Original Article

Role of Ras-related Nuclear Protein/Polypyrimidine Tract Binding Protein in Facilitating the Replication of Hepatitis C Virus

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Abstract

Background and Aims: Ras-related nuclear (RAN) protein is a small GTP-binding protein that is indispensable for the translocation of RNA and proteins through the nuclear pore complex. Recent studies have indicated that RAN plays an important role in virus infection. However, the role of RAN in hepatitis C virus (HCV) infection is unclear. The objective of this study was to investigate the role and underlying mechanisms of RAN in HCV infection. ***Methods:*** Huh7.5.1 cells were infected with the JC1-Luc virus for 24 h and then were incubated with complete medium for an additional 48 h. HCV infection and RAN expression were determined using luciferase assay, quantitative reverse transcription-PCR and western blotting. Small interfering RNA was used to silence RAN. Western blotting and immunofluorescence were used to evaluate the cytoplasmic translocation of polypyrimidine tract-binding (PTB), and coimmunoprecipitation was used to examine the interaction between RAN and PTB. ***Results:*** HCV infection significantly induced RAN expression and cytoplasmic redistribution of PTB. Knockdown of RAN dramatically inhibited HCV infection and the cytoplasmic accumulation of PTB. Colocalization of RAN and PTB was determined by *immunofluorescence, and* a direct interaction of RAN with PTB was demonstrated by coimmunoprecipitation. ***Conclusions:*** PTB in the host cytoplasm is directly associated with HCV replication. These findings demonstrate that the involvement of RAN in HCV infection is mediated by influencing the cytoplasmic translocation of PTB.

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, affecting approximately 71 million persons according to recent estimates.[1] HCV therapy has been revolutionized with the introduction of direct-acting antiviral agents (commonly known as DAAs), which can achieve viral eradication in >95% of cases with minimal toxicity and overall good tolerability.[2] However, the implementation of these approaches is complicated by the cost, potential for reinfection, generation of drug-resistant viruses, reactivation of other viruses, and end-stage liver disease progression that occurs even after virus clearance.[3,4] HCV also remains the sole hepatitis virus for which a vaccine is not yet available. Thus, novel prophylactic and therapeutic approaches for HCV are still necessary. Identification of host factors involved in HCV replication is critical to understand the molecular mechanism of the viral life cycle, which has significant implications for the development of host-directed strategies to interrupt this cycle.

The ras-related nuclear (RAN) protein is a small GTP-binding protein belonging to the RAS superfamily. It has a well-established role in regulating the transport of macromolecules across the nuclear envelope[5,6] and has also been implicated in mitotic spindle assembly, cell cycle progression, and nuclear envelope formation.[7,8] In eukaryotic organisms, the active transport of macromolecules between the nucleus and cytoplasm is an essential cellular process.[9] Dysregulated protein levels of RAN could cause aberrant nucleo-cytoplasmic transport of RNA and proteins, possibly leading to the initiation and progression of many diseases.

Cellular protein polypyrimidine tract-binding protein (PTB) has been shown to enhance HCV translation by binding to the 5'-untranslated region (UTR) and the 3'-end 98 nucleotides (X region) of HCV RNA.[10–12] The immunodepletion of PTB could completely inhibit HCV translation.[13] These results present evidence for the functional requirement of PTB during HCV translation initiation. In addition to the conserved X region of the 3' UTR, PTB also binds to the poly(U) tract of the 3'UTR.[14,15] And UV cross-linking studies showed that the PTB-5'-UTR binding was much weaker than the PTB-3'-UTR binding.[16,17] The strong and preferential binding of PTB to the 3' UTR suggests that it may be recruited to participate in the initiation of HCV RNA replication.[17] The earlier work found that silencing of PTB by small interfering RNA (siRNA) substantially blocked HCV replication.[18,19] And HCV RNA synthesis could be inhibited by anti-PTB antibody in a cell-free, de novo HCV RNA synthesis system.[18] The direct evidence that PTB is required for HCV RNA replication is that PTB co-

Table 1. Primer sequences used in the study

Gene	Upstream primer (5'→3')	Downstream primer (5'→3')
HCV	GCGTTAGTATGAGTGTCGTG	TCGCAAGCACCCTATCAG
RAN	GTGAAGGCGAAATCCATTGT	TCCTAGCAAGCCAGAGGAAG
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

localizes with the viral replicase complex.[20] These studies indicate that PTB is a part of the HCV RNA replication complex and participates in viral RNA synthesis. The above results together indicate that PTB has dual functions in HCV life cycle, including translation and RNA replication.

Our initial study showed that HCV infection significantly induced the expression of RAN. Additionally, infectious HCV cell culture systems have been developed, enabling further investigations of the molecular mechanism of HCV infection.[21,22] Thus, this study was conducted to investigate the role and underlying mechanisms of RAN in HCV infection in an HCV cell culture system using a JC1-Luc chimeric virus.

