assigned from 2D NOESY and 2D TOCSY spectra. In addition, 2D- F2- filtered and F1/F2 – filtered NOESY experiments were recorded with AU and GC ^{13}C, ^{15}N- labeled HCV IRES RNA for unambiguous assignments.[7] The ^{1}H, ^{13}C 2D HSQC spectra were used to monitor chemical shift changes upon binding of peptide to HCV IRES RNA. To identify peptide-binding residues of IRES domain IV, the peptide 1' (LKKLLKLLKKLLK) was added to 0.1 mM RNA in 10 mM sodium phosphate buffer (pH 6.5). The final concentrations of peptide were 0, 0.1, and 0.3 mM. NMR spectra were processed and analyzed using Topspin 3.5 (Bruker) and Sparky 3.110.

Results

It has been reported that a lysine- and leucine- rich short peptides bind to the HCV IRES domain IV, where the peptide 1 (LKKLLKLLKKLLKLKG) showed the highest binding affinity (K_d = ~ 67 nM) to the HCV IRES domain IV.[5] It has been implicated that the internal residues of the peptide 1 is associate with RNA binding.[5] We deleted three amino acids at the C-terminus of peptide 1 and made a truncated construct of peptide 1' (LKKLLKLLKKLLK) to

characterize the tertiary interaction between the peptide 1' and HCV IRES domain IV.

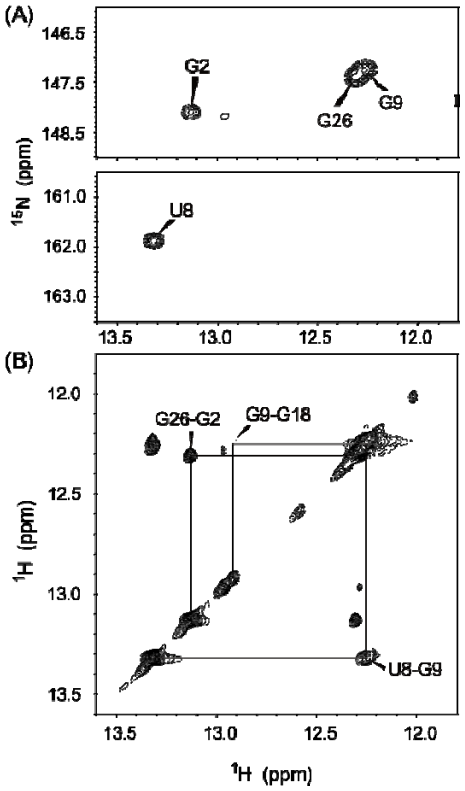

Figure 2. NMR spectra of the domain IV of HCV IRES RNA showing imino proton resonances. (A) Imino proton region of 1H-15N HSQC spectra of IRES RNA that shows the base paired G (top) or U (bottom) (B) 2D 1H-1H NOESY spectrum acquired at 283K with 300ms mixing time. The lines that connect cross peaks indicate imino proton walks between the neighboring imino protons in the helical region of RNA.

In the protein-free form of HCV IRES, the domain IV forms a small hairpin where the start codon AUG is located at the apical loop (Figure 1A).[3] For NMR studies, the hairpin RNA construct of IRES domain IV was designed as shown in Figure 1B. The two GC base pairs at the terminal were added to promote the transcription yield and to increase the stability of the hairpin. To determine the secondary structure of the designed RNA in solution, we examined the 2D NOESY spectra of HCV IRES in the imino NH region (Figure 2). First, to distinguish the imino

