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Hepatitis C virus non-structural 5A protein can enhance full-length core protein-induced nuclear factor-κB activation

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Abstract

AIM: To study the effects of hepatitis C virus (HCV) core and non-structural 5A (NS5A) proteins on nuclear factor-κB (NF-κB) activity for understanding their biological function on chronic hepatitis caused by HCV infection.

METHODS: Luciferase assay was used to measure the activity of NF-κB in three different cell lines cotransfected with a series of deletion mutants of core protein alone or together with NS5A protein using pNF-κB-Luc as a reporter plasmid. Western blot and indirect immunofluorescence assays were used to confirm the expression of proteins and to detect their subcellular localization, respectively. Furthermore, Western blot was also used to detect the expression levels of NF-κB/p65, NF-κB/p50, and inhibitor κB-a (IκB-a).

RESULTS: The wild-type core protein (C191) and its mutant segments (C173 and C158) could activate NF-κB in Huh7 cells only and activation caused by (C191) could be enhanced by NS5A protein. Moreover, the full-length core protein and its different deletion mutants alone or together with NS5A protein did not enhance the expression level of NF-κB. The NF-κB activity was augmented due to the dissociation of NF-κB-IκB complex and the degradation of IκB-a.

CONCLUSION: NF-κB is the key transcription factor that can activate many genes that are involved in the cellular immune response and inflammation. Coexpression of the full-length core protein along with NS5A can enhance the NF-κB activation, and this activation may play a significant role in chronic liver diseases including hepatocellular carcinoma associated with HCV infection.

Key words: HCV; NS5A; Core protein; NF-κB

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INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent for acute and chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma[1,2]. HCV is an enveloped positive-sense RNA virus of the *Flaviviridae* family. The viral genome encodes a single polyprotein precursor of ~3 010 amino acids, which is cleaved by both host and viral proteases to produce three structural proteins at the amino terminus (Core, E1, and E2) and six nonstructural proteins at the carboxyl terminus (NS2, NS3, NS4A, NS4B, non-structural 5A (NS5A), and NS5B)[3-5].

HCV core protein is the viral nucleocapsid protein that binds to and packages the viral RNA genome. Core protein is a multifunctional protein that can interact with many cellular factors such as lymphotoxin-β receptor, tumor necrosis factor receptor (TNFR), heterogeneous nuclear ribonucleoprotein and LZIP[6-10]. Core protein also can modulate the expression of some genes like interleukin-2 (IL-2), p53 and p21[11-14]. NS5A protein is a phosphoprotein that exists in differentially phosphorylated forms of 56 and 58 ku with modifications of serine residues[15]. NS5A protein can directly interact with double-stranded RNA-dependent kinase and inactivate its function, thus modulating the IFN-stimulated antiviral response[16].

Nuclear factor-κB (NF-κB) belongs to a highly conserved Rel-related protein family, which includes RelA (p65), Rel B, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). Of these, the p50/p65 heterodimer, commonly called NF-κB, is the most abundant and ubiquitous. NF-κB is the key transcription factor activating many genes involved in the cellular immune responses and inflammation, such as interferon-β, TNF-α, IL-2, IL-6, and IL-8[17].

Many researchers have reported that HCV core protein can modulate the activity of NF-κB in mammalian cells[18-21]. This phenomenon has also been found in NS5A protein[22-24]. Core protein can interact with NS5A protein both *in vitro* and *in vivo*[25]. However, whether this interaction has effect on NF-κB activation has not yet been determined. In this study, we investigated the effect of core protein and NS5A protein coexpression on NF-κB activity in three different cell lines. Our results showed that NS5A protein could

enhance the effect of full-length core protein on NF-κB activation in Huh7 cell line only. This activation is associated with the degradation of inhibitor κB-α (IκB-α).

