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hnRNP L is required for the translation mediated by HCV IRES

Byounghoon Hwang [a], Jong Hoon Lim [a], Bumsuk Hahm [b], Sung Key Jang [b], Seong-Wook Lee [a,*]

[a] Department of Molecular Biology, Institute of Nanosensor and Biotechnology, Dankook University, 126, Jukjeon-dong, Suji-gu, Yongin 448-701, Republic of Korea
[b] Department of Life Science, Pohang University of Science and Technology, Kyungbuk 790-784, Republic of Korea

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ABSTRACT

Translation of hepatitis C virus (HCV) RNA is initiated by internal loading of the ribosome into the HCV internal ribosome entry site (IRES). Previously, heterogeneous ribonucleoprotein L (hnRNP L) was shown to bind specifically to the 3′ border region of the HCV IRES and enhance HCV mRNA translation. Here, we provide evidence for the functional requirement of hnRNP L for the HCV IRES-mediated translation initiation using specific RNA aptamers. *In vitro* selection techniques were employed to isolate RNA aptamers against hnRNP L, which were shown to contain consensus sequences with repetitive ACAC/U. The hnRNP L-specific RNA aptamers efficiently inhibited the *in vitro* translation reactions mediated by the HCV IRES in rabbit reticulocyte lysates. RNA ligands with only (ACAU)5 or (AC)10 nucleotide sequences could also specifically bind to hnRNP L, and specifically and effectively impeded *in vitro* translation reactions controlled by the HCV IRES. Importantly, the hnRNP L-specific RNA aptamers inhibited the HCV IRES function in cells in a dose-dependent manner, and the aptamer-mediated inhibition of the HCV IRES was considerably relieved by the addition of hnRNP L-expressing vector. These results strongly demonstrate the functional requirement of cellular hnRNP L for the HCV IRES activity.

HCV, a member of family *Flaviviridae*, is the main pathogen causing chronic hepatitis, liver cirrhosis, and in some instances, hepatocellular carcinoma [1]. HCV contains a single positive-stranded RNA genome of about 9600 nucleotides (nt) in length encoding a single polyprotein of 3010 amino acids [2]. This polyprotein precursor is co- or post-translationally processed into at least three mature structural (C, E1, and E2) and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) by cellular and viral proteases [2,3].

The translation initiation of the HCV RNA genome occurs by cap-independent internal ribosome binding into the highly conserved [4] HCV IRES element which consists of 341-nt long 5′ nontranslated region (5′NTR) and part of the N-terminal coding sequence of the core [5–8]. Several trans-acting cellular factors were identified to interact with the HCV IRES element and assist in internal initiation of translation. Several subunits of the eukaryotic initiation factor, eIF-3, have been reported to bind the apical half of domain III of the 5′NTR [9,10]. Polypyrimidine tract-binding protein (PTB) has been shown to bind to multiple sites of the HCV IRES, and the PTB binding is required for the IRES function [11–13]. La antigen was shown to bind the context of initiation codon of the HCV IRES, which is functionally required for the HCV IRES-dependent translation [14,15]. Previously, we reported that another cellular protein, hnRNP L, specifically interacts with the 3′ end of the HCV IRES. The binding of hnRNP L to the HCV IRES correlated well with the translational efficiencies of corresponding mRNAs [16], which suggested that hnRNP L may play a critical role in the translation of HCV mRNA through the IRES element. Moreover, hnRNP L has been proposed as a global regulator on the level of mRNA processing including alternative splicing [17,18], mRNA export [19,20], mRNA stability [21,22], and alternative poly(A) selection [23].

In the present study, we employed *in vitro* selection techniques, called systematic evolution of ligands by exponential enrichment (SELEX) [24,25], and isolated RNA aptamers against hnRNP L to address the biological requirement of hnRNP L for the HCV IRES. These RNAs were shown to bind hnRNP L with high specificity. Of note, the RNA aptamers efficiently and specifically interfered with cap-independent translation directed by the HCV IRES not only in the translation assay *in vitro* but also in liver cells.

Materials and methods

Expression and purification of the his-tagged hnRNP L. A recombinant protein of hnRNP L was cloned into pET21 expression vector (Novagen), which expresses recombinant proteins tagged with a hexahistidine at C-terminus. Proteins were overexpressed in *Escherichia coli* BL21 (DE3) strain and purified with Ni–nitrilotriacetic acid (NTA) agarose column (Qiagen) and a poly(U)-Sepharose column (Amersham Pharmacia) as described [16].