Methods

Cell culture and virus plasmids

Human hepatoma Huh7.5.1 cells were grown at 37°C in a 5% carbon dioxide atmosphere with Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 10% fetal bovine serum. The plasmid pFL-JC1 was a kind gift from Apath (St. Louis, MO, USA). The chimeric full-length construct pFL-JC1 has been described elsewhere.[21,23,24] To facilitate the detection of HCV infection, reporter viruses were constructed by inserting the firefly luciferase gene into the carboxyl-terminal region of NS5A in the JC1 genomes.[23,25]

RNA transfection, HCV infection, and titration

The production of infectious HCV in hepatocytes was performed as described previously. Briefly, Huh7.5.1 cells were mixed with *in vitro*-transcribed RNA and electroporated (Gene Pulser System; Bio-Rad Hercules, CA, USA) using a single square wave at 260 V and a 25-millisecond pulse length. The supernatant was harvested and concentrated using a centrifugal filter (Amicon 100K; Millipore, Billerica, MA, USA). Purified viruses were used for infection and titration.

HCV replication and treatment

Huh7.5.1 cells were seeded into 96-well plates at a density of 5×10^3 cells per well in 100 µL of medium. After incubating overnight for attachment, JC1-Luc virus was added to the wells. After 24 h, the medium was aspirated and replaced with 100 µL of complete medium, followed by an additional 48 h incubation. The HCV infection and RNA replication rates were quantified by measuring the luciferase activity using a microplate luminometer (Veritas microplate luminometer; Turner Biosystems, Sunnyvale, CA, USA).

Quantitative reverse transcription-PCR

Cells were collected by trypsinization, and the total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. cDNA was synthesized using the PrimeScript™ Reverse Transcription Reagent Kit with gDNA Eraser (Takara, Tokyo, Japan). The products were then used for analysis by the PRISM 7900 Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA) and the SYBR® Premix Ex *Taq*™ Kit (Takara). The samples were processed in triplicate and analyzed by the $2-\Delta\Delta Ct$ method. The primers were purchased from Sangon Biotech (Shanghai, China) and are listed in Table 1.

Western blotting

Whole-cell extracts were prepared using RIPA lysis buffer containing the protease inhibitor PMSF. Additionally, the cytoplasmic and nuclear extracts from cells were extracted using the Nuclear and Cytoplasmic Extraction Reagents (Product No. 78835; ThermoFisher Scientific, Waltham, MA, USA). Equivalent amounts of protein (20 µg) were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked in 5% nonfat milk in Tween 20 Tris-buffered saline and incubated with primary antibodies specific for HCV core protein (Abcam, Cambridge, UK), PTB (Abcam), RAN (Abcam), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a cytoplasmic protein marker; Cell Signaling Technology, Danvers, MA, USA) and proliferating cell nuclear Ag protein (PCNA, a nuclear protein marker; Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (SouthernBiotech, Birmingham, AL, USA) for 1 h at room temperature (RT) and visualized with enhanced ECL (ThermoFisher Scientific) following exposure to X-ray films.

Immunofluorescence and confocal analysis

Huh7.5.1 cells plated on glass cover slips (BD Biosciences, San Jose, CA, USA) were infected with JC1-Luc virus for 24 h and then incubated with 100 µL of complete medium. The cells were harvested for immunofluorescence staining after 48 h of incubation. The cells were fixed in 4% paraformaldehyde for 30 m at RT, permeabilized in 0.5% Triton X-100/phosphate-buffered saline for 20 m, and then blocked in 1% bovine serum albumin in phosphate-buffered saline for 1 h at RT to minimize nonspecific adsorption of the antibodies. The cells were then incubated with primary antibodies (PTB or RAN: 1:100; Abcam) in 1% bovine serum albumin/Tween 20 Tris-buffered saline overnight at 4°C, followed by an additional incubation in Alexa fluor-conjugated secondary antibodies (1:500; Life Technologies, Gaithersburg, MD, USA) at RT for 1 h. The nucleus was stained with 4',6'-diamidino-2-phenylindole dihydrochloride (commonly known as DAPI; Sigma-Aldrich, St. Louis, MO, USA), and then the cells were washed three times with phosphate-buffered saline. After the coverslips were mounted on glass slides with mounting medium, the glass slides were photographed using a confocal microscope (Olympus, Tokyo, Japan).

