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Hepatitis C Virus Core Protein Potentiates TNF-α-Induced NF-κB Activation through TRAF2-IKKβ-Dependent Pathway

Young Mee Chung,* Kyu Jin Park,† Soon Young Choi,* Soon Bong Hwang,† and Soo Young Lee*,[1]

**Division of Molecular Life Sciences and Center for Cell Signaling Research, Ewha Womans University, Seoul 120-750, Korea; and †Institute of Environment and Life Science, The Hallym Academy of Sciences, Hallym University, Chuncheon 200-702, Korea*

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Previous work has implicated that the core protein of hepatitis C virus (HCV) may play a modulatory effect on NF-κB activation induced by TNF-α. However, it is unclear how HCV core protein modulates TNF-α-induced NK-κB activation. Here we show that overexpression of HCV core protein potentiates NF-κB activation induced by TNF-α. Expression of dominant negative form of TRAF2 inhibits the synergistic effects of HCV core protein on NF-κB activation, suggesting that HCV core protein potentiates NF-κB activation through TRAF2. Moreover, we demonstrate that HCV core protein potentiates TRAF2-mediated NF-κB activation via IKKβ. In addition, HCV core protein associates with TNF-R1-TRADD-TRAF2 signaling complex, resulting in synergistically activation of NF-κB induced by TNF-α. Thus, these observations indicate that HCV core protein may play an important role in the regulation of the cellular inflammatory and immune responses through NF-κB.

Key Words: **HCV core protein; TNF-α; NF-κB; TRAF2; IKKβ.**

TNF-α is a pleiotropic proinflammatory cytokine secreted by activated macrophages and T lymphocytes in response to viral infections, which may inhibit viral replication or induce apoptosis, and some viruses have evolved strategies to block the antiviral effect of TNF-α (1, 2). Intracellular signal transduction from TNF receptor 1 (TNF-R1) occurs through a controlled series of protein–protein interactions. Following TNF-α-induced trimerization of the receptor, TNF receptor-associated death domain-containing protein (TRADD) is recruited to a region of TNF-R1 to which the cytotoxic function has been mapped, namely, the death domain (3). TRADD subsequently recruits other effector proteins into the complex. FADD/MORT1, TRAF2, and RIP are not only recruited but also have been shown to interact directly with TRADD (4, 5). While FADD/MORT1 is essential for TNF-α-induced apoptosis, TRAF2 and RIP seem to be involved in both the transcription factor nuclear factor (NF)-κB and the c-Jun NH_2-terminal kinase (JNK) activation (6–8). Among these signaling events induced by TNF-α, the NF-κB regulates expression of many inflammatory and innate immune response genes, including proinflammatory cytokines, chemokines, and adhesion molecules (9–12).

Hepatitis C virus (HCV) is a positive-strand RNA virus that has been identified as the major cause of posttransfection non-A, non-B hepatitis worldwide, which often leads to liver cirrhosis and hepatocellular carcinoma (13). Although humoral and cellular immune responses to HCV have been detected, a proper understanding of viral persistence is not known. Viral infection may often induce host immune responses, and many viruses, in turn, encode proteins which inhibit this defense mechanism (14). These alterations in cell survival contribute to the pathogenesis of a number of human diseases, including viral oncogenesis (15). Of at least 10 viral proteins encoded by the HCV genome, its nucleocapsid core protein has regulatory roles in viral and cellular genes and also has effects on cell growth and proliferation (16–18). Recently, it is known that HCV core protein can associate with TNF receptor-related lymphotoxin-β receptor (LT-βR) and TNF-R1, respectively (19, 20). This interaction modulates one of the biological activities, i.e., cytolytic activity, of LT-βR triggering by its recombinant ligand (LT-$\alpha 1\beta 2$) in certain cell line. Interaction of HCV core protein with the cytoplasmic tail of TNF-R1 also modulates TNF-α-induced NF-κB activation although its positive or negative effects on TNF-α-induced NF-κB activation still remains controversial depending on cell lines (21, 22).

[1] To whom correspondence should be addressed at Division of Molecular Life Sciences and Center for Cell Signaling Research, Ewha Womans University, 11-1 Daehyun-dong, Seodaemoon-gu, Seoul 120-750, Korea. Fax: 82-23277-3760. E-mail: leesy@mm.ewha.ac.kr.