* Corresponding author. Fax: +82 31 8005 4058.
E-mail address: SWL0208@dankook.ac.kr (S.-W. Lee).

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Selection procedure. In vitro selection was performed as described previously [26], with a few modifications. RNA library was produced using *in vitro* transcription of randomized synthetic DNA oligonucleotides with NTPs and T7 RNA polymerase. The sequence of the RNA library is 5′-GGGAGAGCGGAAGCGUGCUG GGCCN$_{40}$CAUAACCCAGAGGUCGAUGGAUCCCCCC-3′, where N_{40} represents 40 nucleotide (nt) with equimolar incorporation of A, G, C, and U at each position. First, 1.44 μM of the RNA library was preincubated with 20 μl of Ni–NTA agarose beads in 100 μl binding buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, and 1% BSA) for 30 min at room temperature with shaking. The RNAs that nonspecifically bound to the beads were then discarded. The precleared RNAs were further incubated with 133 nM of his-tagged hnRNP L for 30 min at room temperature. RNAs which bound to hnRNP L were recovered, amplified with RT-PCR and *in vitro* transcription, and used for next rounds of selection. In subsequent rounds, hnRNP L concentration was reduced by 3- to 4-folds at every 3 or 4 round for more stringent condition. After 10 rounds of selection, the selected RNA pools were amplified, and the amplified DNA was cloned and sequenced.

Binding analysis of selected RNA aptamers. RNAs were radiolabeled internally or at their 5′-ends and isolated as described [26–29]. Purified RNAs were incubated with proteins as described above. RNA–hnRNP L complexes were precipitated with Ni–NTA agarose beads. Bound RNAs were extracted from the pellets, and then analyzed on a 6% polyacrylamide gel with urea. Alternatively, purified RNAs were incubated with hnRNP L in 40 μl binding buffer with 1 μg tRNA. The RNA–hnRNP L complexes were then analyzed on a nondenaturing polyacrylamide gel containing 2% glycerol for gel shift analysis.

Cell-free translation. In vitro translations were performed in 12.5 μl reaction mixtures containing 40 nM template RNA in the presence of [^{35}S] methionine (NEN) using rabbit reticulocyte lysates (RRL, Promega) as described [16]. Translation reactions were performed at 30 °C for 1 h and analyzed by SDS–15% polyacrylamide gel electrophoresis. The intensities of the autoradiographic images produced by ^{35}S were enhanced by fluorography with salicylic acid. Gels were dried and then exposed to Kodak XAR-5 for 12–18 h.

Cells and RNA transfection. A human hepatoma cell line (Huh7) was utilized for the co-transfection assay. The cells (1×10^6) were transfected with *in vitro* transcribed capped dicistronic dual-luciferase RNA using by lipotransfection with 6 μl DMRIE-C (Gibco-BRL). To demonstrate the inhibitory activity of RNA aptamer on the HCV IRES-mediated translation, the cells were co-transfected with the reporter RNAs along with or without the RNA aptamer. After 24 h of transfection, the cells were harvested and reporter gene expression activities were determined by measuring relative light units using a luminometer TD-20/20 (Turner Designs Instrument) and dual-luciferase reporter assay system (Promega).

Results and discussion

In vitro selection of hnRNP L-specific RNA aptamers

To isolate specific RNA ligands that bound the hnRNP L protein, we employed SELEX procedure using combinatorial RNA library