Table 2. siRNA sequences used in the study

siRNAs	Sense, 5'→3'	Antisense, 5'→3'
003	ACAGGAAAGUGAAGGCGAA dTdT	dTdT UGUCCUUUCACUUCCGCUU
004	GACCUUCGUGAAACGUCAU dTdT	dTdT CUGGAAGCACUUUGCAGUA
005	GUAUGUAGCCACCUUGGGU dTdT	dTdT CAUACAUCGGUGGAACCCA

Protein-protein interaction analysis

For coimmunoprecipitation, the cells were lysed with Cell Lysis Buffer (1×; Cell Signaling Technology) containing 1 mM PMSF, 10 mM nicotinamide and 10 µM trichostatin A The lysed cells were centrifuged (13,000 × *g*, 4°C, 10 m), and the supernatants were used for immunoprecipitation. Next, 50 µL of fresh protein G magnetic beads (Millipore) was incubated with anti-RAN or anti-PTB for 10 m with continuous mixing at RT. The lysates (400 µg) and the immobilized capture antibody were then incubated at 4 °C under constant rotation overnight. The beads were washed three times with 1 mL of cold lysis buffer containing protease inhibitors. Finally, the beads were resuspended in 80 µL of 2× Laemmli sample buffer and heated to 95°C for 10 m. The beads were then centrifuged for 1 m at 1,000 × *g*, and the supernatant was collected and used for western blotting.

RNA interference

Small interfering (si)RNAs against human RAN (Ribobio, Guangzhou, China) and control scrambled siRNA (Ribobio, Guangzhou, China) were predesigned and synthesized. Huh7.5.1 cells (at 30% to 40% confluence) were transfected with 100 nM RAN siRNAs using the Lipofectamine® RNAiMAX Reagent (Invitrogen), according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were infected with JC1-Luc virus for 24 h and subsequently incubated with fresh medium for 48 h. The cells were then collected and lysed for luciferase assays, quantitative reverse transcription-PCR and western blotting. The sequences used here are listed in Table 2.

Data analysis

All the data were processed using SPSS 19.0 software and presented as mean ± standard error. Analysis of variance (commonly known as ANOVA) and the least significant difference test were used for comparisons among the groups. When the data were not normally distributed, the Mann-Whitney *U* test and Kruskal-Wallis test were used. A *p*-value less than 0.05 was considered significant.

Results

HCV infection induces RAN expression and cytoplasmic redistribution of PTB

After Huh7.5.1 cells were infected with the JC1-Luc virus, the HCV infection and replication efficiencies were analyzed using the luciferase assay and western blotting. The luciferase activity and core protein expression levels were markedly increased when the cells were infected with the JC1-Luc virus ($p<0.01$; Fig. 1A, B, D) compared with the control group. RAN expression was also examined by quantitative reverse transcription-PCR and western blotting. JC1 virus

infection substantially induced the RAN expression compared with the control ($p<0.01$; Fig. 1B–D).

As mentioned above, studies have shown that PTB binds to HCV RNA at several different sites and participates in viral replication or translation. The cytoplasmic translocation of PTB was determined by western blotting in our study. The results revealed that PTB was predominantly localized in the nucleus in the absence of HCV infection; however, strong cytoplasmic accumulation of PTB was observed following HCV stimulation (Fig. 2A, C; $p<0.01$). The distribution of PTB in HCV-infected cells was also evaluated by immunofluorescence assay. The cytoplasmic distribution of PTB was identified when cells were infected with the JC1-Luc virus (Fig. 2B). These data together illustrated that HCV infection induced the cytoplasmic accumulation of PTB.

### Knockdown of RAN inhibits HCV infection and cytoplasmic accumulation of PTB

The silencing effects of siRNAs on the mRNA and protein expression levels of RAN were evaluated using quantitative reverse transcription-PCR and western blotting. Compared with the MOCK-treated group, the RAN mRNA and protein levels in RAN siRNA-treated cells were significantly decreased ($p<0.01$; Fig. 3). Inhibition of the replication of HCV was measured using the luciferase assay, quantitative reverse transcription-PCR and western blotting. The luciferase activity and both HCV RNA and core protein expression levels were dramatically inhibited by RAN siRNA ($p<0.01$; Fig. 4A–C) compared with the MOCK-treated group. Additionally, the cytoplasmic accumulation of PTB in the RAN-silenced group was significantly decreased (Fig. 4D, E).

RAN colocalizes with PTB

To verify whether RAN colocalizes with PTB, the subcellular localization of RAN and PTB were evaluated by immunofluorescence staining with anti-RAN (red) and anti-PTB (green) antibodies in Huh7.5.1 cells infected with the JC1 virus. The merged image in yellow indicates the combination of PTB fluorescence intensity with the RAN fluorescence intensity. PTB and RAN staining was mainly distributed in the nucleus of Huh7.5.1 cells, but strong cytoplasmic RAN and PTB immunofluorescence was identified when cells were infected with the JC1 virus (Fig. 5). Additionally, double-labeling of RAN and PTB was observed in both the cytoplasm and nucleus. The above results revealed that HCV infection induces the cytoplasmic distribution of RAN and PTB and RAN colocalizes with PTB.