MATERIALS AND METHODS

Plasmid construction

The recombinant plasmids with different deletion mutations of HCV core or NS5A genes were constructed by inserting the corresponding DNA fragments into the eukaryotic expression vector pcDNA3 under the immediate early CMV promoter. The DNA fragments were amplified by PCR from pBRTM/HCV1-3011 plasmid (genotype 1a, a generous gift from Dr. Charles M Rice, University of Washington) using primers in Table 1. Primers 1a and 1b were used to generate pcDNA-core (for C191) containing the full-length core protein, 1a and 1c primers were used to generate pcDNA-Δcore519 (for C173) with a deletion of 54 nt at its 3'-end, 1a and 1d primers were used to generate pcDNA-Δcore474 (for C158) with a deletion of 99 nt at its 3'-end, 2a and 1b primers were used to generate pcDNA-Δcore [76-573, for C(26-191)] with a deletion of 75 nt at its 3'-end, 2a and 1d primers were used to generate pcDNA-Δcore [76-474, for C(26-158)] with a 75 and 99 nt deletion at the 5'-end and 3'-end, respectively, 3a and 3b primers were used for pcNS5A with full-length 5A gene. All the constructed plasmids were confirmed by restricted digestion and sequencing.

Table 1 Primers used in this study

Primers	Oligonucleotides
1a	5′-AAG CTT *GAATTC* GCG **ATG** AGC ACG AAT CCT-3′ (*Eco*RI)
1b	5′-GTC GAC *TCTAGA* **CTA** GGC CGA AGC GGG CAC-3′ (*Xba*I)
1c	5′-GTC GAC *TCTAGA* **CTA** AGA GCA ACC AGG AAG-3′ (*Xba*I)
1d	5′-GTC GAC *TCTAGA* **CTA** CAG AAC CCG GAC GCC-3′ (*Xba*I)
2a	5′-AGT ACT *GAATTC* GCG **ATG** GGT GGC GGT CAG-3′ (*Eco*RI)
3a	5′-AGA TCT *AAGCTT* **ATG** GCT TCC GGC TCC TGG-3′ (*Hin*dIII)
3b	5′-TGG TAC *TCTAGA* **TCA** CAT TCA GCA GCA-3′ (*Xba*I)

Italic nucleotides represent the enzymes used for the ligation and the enzymes are also marked in the end of each primer. Bold nucleotides ATG and CTA represent the start and stop codon, respectively.

Cell culture and transfection

Cos-7, HeLa, and Huh7 cell lines were grown in Dulbecco's modified Eagle's medium (Hyclone) with 10% fetal bovine serum (Gibco) at 37 °C in 50 mL/L CO_2. DNA transfection experiment was performed using Lipofectamine™ 2000 (Gibco) according to the manufacturer's instructions.

Luciferase assay

The pNF-κB-Luc (Stratagene) vector containing the *Photinus pyralis* (firefly) luciferase reporter gene driven by a basic promoter element (TATA box) plus five repeats of κB *cis*-enhancer element (TGGGGACTTTCCGC) was used in this experiment. Approximately 5×10^4 cells were seeded into a 24-well tissue culture plate 24 h before transfection. The cells were cotransfected with 0.8 μg plasmids as four kinds of combination: (1) 0.4 μg pNF-κB-Luc and different concentrations (0.1, 0.2, and 0.4 μg) of core mutations or pcNS5A; (2) 0.4 μg pNF-κB-Luc and the same concentration of core mutations (0.2 μg) and pcNS5A (0.2 μg); (3) 0.3 μg pNF-κB-Luc and the same concentration of pcDNA-core (0.2 μg) and different concentrations (0.1, 0.2, and 0.3 μg) of pcNS5A; (4) 0.3 μg pNF-κB-Luc and the same concentration of pcNS5A (0.2 μg) and different concentrations (0.1, 0.2, and 0.3 μg) of pcDNA-core. Cells were cotransfected with pNF-κB-Luc and vector pcDNA3 as a negative control. The total concentration of the transfected plasmids was kept constant with pcDNA3 vector. Cells were harvested at 24 h after transfection and then lysed in the reporter lysis buffer (Promega). The luciferase activity was measured by TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA, USA), and normalized with respect to the protein concentration of the cell lysates. Each experiment was repeated at least thrice.