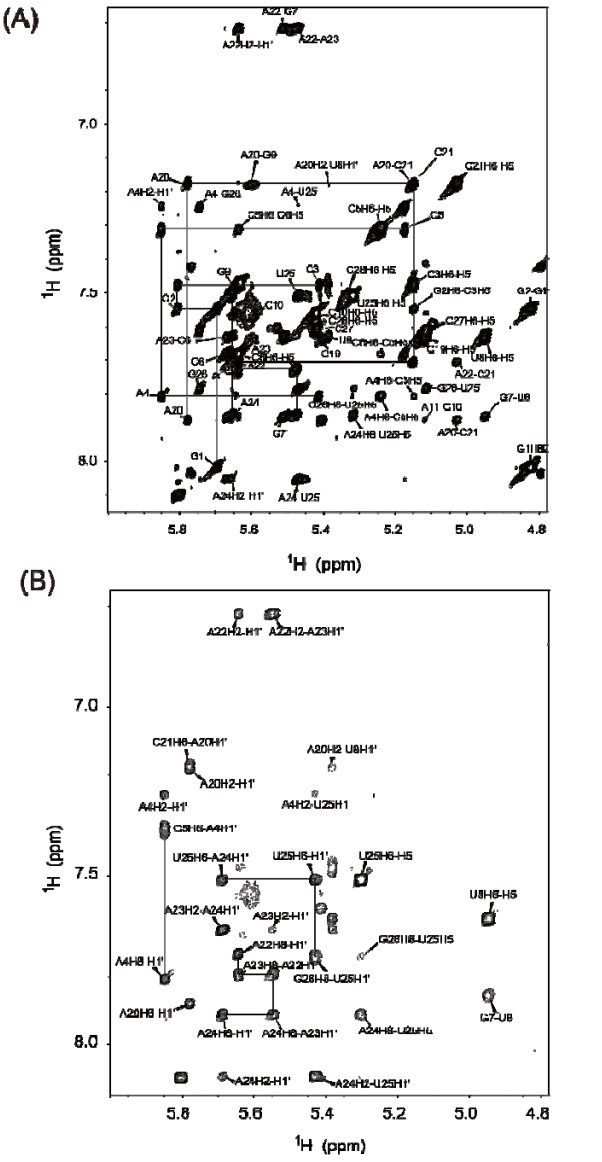

Figure 3. (A) 2D NOESY spectrum and assignments of the sugar-base proton resonance of HCV IRES domain IV. (B) Filtration of GC resonances in F2 dimension reduce the number of cross peaks in 2D NOESY spectrum

protons of G (H1) from U (H3) 2D ^{1}H-, ^{15}N- HSQC spectra were examined. The characteristic resonance frequencies of the N1 and N3 of G and U were identified (Figure 2A). Next, the secondary structure formation was examined by analyzing the 2D NOESY spectra of the imino region (Figure 2B). The presence of a NOE between the two imino protons of G2 and G26 indicates that the bottom stem of the