RAN directly interacts with PTB

We investigated the potential crosstalk between RAN and PTB in Huh7.5.1 cells. Therefore, endogenous protein-protein interaction in cells was examined by co-immunoprecipitation experiments using anti-RAN and anti-PTB antibodies. Cells were extracted and immunoprecipitated with anti-RAN anti-
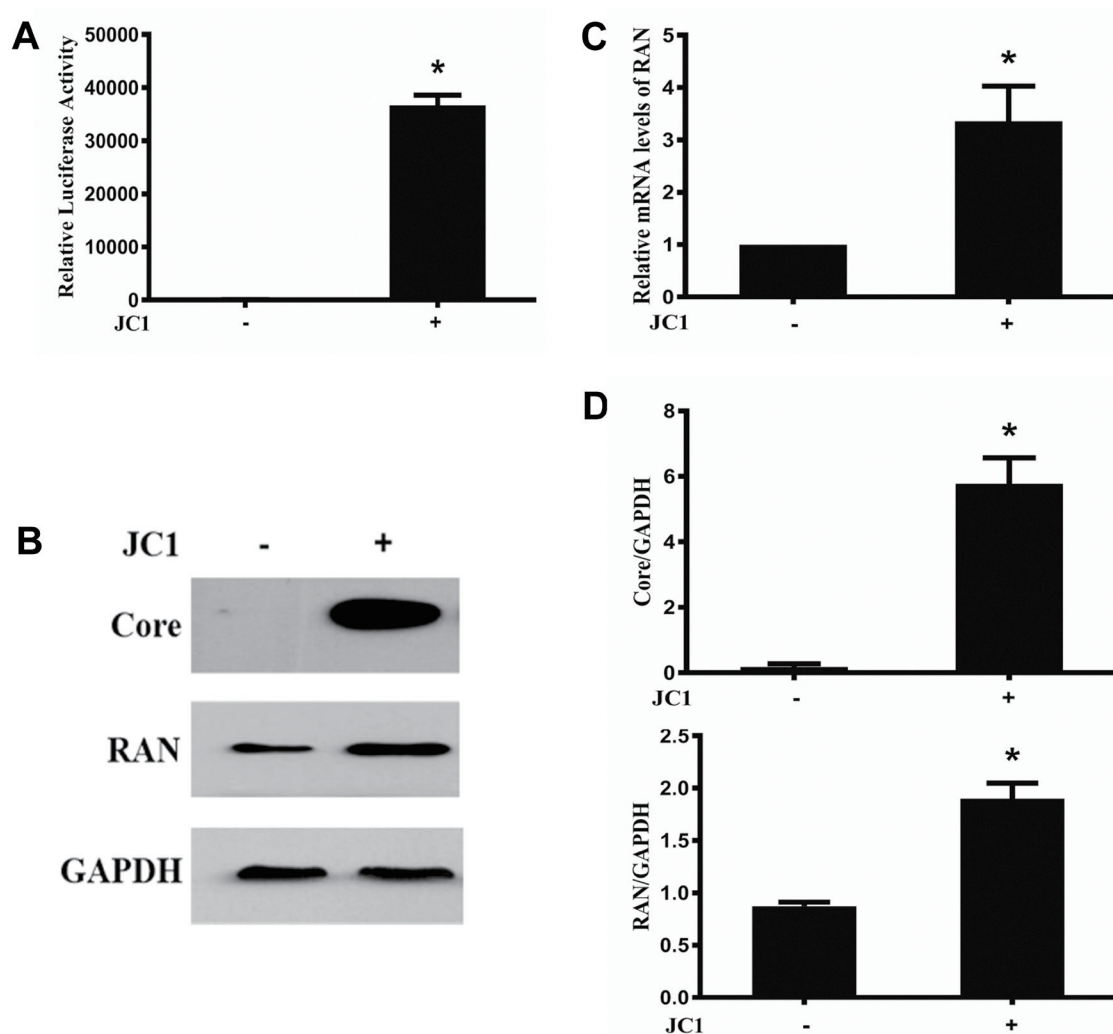
Fig. 1. HCV infection induces the expression of RAN. Huh7.5.1 cells were infected with the JC1 virus. Naïve Huh7.5.1 cells were used as a MOCK-infected control. (A) The replication of HCV was examined using the luciferase assay. (B) Intracellular core and RAN levels were analyzed using western blotting at 48 h post-infection. (C) Intracellular RAN levels were determined using quantitative reverse transcription-PCR at 48 h post-infection. (D) The ratios of core/GAPDH and RAN/GAPDH are shown. Representative images of three independent experiments are presented. $^*p<0.01$, compared with control cells.

body, and the immune complexes were analyzed by western blotting with the anti-PTB antibody. RAN interacted with PTB in Huh7.5.1 cells (Fig. 6), confirming their crosstalk.