Western blot analysis

Twenty-four hours after transfection, the total cell proteins were separated by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The blots were first blocked with 5% non-fat milk in Tris buffer saline (TBS) containing 1 g/L Tween-20 and then probed with the first antibodies against HCV core protein (ViroStat, Inc., 1851), HCV NS5A protein (a generous gift from Dr. Stephene J Polyak, University of Washington), actin (Santa Cruz Biotechnology, Inc., sc-1616), NF-κB/P65 (Santa Cruz Biotechnology, Inc., sc-800), NF-κB/P50 (Santa Cruz Biotechnology, Inc., sc-114), IκB-α (Santa Cruz Biotechnology, Inc., sc-847) for 1 h at 37 °C After extensive washes, secondary antibodies conjugated with horseradish peroxidase were applied onto the blots for at least 1 h at 37 °C. Blots were washed five times with TBS-1 g/L Tween-20. Reagents for enhanced chemiluminescence were applied to the blots and the light signals were detected by X-ray film.

Indirect immunofluorescence assay

Sterile coverslips were placed in six-well tissue culture plates before the Cos-7 cells were plated on them. Twenty-four hours after transfection, the coverslips were rinsed twice with PBS, and the cells were fixed in methanol-acetone (1:1) at -20 °C for 5 min. After washing thrice with PBS, the fixed cells were covered with anti-core monoclonal or anti-NS5A mAbs for 1 h at 37 °C, and then washed thrice with PBS. Secondary antibodies (FITC-conjugated goat anti-mouse IgG) were then applied on the cells for 1 h at 37 °C in the dark. After washing, the cells were stained with propidium iodide to visualize the nuclear DNA and mounted in antifade mounting medium. The stained cells were visualized under a Leica confocal microscope (TCS NT SN 1500001, Leica Laser Technik, Germany). For the colocalization of full-length core and NS5A proteins, rabbit anti-core polyclonal antibody (produced in our laboratory) and mouse NS5A mAb were used as primary antibodies and TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG were used as secondary antibodies, respectively.

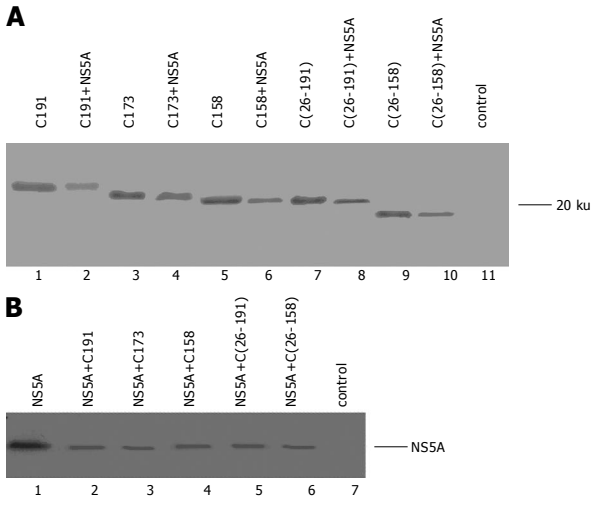

Figure 1 Expression of core mutant fragments **(A)** and NS5A protein **(B)**.

RESULTS

Identification of core mutants and NS5A protein

In this study, four core deletion mutants (C173, C158, C (26-191), C(26-158) were constructed besides the wild-type core protein (C191). C173 is the mature form of core protein with deletion of 18aa at its C-terminus. C158 is a mutant fragment with deletion of the hydrophobic amino acids at its C-terminus. C(26-191) is a mutant fragment without the reported functional domain at its N-terminus. C(26-158) is a mutant fragment without functional and hydrophobic domains at its N- and C-terminus, respectively. The expression of core mutants and NS5A protein in Huh7 was measured by Western blot. As shown in Figure 1A, core mutant fragments were expressed with a correct molecule mass of 21 ku for C191, 19 ku for C173, 18 ku for C158, 18 ku for C(26-191) and 15 ku for C(26-158) in the cells transfected with either core mutation fragments alone (lanes 1, 3, 5, 7, and 9) or together with NS5A protein (lanes 2, 4, 6, 8, and 10). The expression of NS5A protein is shown in Figure 1B. We got the correct band of 56 ku in the cells transfected with NS5A protein alone (lane 1) or together with core mutations (lanes 2-6). This experiment showed that core mutants and NS5A protein were expressed successfully from their respective recombinant plasmids.