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Here we show that HCV core protein potentiates TNF-α-induced NF-κB activation via a TRAF2-dependent pathway. In addition, we show that HCV core protein potentiates TRAF2-mediated NF-κB activation via IKK-β. Importantly, HCV core protein associates with TNFR1-TRADD-TRAF2 signaling complex, resulting in synergistically activation of NF-κB induced by TNF-α. These findings suggest that HCV core protein may act as a potential regulator of the host immune system.

MATERIALS AND METHODS

Reagents and cell line. Rabbit antisera specific for HCV core have been described previously (23). Antibodies (Abs) specific for TRAF2 (C-20), and TRADD (H-278) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against the Flag epitope were from Sigma. Recombinant human TNF were from R & D Sys. Inc. (Minneapolis, MN). 293 cells were obtained from Y. Choi (The Rockefeller University, NY) and were cultured in Dulbecco's modified Eagle medium (DMEM).

Recombinant plasmids. The mammalian expression plasmids, pcDEF-core, expressing HCV core protein (aa 1–191) from Korean isolate and the bacterial expression plasmids for GST-TNF-R1 (aa 205–426) were described previously (23). The mammalian expression plasmids for TRAF2 and TRAF2.DN (aa 241–501), TRAF5 and TRAF5.DN (aa 236–559), TRAF6 and TRAF6.DN (aa 289–522), TRAF1, and TRADD have been described previously (24). Flag-tagged IKKα and IKKβ and their inactive mutants IKKα (KM) and IKKβ (KM) were a kind gift from Dr. Hiroyasu Nakano (Jutendo University, Japan).

Transfection and reporter assays. 293 cells were transfected in 6-wells dishes by calcium phosphate precipitation as described (24). Each transfection maintained an equal amount of total DNA by adding appropriate amount of the control vector, pcDNA3.1 (Invitrogen). Thirty hours after transfection, luciferase activity was determined and normalized relative to β-galactosidase activity as described (24).

Immunoprecipitation and kinase assay. Cells harvested from each transfection were lysed by homogenization on ice in buffer A containing 100 mM NaCl, 50 mM Tris (pH 8.), 1 mM sodium orthovanadate, 1 mM NaF, 0.5 mM β-glycerophosphate, and protease inhibitors. Each of cell lysates containing 200 μg of protein was incubated with the anti-Flag mAb M2 for IKKα or IKKβ for 4 h at 4°C. The immunoprecipitation complex was extensively washed, and kinase reaction mixture (10 μCi [γ-^{32}P]ATP, 2 mM $MgCl_2$, 1 mM DTT in buffer A) was added to the protein G beads and incubated at 30°C for 30 min. For IKKα and IKKβ kinase activity assay, the substrates were 5 μg of GST-IκBα (1–54) fusion protein. The reaction mixtures were then separated by SDS–PAGE followed by autoradiography.

Precipitation of GST fusion proteins and Western blot analysis. 293 cells were transfected with various combinations of expression vectors as indicated. Thirty hours after transfection, cells were harvested in phosphate-buffered saline/1 mM phenyl-methylsulfonylfluoride (PMSF), lysed in lysis buffer (20 mM Hepes [pH 7.9], 100 mM KCl, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, plus protease inhibitors) and cleared centrifugation to obtain whole-cell extracts. After lysis, aliquots of cell lysates were incubated for 2 h at 4°C with 2 μg of GST-TNF-R1 (aa 205–426) fusion protein expressed in *E. coli* BL21. The glutathione-Sepharose (Pharmacia, Piscataway, NJ) beads were then washed five times with the lysis buffer. The resultant proteins were separated in 12% SDS–PAGE and transferred to Immobilin P (Millipore Corp., Bedford, MA). The blot was subjected to Western blot analysis using enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL).