Discussion

RAN is a critical player in nucleo-cytoplasmic transport that is mainly localized in the nucleus and cycles between the GDP-bound inactive and GTP-bound active state.[26] It is now well established that RAN plays an important role in cancer development and progression.[27,28] It is overexpressed in various cancers and correlated with increased aggressiveness of the cancer cells *in vitro* and *in vivo*.[29–31] Recent studies have indicated that RAN also plays an important role in virus infection. A study showed that the microRNA miR-134 regulated poliovirus replication via the modulation of RAN.[32] Additionally, the reduced production of RAN by RNA interference markedly reduced the synthesis of EV71-encoded viral proteins and virus titers.[33] However, the role of RAN in HCV infection remained unclear.

The above data prompted us to investigate the function of RAN in the regulation of HCV infection. Our results showed that HCV infection significantly induced RAN expression. Additionally, the knockdown of RAN expression with siRNAs significantly reduced HCV replication. RAN silencing has been shown to cause aberrant nucleo-cytoplasmic transport of tumor suppressors and oncogenes, possibly leading to the initiation of cancer.[34] Because RAN plays a key role in controlling nucleo-cytoplasmic trafficking, we hypothesized that HCV-induced upregulation of RAN expression might be involved in the development of HCV by influencing the essential viral proteins or host proteins for viral replication.