HCV core protein activated NF-κB and was enhanced by NS5A protein in Huh7 cells

To study the effect of the coexpression of HCV core and NS5A proteins on NF-κB activity, the expressed plasmids, pcDNA-core, pcDNA-Δcore519, pcDNA-Δcore474, pcDNA-Δcore (75-573), pCDNA-Δcore (75-474), alone or together with pcNS5A were cotransfected with pNF-κB-Luc into three different cell lines Cos-7, HeLa, and Huh7. Luciferase activity was measured 24-h post-transfection. Our results showed a significant change of relative luciferase activity in Huh7 cells (Figure 2A) but not in Cos-7 cells (Figure 2B) or HeLa cells (Figure 2C). In Huh7 cells, the expression of C191 resulted in a significant increase in the NF-κB activity (sevenfold) compared to control cells transfected with vector alone. Expression of C173 and C158 also resulted in an increase in NF-κB activity with about six- and fourfold, respectively, compared to control. Furthermore, the luciferase activity was increased in a dose-dependent manner in core-expressing cells. In contrast, there was no significant change in the relative luciferase activity in the cells transfected with pcDNA-Δcore (75-573) or pcDNA-Δcore (75-474), in which core proteins had 25 amino acids deleted at the N-terminus. This suggested that the N-terminal region (1-25 aa) of core protein is important for its activation on NF-κB, which is consistent with the previous report[20]. The different augmentation of luciferase activity caused by C191, C173, and C158 suggested that

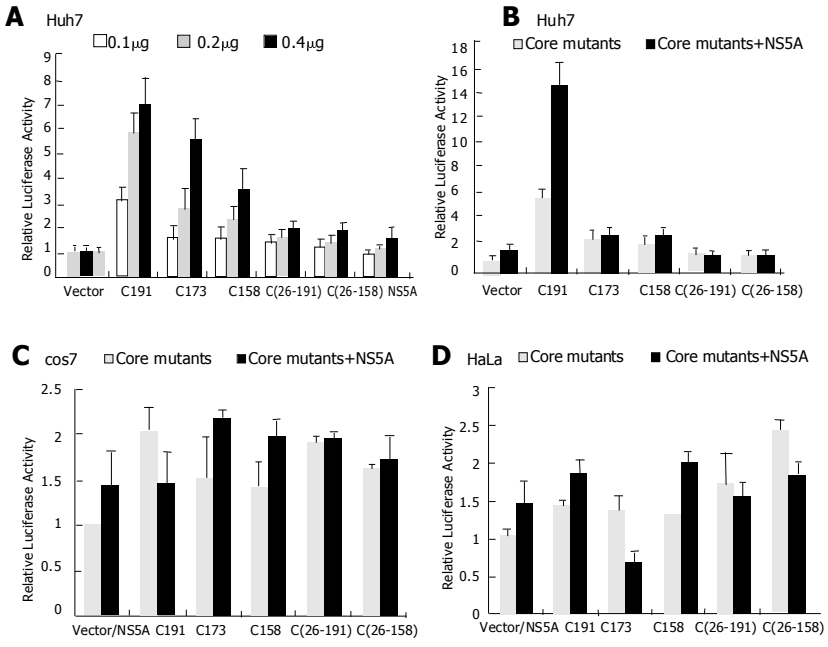

Figure 2 Effect of HCV core and NS5A proteins on NF-κB activity. **A:** NF-κB activation capacity of the core mutant fragments and NS5A protein individually expressing in Huh7 cell line; **B:** NF-κB activation capacity of the core mutants and NS5A protein expressing in Huh7 cells, **C:** Cos-7 cells; **D:** HeLa cells.

the activation of NF-κB by core protein was related to the hydrophobicity of C-terminus of core proteins (C191> C173>C158).

When NS5A protein was expressed alone, it had no effect on NF-κB activation (Figure 2A). But co-expression of C191 and NS5A protein resulted in a significant increase in the luciferase activity with about 15-fold compared to the control and twofold compared to the C191-expressing cells only. However, the relative luciferase activity in the cells co-expressing NS5A protein and other core mutants was not augmented (Figure 2B). These results indicated that NS5A protein could enhance C191-induced NF-κB activation.