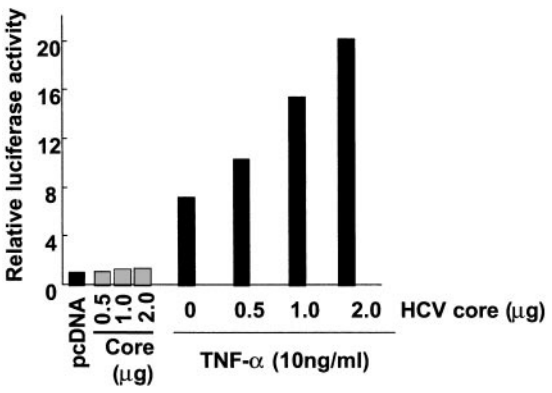

FIG. 1. A dose-dependent effect of HCV core expression on TNF-α-induced NF-κB activation. 293 cells were transfected with 50 ng of p(κB)$_3$-IFN-LUC in the presence of the indicated amounts of HCV core expression vectors. Controls were transfected with pcDNA3.1 empty vectors. All the transfections included 25 ng of pCMV-β-Gal plasmids and DNA concentration was held constant by the addition of empty vector. Twenty-four hours after transfection, cells were left untreated or treated for 6 h with human TNF-α (10 ng/ml) and then cell lysates were prepared and used for luciferase assay. All values represent luciferase activities normalized to β-Gal activities, and the results are displayed as a fold induction over vector alone. A representative result of three independent experiments is shown.

RESULTS AND DISCUSSION

HCV core protein potentiates TNF-α-induced NF-κB activation. Since TNF-α plays prominent roles in the host defense against viruses, we decided to determine whether HCV core protein was capable of modulating TNF-α-induced NF-κB activation in luciferase reporter gene assays. 293 cells were cotransfected with HCV core expression vectors with luciferase reporter constructs to monitor NF-κB transcriptional activity. When expressed alone, HCV core protein did not activate NF-κB, whereas TNF-α (10 ng/ml) induced a ~sevenfold increase of NF-κB-dependent reporter gene activity (Fig. 1). Interestingly, HCV core expression potentiated TNF-α-induced NF-κB activation in a dose-dependent manner. These data suggest that HCV core expression potentiates TNF-α-induced NF-κB activation while core itself, regardless of its protein expression level, had no effects on endogenous NF-κB activation.

Potentiation of TNF-α-induced NF-κB activation by HCV core protein is mediated through TRAF2. Because TRAF2 interacts with TRADD and functions downstream of TRADD in TNF-R1-induced NF-κB activation, we determined whether HCV core expression modulate TRAF2-mediated NF-κB activation in 293 cells. An empty control plasmid or overexpression of TRAF1 in the presence of HCV core did not activate NF-κB. However, overexpression of HCV core protein