PTB is primarily localized in the nucleus. However, it can shuttle from the nucleus to the cytoplasm in response to specific signals, such as viral infection.[25] As a ubiquitous RNA-binding protein, PTB can function as both a repressor and activator of RNA metabolism by restructuring RNA to promote or inhibit the binding of other factors, processes known to be important for the life cycle of many viruses.[35] The necessity of PTB for HCV replication and translation has been proposed. Studies have shown that the recognition of
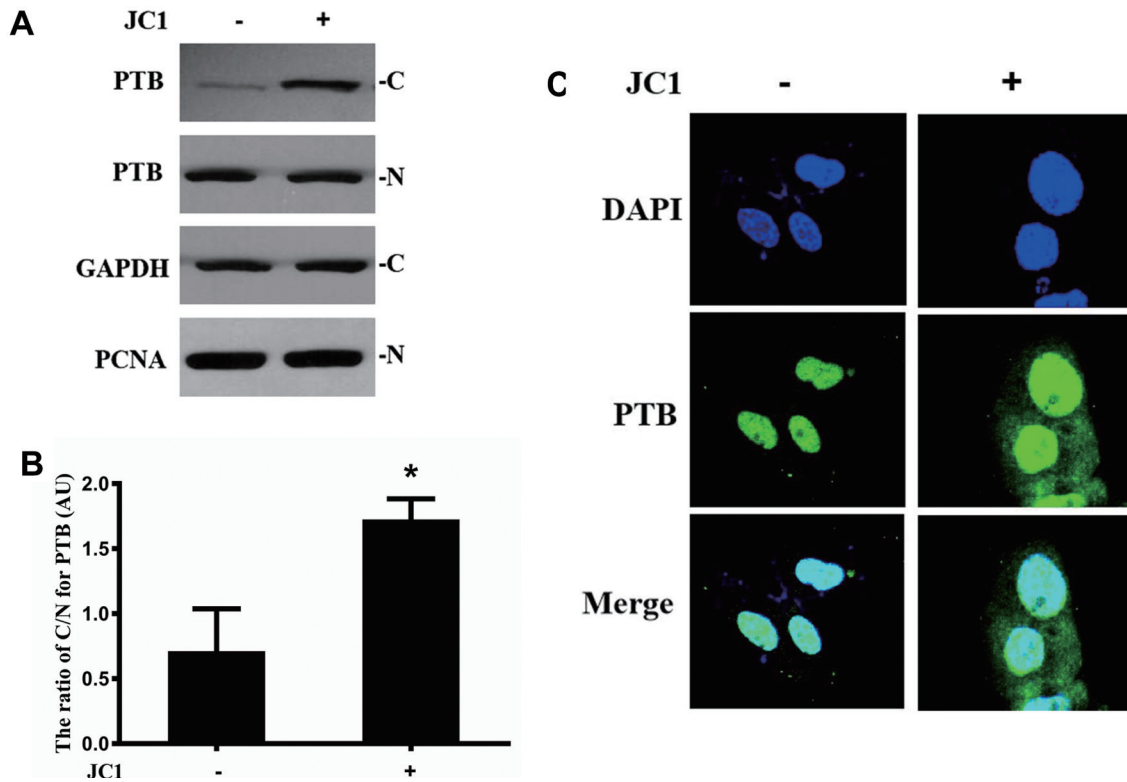
Fig. 2. HCV infection induces the nucleo-cytoplasmic shuttling of PTB. (A and B) The cytoplasm to nuclear ratio for PTB expression levels was detected using western blotting. C, cytoplasm; N, nucleus; PCNA, proliferating cell nuclear Ag. The representative blots of three independent experiments are shown. (C) Imaging of PTB fluorescence intensity. Cellular PTB was stained with anti-PTB mouse antibody and then stained with Alexa fluor 488 anti-rabbit secondary antibody (green color). The nucleus was stained with DAPI (blue color). Merge indicates the combination of PTB fluorescence intensity with nuclear fluorescence intensity. *$p<0.01$, compared with control cells.