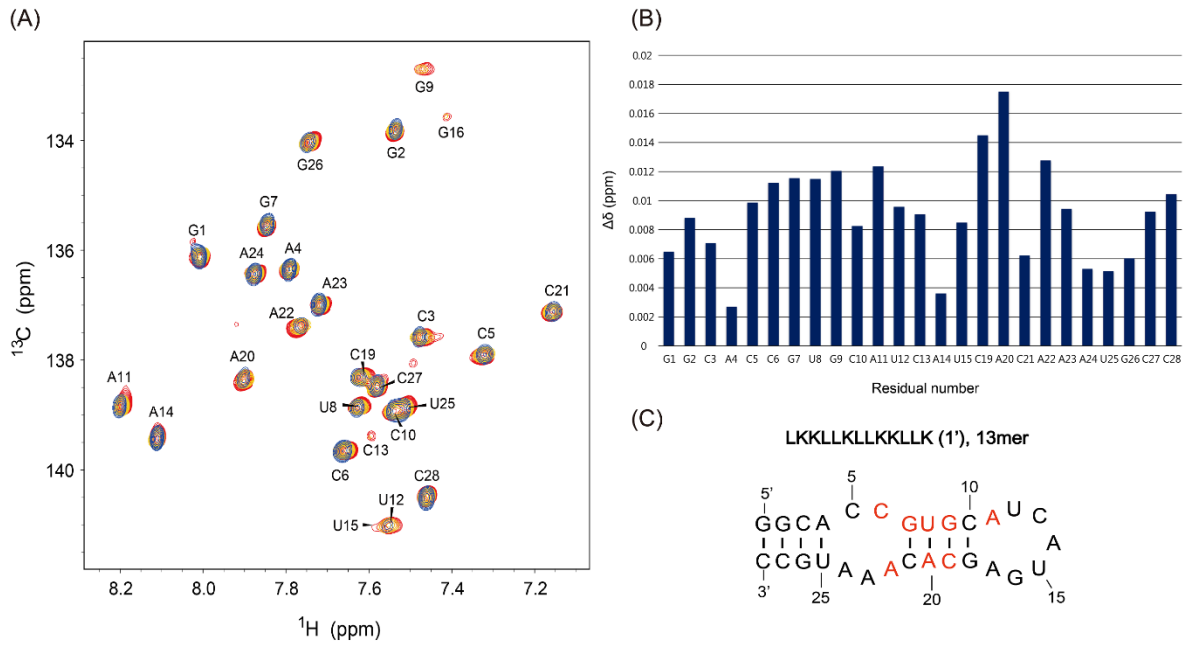

Figure 4. NMR binding study of IRES domain 4 RNA with peptide 1'. (A) Aromatic region (C6-H6 and C8-H8) of ^{1}H-^{13}C HSQC spectrum of IRES D4 (0.1mM) after addition of peptide 1' (a final concentration: red,0; yellow, 0.1; blue, 0.3 mM). (B) Chemical shift perturbations upon addition of peptide 1' to IRES domain IV. The equation, $\Delta\delta$ ppm = $[(\Delta\delta\ 1H\ ppm)^2 + (\Delta\delta\ 13C\ ppm/4)^2]^{1/2}$, was used to measure chemical shift perturbations. (C) Residues influenced by peptide 1′ in NMR spectra are colored red ($\Delta\delta > 0.01$ ppm). The sequence of peptide 1′ is shown.

hairpin was formed. Also the sequential NOEs between the imino protons of U8 –G9 – G18 suggested that the upper stem of IRES domain IV was formed. After the secondary structure of IRES domain IV was confirmed, we assigned H1' protons of the sugar and aromatic protons (H8/H6/H2) of the bases using 2D NOESY experiments (Figure 3A and B). In this spectrum the sequential NOEs between H8/H6 and H1' were used for assignments. In addition, for unambiguous assignment a series of base-specific 2D filter/edited NOESY spectra were analyzed (Figure 3B).[8, 9]

The tertiary interaction between the peptide 1' and IRES domain IV was monitored in the aromatic C8H8/C6H6 region of 2D ^{1}H-, ^{13}C- HSQC spectra. Specifically, the chemical shift changes of the IRES domain IV were monitored as the ratio of RNA to peptide 1' increased from 1 to 3 (Figure 4A). The results showed that the most perturbed residue was A20 and the chemical shifts of the neighboring residues were more shifted compared to others, indicating that peptide 1' preferentially interacts with the upper stem of IRES domain IV.

Discussion

In this study, we examined the binding interaction of L- and K- rich 13mer peptide (peptide 1') with the HCV IRES domain IV RNA using the CSP method. We expected a large CSP with peptide 1', since we only truncated three amino acid residues at the C'-terminus of peptide 1 that showed a high affinity to IRES domain IV. However, the changes of chemical shifts were small, suggesting that the binding affinity of peptide 1' to RNA is much weaker than peptide 1, and that the C-terminal residues would be important for binding to RNA. In spite of the small CSPs, our results show that peptide 1' binds to the upper stem of IRES domain IV, so the binding

of peptide would prevent the initiation of translation by interfering with the recognition of AUG start codon located in apical loop of IRES domain IV. Although it is necessary to further test cellular permeability of the peptide and to investigate peptide binding to IRES RNA in cells, peptide would replace the small molecules as an alternative new drug.
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Acknowledgements

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