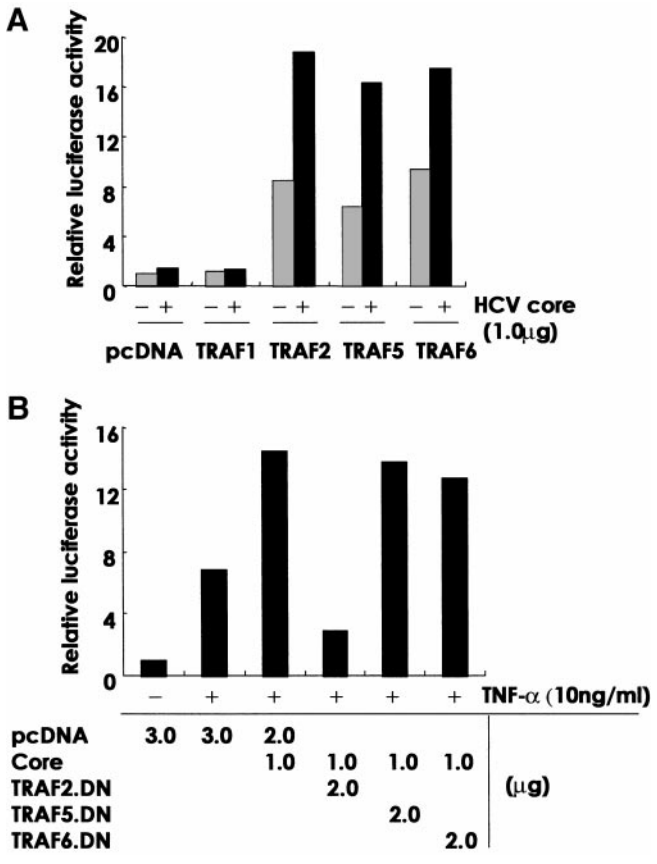

FIG. 2. HCV core protein potentiates TRAF2-mediated NF-κB activation. (A) 293 cells were transfected with 1.0 μg of each TRAF1, TRAF2, TRAF5, and TRAF6 expression vector together with 50 ng of $p(\kappa B)_3$-IFN-LUC in the presence or absence of 1.0 μg of HCV core expression vectors. Thirty hours after transfection, cell lysates were prepared and used for luciferase assay. Luciferase activity was determined as described in the legend to Fig. 1. A representative result of three independent experiments is shown. (B) TRAF2.DN (241–501) inhibits the synergistic activation of NF-κB by HCV core during TNF-α stimulation. 293 cells were transfected with 1.0 μg of HCV core expression plasmids together with 50 ng of $p(\kappa B)_3$-IFN-LUC in the presence 2.0 μg of TRAF2.DN, TRAF5.DN, and TRAF6.DN expression vectors, respectively. A representative result of three independent experiments is shown.

could potently activate TRAF2-mediated NF-κB activation (Fig. 2A). Surprisingly, we found that HCV core expression also potentiated TRAF5- and TRAF6-mediated NF-κB activation. It is known that TRAF5 plays a role in NF-κB activation in signaling through CD40, as well as CD30, RANK, and LT-β receptor (25). TRAF6 also participates in NF-κB activation signaled by CD40, RANK, and IL-1 receptor (26). The fact that HCV core protein interacts with LT-β receptor (19) may explain the observation that HCV core expression potentiated TRAF5-mediated NF-κB activation. Like TNF-α, IL-1 that is one of major proinflammatory cytokines also activates NF-κB through TRAF6. We have found that overexpression of HCV core protein also potentiated IL-1-induced NF-κB activation (data not shown), which is mainly mediated through TRAF6 (26). The precise role of HCV core protein in modulating TRAF5- and TRAF6-induced NF-κB activation has yet to be determined.

To further confirm whether HCV core expression is involved in TNF-α-induced NF-κB activation, we examined the effects of dominant negative forms of TRAF2, TRAF5, and TRAF6 on the synergistic activation of NF-κB induced by TNF-α and HCV core protein. As shown in Fig. 2B, TRAF2.DN (241–501) but not TRAF5.DN (236–559) and TRAF6.DN (289–522) inhibited the synergistic effects of HCV core on TNF-α-induced NF-κB activation. Taken together, these data suggest that HCV core protein contribute to TNF-α-induced NF-κB activation through a TRAF2-dependent pathway.

Synergistic effect of HCV core on TNF-α-induced NF-κB activation is mediated through TRAF2-IKKβ-dependent pathway. To determine whether IKKα and IKKβ are required for the synergistic activation of TRAF2-mediated NF-κB by HCV core protein, we transfected 293 cells with Flag-tagged IKK-α or IKK-β in the presence or absence of HCV core expression vectors. The transiently expressed IKK-α and IKK-β were immunoprecipitated using an anti-Flag monoclonal antibody, and their kinase activity was measured by an *in vitro* kinase assay using a substrate containing the N-terminal portion of IκBα fused to glutathione S-transferase. When expressed alone, IKKα or IKKβ exhibited low basal kinase activity. However, as previously reported (26), TRAF2 coexpression led to marked enhancement in IKKα or IKKβ catalytic activity. Increasing amounts of HCV core protein did not enhance TRAF2-mediated IκBα phosphorylation by IKKα (Fig. 3A). However, we found that the phosphorylation of IκBα by IKKβ was significantly enhanced by HCV core expression in a dose-dependent manner (Fig. 3B).

Next, we addressed whether dominant negative mutants of IKKα and IKKβ could inhibit the synergistic effects of HCV core protein on TNF-α-induced NF-κB activation in a luciferase reporter gene assays. As shown in Fig. 3C, IKKβ (KM), a dominant negative mutant of IKKβ potently inhibited the synergistic effects of HCV core protein on TNF-α-induced NF-κB activation, whereas IKKα (KM) partially inhibited. These results suggest that the synergistic effects of HCV core protein on TNF-α-induced NF-κB activation was mediated through TRAF2-IKKβ-dependent pathway. Our data are consistent with the observation that IKKβ, but not IKKα, plays the major role in IKK activation and induction of NF-κB activity by proinflammatory stimuli (28).