NS5A protein enhanced core protein-induced NF-¦ÊB activation in a dose-dependent manner

In order to understand, how NS5A protein affects the NF-κB activation caused by C191 core protein, we designed two kinds of combination between pcNS5A and pcDNA-core expression vectors for luciferase assay. In the first one, 0.2 μg of pcNS5A vector was co-transfected with different concentrations of pcDNA-core (0.1, 0.2, and 0.3 μg), while in the other combination, 0.2 μg of pcDNA-core was co-transfected with different concentrations of pcNS5A (0.1, 0.2, and 0.3 μg). Result is shown in Figure 3. When pcNS5A transfection was certain, more pcDNA-core was transfected, the higher luciferase activity could be detected, while the enhanced increasing of activity in number was almost the same (ninefold) compared to cells expressing C191 alone (Figure 3, right), which meant that the same amount of NS5A protein exerted the same effect on different amounts of C191. When pcDNA-core transfection was certain, more pcNS5A was transfected, higher enhanced increasing of luciferase activity could be detected (Figure 3, left). These results showed that NS5A protein could enhance core protein-induced NF-κB activation in a dose-dependent manner.

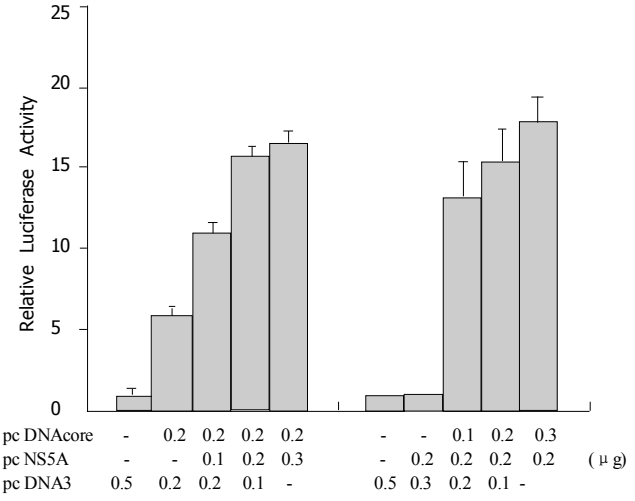

Figure 3 Enhancement of core protein-induced NF-kB activation in a NS5A protein dose-dependent manner.

Activation of NF-κB by core protein and NS5A was associated with IκB−α degradation

We further studied whether NF-κB activation occurred at translational or post-translational levels. To do this, the expression levels of NF-κB/p65, NF-κB/p50, and IκB-α in C191-expressing Huh7 cells either alone or together with NS5A were studied. As shown in Figure 4, at the same concentration of cell lysates as confirmed by actin (Figure 4D), there was no change in the p65 and p50 expression profile (Figures 4A and B). While in case of the IκB-α, the expression level was contrary to the NF-κB activity. In the control, cells transfected with pCDNA3 vector had the highest expression level of IκB-α (Figure 4C, lane 1), while in the cells co-expressing C191 and NS5A with the highest NF-κB activity had the lowest expression of IκB-a (Figure 4C, lane 3). Cells expressing C191 also had a much lower expression of IκB-α (Figure 4C, lane 4), while cells expressing NS5A protein had a much higher expression of IκB-α (Figure 4C, lane 2). It was more likely that when C191 and NS5A protein were co-expressed, they activated IκB kinase (IKK) through some signal pathway, resulted in the phosphorylation and then degradation of IκB, leading to the activation of NF-κB. These results suggested that C191 alone or together with NS5A protein did not enhance the expression of NF-κB. Thus, the NF-κB activity was augmented due to the dissociation of NF-κB¨CIκB complex and IκB degradation.

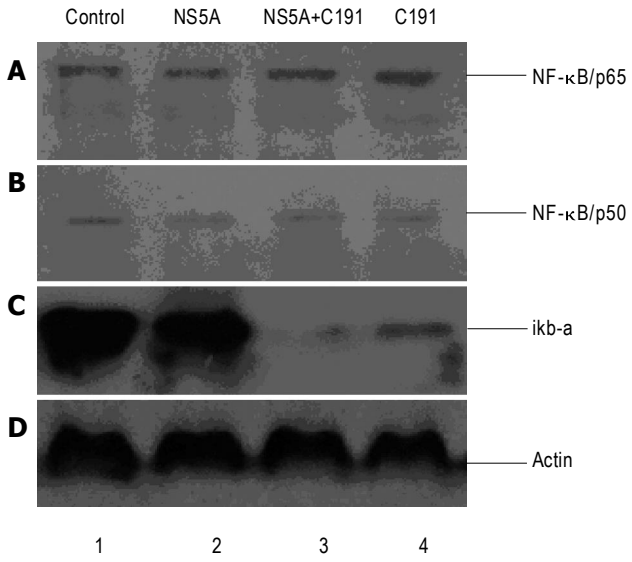

Figure 4 Expression of NF-κB/p65, NF-κB/p50 and IkB-a.