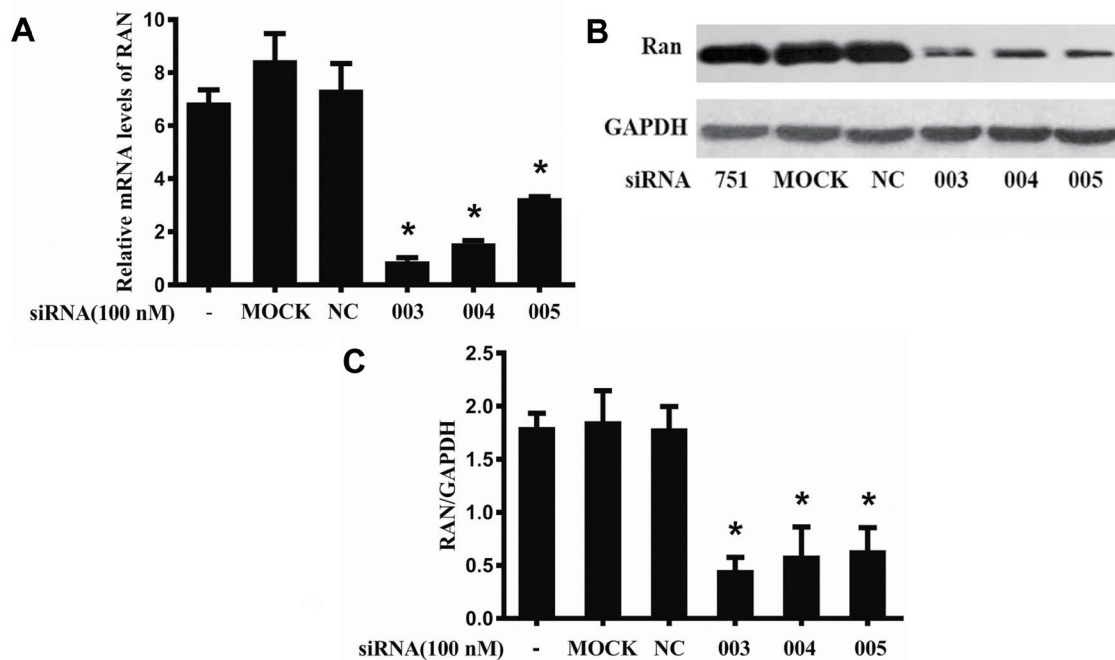
Fig. 3. Silencing effects of siRNAs on the mRNA and protein expression levels of RAN. Huh7.5.1 cells were treated with siRNAs specific to RAN (003, 004 and 005); an irrelevant siRNA (NC) was used as a control in each experiment. (A–B) The expression levels of RAN after siRNA treatment were determined by quantitative reverse transcription-PCR and western blotting. GAPDH mRNA and protein were used for normalization. (C) The ratio of RAN/GAPDH is shown. The results represent three independent experiments. *$p<0.01$, compared with MOCK-treated cells.
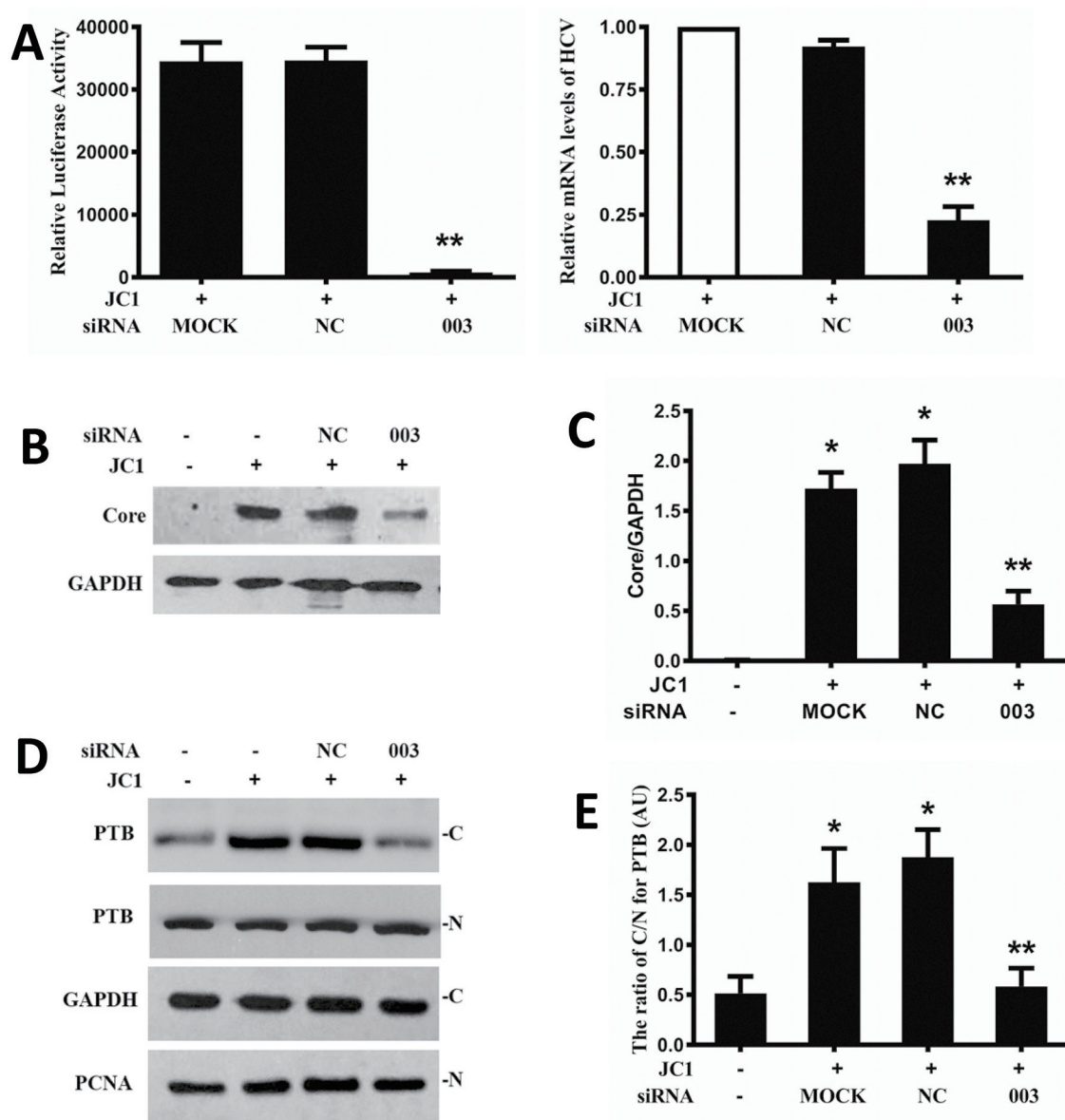
Fig. 4. Knockdown of RAN inhibits HCV replication and the cytoplasmic redistribution of PTB. (A–C) Inhibition of HCV replication was investigated using the luciferase assay, quantitative reverse transcription-PCR and western blotting. Naïve Huh7.5.1 cells were used as a control. (D–E) Inhibition of the cytoplasmic redistribution of PTB was studied using western blotting. $^*p<0.01$, compared with control cells. $^{**}p<0.01$, compared with JC1 virus-infected cells.