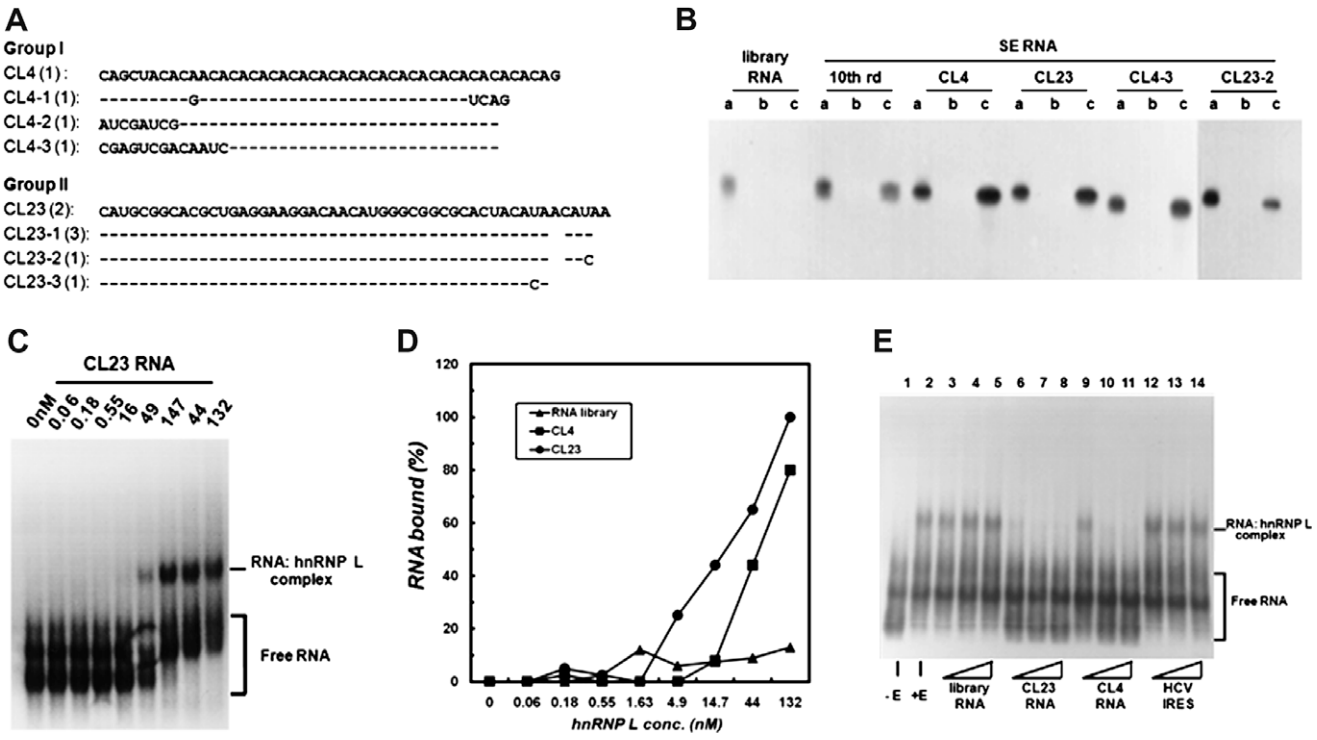

Fig. 1. RNA aptamer sequences and their strong and specific binding to hnRNP L. (A) After 10 rounds of *in vitro* selection, the sequence of 11 selected RNAs was determined. Two different RNA sequence groups were found in these clones with each being present multiple times (numbers in parentheses). The line drawn for sequences CL4-1,2,3 or CL23-1,2,3 indicates that nucleotides found at these positions are identical to those shown for sequence CL4 or CL23, respectively. (B) Internally radiolabeled RNAs were incubated with (lane c) or without (lane b) hnRNP L (44.1 nM) and the RNA–protein complexes were precipitated with Ni–NTA agarose beads. Bound RNAs were extracted and analyzed on 6% polyacrylamide gel with urea. Lane a contains 10% of labeled RNA (664 pM) used in this study. (C) Radiolabeled CL23 RNA aptamer (50 pM) was incubated with increasing amount of hnRNP L (0–132 nM). The RNA–protein complexes were electrophoresed on a 6% nondenaturing polyacrylamide gel. (D) The percentage of RNA to hnRNP L was calculated by determining the fraction of radioactivity present in the RNA–hnRNP L complexes. The plotted numbers have been normalized to the bound RNA fraction seen on 132 nM concentration of the protein. Values shown represent means of three separate measurements. (E) Radiolabeled CL23 RNA aptamer (50 pM) was incubated with (lanes 2–14) or without (lane 1) hnRNP L (14.7 nM). The RNA–protein complexes were electrophoresed on a 6% nondenaturing polyacrylamide gel. Increasing amount (2.5, 25, and 250 nM) of unlabeled library RNA (lanes 3–5), CL23 (lanes 6–8), CL4 RNA aptamer (lanes 9–11), or HCV IRES (332–402) RNA (lanes 12–14) was added to the binding reaction.