Subcellular localization of C191 and NS5A proteins in Cos-7 cell line

We then analyzed the subcellular localization of C191 and NS5A proteins using indirect immunofluorescence assay in Cos-7 cell line. As shown in Figure 5, C191 was localized predominantly in the cytoplasm (Figures 5A-C). NS5A protein was also localized in the cytoplasm with fluorescence signals surrounded the nuclear membrane (Figures 5D-F). On the other hand, the co-expression of C191 and NS5A proteins did not alter the localization of C191, but redistributed the NS5A protein to the same localization pattern of C191 (Figures 5G-I). All these results are consistent with previous reports[25-27]. Thus, the same

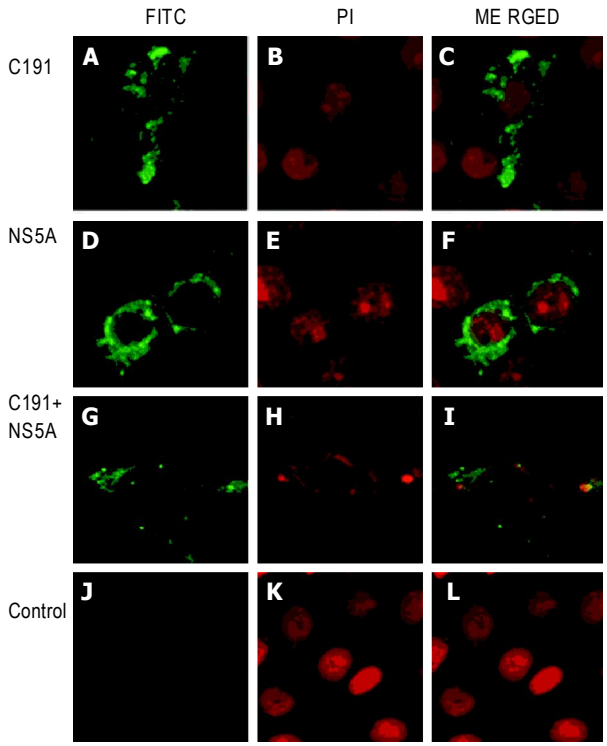

Figure 5 Localization of C191 and NS5A proteins. **A-C:** C191; **D-F**: NS5A protein; **G:** NS5A protein (FITC-labeled); **H:** C191 protein (TRITC-labeled); **I:** merged localization of C191 and NS5A proteins; **J-L:** pcDNA3 vector alone as a negative control.

cytoplasmic localization pattern of C191 and NS5A protein suggested that NF-κB activation caused by co-expression of C191 and NS5A protein was more likely due to a physical interaction between them or the interaction with the same cellular factors.

DISCUSSION

NF-kB is a critical regulator of the immediate early pathogen response, and plays an important role in promoting inflammation and in regulation of cell proliferation and survival[17,28]. NF-κB is highly activated at sites of inflammation in diverse diseases and induces transcription of pro-inflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases, cyclooxygenase 2, and inducible nitric oxide synthase[29]. Moreover, NF-κB can also activate the expression of cyclin D1, c-myc, other regulators of the cell cycle and enhance cell survival by switching on genes that inhibit pro-apoptotic signals[30]. Because of the multifunction of NF-κB, many viruses including several human pathogens such as HIV-1, human T-cell leukemia virus, herpes simplex virus, HBV, and Epstein-Barr virus have evolved different strategies to modulate the activity of NF-κB[31-34]. Some of them modulate the NF-κB activity through the binding of the viral particles to their receptor, some of them modulate through viral proteins. The activation of NF-κB may be a strategy of viruses to block apoptosis and prolong survival of the host cells in order to gain time for replication and increase viral progeny production[35].