the 3'-UTR by PTB was necessary for the efficient replication of HCV RNA.[14,18,36] Other studies have also indicated that the interaction of PTB with the 5'-UTR and 3'-UTR of the HCV RNA was required for the initiation of translation.[12,13]

Our previous results indicated that the cytoplasmic accumulation of PTB was directly associated with HCV replication, and blocking the cytoplasmic redistribution of PTB could inhibit HCV replication.[37] However, the mechanism of nucleo-cytoplasmic translocation of PTB is not clear. As the major cellular function of RAN is to regulate nucleo-cytoplasmic transport of molecules through the nuclear pore complex, we consider that RAN might interact with PTB and facilitate its nucleo-cytoplasmic translocation.

Based on the above research assumptions, our research continued and found that increased nucleo-cytoplasmic translocation of PTB in response to HCV infection was dra-

matically inhibited by RAN silencing. RAN interacted with PTB, as demonstrated by coimmunoprecipitation studies, and facilitated its nucleo-cytoplasmic translocation. Because PTB in the host cytoplasm is directly associated with HCV replication, the involvement of RAN in HCV replication can be the result of the cytoplasmic accumulation of PTB.

Conclusions

In summary, our results demonstrate that the involvement of RAN in HCV infection is mediated by interacting with PTB and then influencing the cytoplasmic translocation of PTB. Our work uncovers a new mechanism responsible for host cellular factors involved in HCV infection and indicates that targeting of the nucleo-cytoplasmic translocation of the host PTB protein
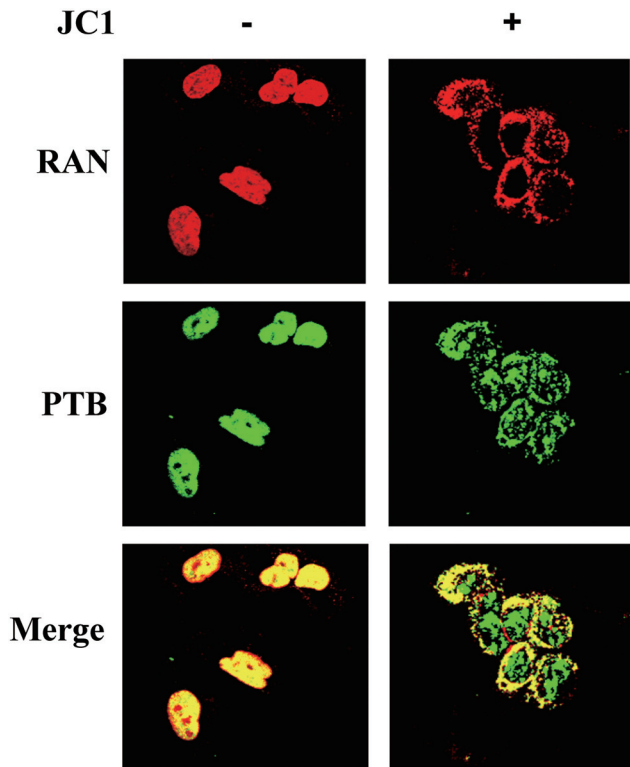
Fig. 5. Colocalization of RAN with PTB on Huh7.5.1 cells infected with JC1 virus. Cells infected with JC1 virus were fixed with ice-cold methanol at 48 h post-infection. The fixed cells were incubated with anti-RAN antibody or anti-PTB antibody followed by a secondary antibody labeled with Alexa fluor 647 (red, RAN) or Alexa fluor 488 (green, PTB). The colocalized signal (yellow) was visualized by merging the two images. All the experiments were performed in triplicate.

could be a novel strategy against HCV. Antiviral agents acting through this mechanism might inhibit viral infection with no or a decreased chance of drug-resistant mutations. Ideally, the combination of a RAN suppressor with known anti-HCV drugs might provide a variety of drug regimens that are appropriate for different patients and provide the potential advantage for preventing or decreasing drug-resistant mutations.
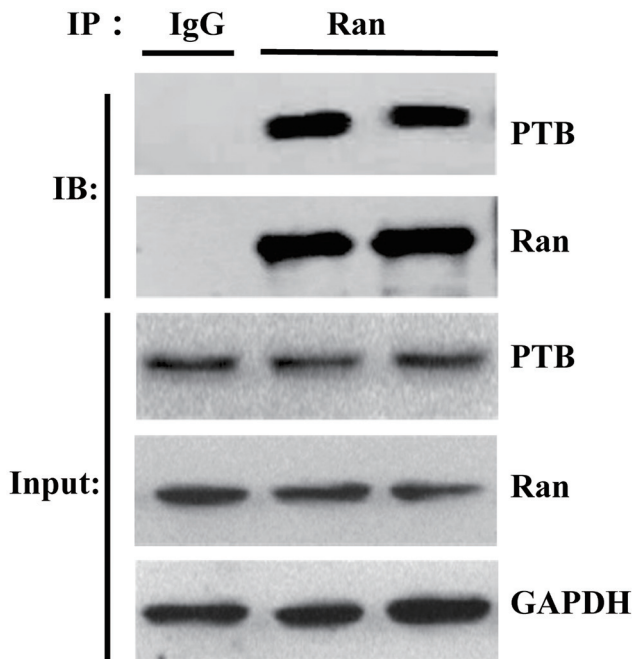
Acknowledgments

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Conflict of interest

The authors have no conflict of interests related to this publication.

Fig. 6. RAN physically interacts with PTB in Huh7.5.1 cells. Cells were extracted and immunoprecipitated with the anti-RAN antibody, and the immunocomplexes were analyzed by western blotting using the anti-PTB antibody. The IgG antibody was used as a negative control.

Author contributions

Study concept and design (JL, YG), acquisition of data (JX, JC), analysis and interpretation of data (JX, XM), drafting of the manuscript (JX, YS), critical revision of the manuscript for important intellectual content (XM, JC), administrative, technical, or material support, and study supervision (HY).

Data sharing statement

All data are available upon request.

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