Since HCV core protein can interact with TNF-α by yeast two-hybrid system[8], NF-κB, the main effective factor in the TNF-α signaling pathway, has become a hot factor in the functional studies of core protein. Core protein can modulate NF-κB at the basal level in many mammalian cell lines, although the modulation is different from HCV genotype and cell lines[19-21].

In this study, we found that core protein could increase the activity of NF-κB only in Huh7 cell line, but not in Cos-7 and HeLa cell lines. This augmentation was in a core protein dose-dependent manner. Core protein can interact with NS5A protein both *in vitro* and *in vivo*[25]. NS5A protein can also modulate the activity of NF-κB[23]. Since this modulation is also different from different cell lines[36], we want to know if this interaction between core and NS5A proteins has certain effect on the regulation of NF-κB. Our results showed that when C191 and NS5A proteins were co-expressed in Huh7 cell line, they activated NF-κB and this activation was in a NS5A protein dose-dependent manner. Moreover, the full-length core protein was required for the interaction between core and NS5A proteins, because no increase in the NF-κB activity was observed in the cells co-expressing any of the core mutant fragments and NS5A protein. Further experiments have confirmed that this activation is associated with the NF-κB-IκB complex dissociation and IκB degradation but not with the change in the expression level of NF-κB.

There are two signaling pathways leading to NF-κB activation. The classical NF-κB pathway, based on IKK-β-dependent IκB degradation, is essential for innate immunity. The alternative NF-κB pathway, based on IKK-α processing of NF-κB2/p100 into NF-κB2/p50, is related to lymphoid organ development and adaptive immunity[29]. The activation of NF-κB caused by co-expression of core and NS5A proteins may occur in the classical NF-κB pathway. Proinflammatory cytokines and pathogen-associated molecular patterns, working through different receptors belonging to the TNFR and Toll-like receptor-IL-1 receptor superfamilies, cause activation of IKK. The activated IKKs, predominantly acting through IKK-β in an IKK-γ-dependent manner, catalyze the phosphorylation of IκBs, polyubiquitination and subsequent degradation by the 26S proteasome[29]. As previously reported, core protein can activate NF-κB through TNF receptor-associated factor 2-IKK-β-dependent pathway[37]. The phosphorylation of I κB-α by IKK-β can be significantly enhanced by HCV core protein expression in a dose-dependent manner. NS5A protein can activate NF-κB by altering intracellular calcium levels and then inducing oxidative stress, through tyrosine phosphorylation of IκB-α at Tyr^{42} and Tyr^{305} residues instead of $Ser^{32,36}$ phosphorylation of IκB-α caused by IKK, thus inducing IκB-α degradation[24]. We found that expression of core protein could activate NF-κB, while this did not happen in case of NS5A protein expression in Huh7 cells. These results of NS5A protein are consistent with one report[38] but inconsistent with other reports[22,24]. This may be due to the different cell lines and/or experimental methods used in their studies. Interestingly, co-expression of C191 and NS5A proteins activated NF-κB significantly.

When NS5A and C191 proteins were co-expressed, this interaction caused the change in the conformation of NS5A protein, which leads to the tyrosine phosphorylation of Iκ B-α, and resulted in core protein-induced NF-κB activation. Further experiments need to be done to elucidate this idea.

HCV causes persistent infection and chronic hepatitis in most infected individuals. Spontaneous recovery following HCV infection is a relatively rare event. The mechanisms responsible for HCV-mediated chronicity and disease progression remain unclear[39]. The activation of NF-κB has been found in both subgenomic replicon and full-length HCV-transfected cells and HCV-infected livers [24,36,39]. This may provide the possible mechanism for HCV pathogenesis.

C191 is produced in the early stage of HCV infection and then cleavaged to its mature form C173 to assist virus assembly[40]. In this study, we found that NS5A protein could enhance C191-induced NF-κB activation but not C173, when NS5A and core proteins were co-expressed in Huh7 cells. Activation of NF-κB always leads to inhibition of apoptosis. In the early stage of HCV infection, NS5A protein functions synergically with C191 on NF-κB activation and this may inhibit apoptosis of host cells; while in late stage of HCV infection, NS5A protein does not function synergically with C173 on NF-κB activation and decreases their ability to inhibit apoptosis of host cells. This may play an important role in the establishment of chronic and persistent liver disease during HCV infection.

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