HCV core protein associates with TNFR1-TRADD-TRAF2 signaling complex. The ability HCV core to bind the TNF-R1 (20) raised the possibility that HCV core may regulate formation of the receptor signaling complex in-

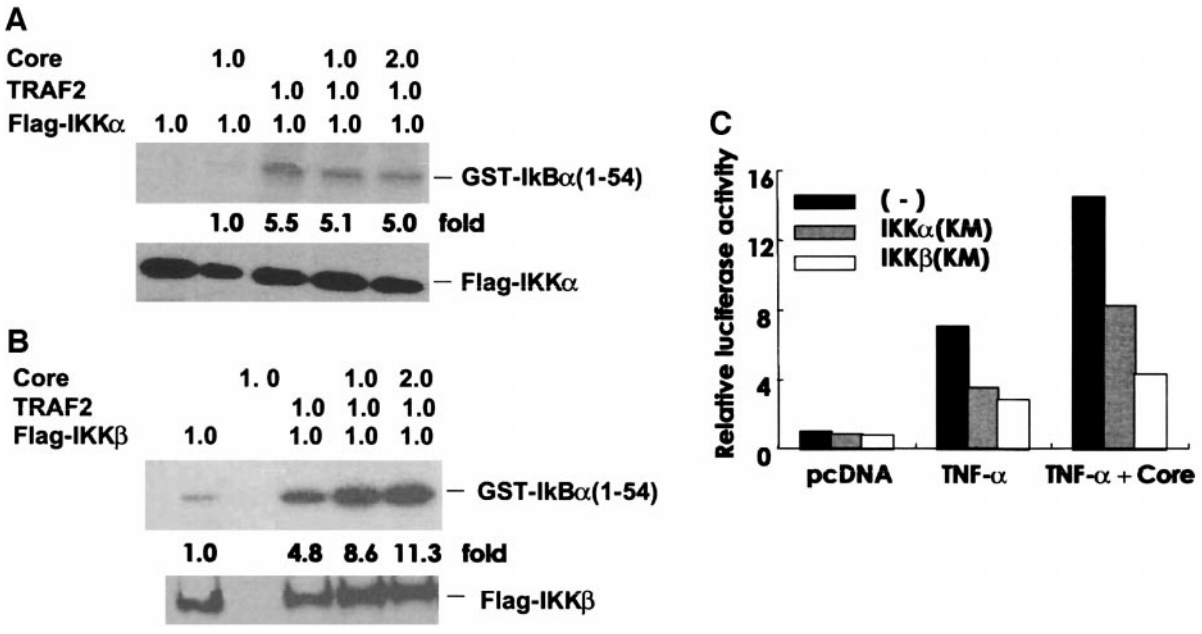

FIG. 3. Synergistic effect of HCV core protein on TNF-α-induced NF-κB activation is mediated through TRAF2-IKKβ pathway. (A) 293 cells were cotransfected with Flag-IKKα, together with TRAF2 and/or HCV core as indicated at the top. Thirty hours after transfection, Flag-IKKα was immunoprecipitated, and its activity was determined by immunocomplex kinase assay with GST-IκBα (1–54) as a substrate as previously described under Materials and Methods. Substrate phosphorylation was quantitated with a phosphoimager. Fold stimulation is indicated (bottom). An aliquot of each lysate was analyzed for its content of IKKα by immunoblotting with anti-Flag monoclonal antibody. (B) 293 cells were cotransfected with Flag-IKKβ, together with TRAF2 and/or HCV core as indicated at the top. Thirty hours after transfection, Flag-IKK-β was immunoprecipitated, and its activity was determined as described for A. (C) Synergistic activation of TNF-α-induced NF-κB by HCV core is mediated through IKKβ. 293 cells were transfected with 50 ng of p$(\kappa B)_3$-IFN-LUC in the presence or absence of inactive IKKα (KM) mutant (2.0 μg), inactive IKKβ (KM) mutant (2.0 μg), HCV core (1.0 μg), or empty vector, as indicated. Twenty-four hours after transfection, cells were left untreated or treated with TNF-α (10 ng/ml) for 6 h. Luciferase activity was determined as described in the legend to Fig. 1. A representative result of three independent experiments is shown.

volving TRADD and TRAF2. Therefore, it was important to determine whether HCV core protein can bind simultaneously to TNF-R1-TRADD-TRAF2 signaling complex. Coexpression of TRADD and TRAF2 with increasing amounts of HCV core protein was not able to prevent recruitment of TRADD-TRAF2 into the TNF-R1 signaling complex. We found that HCV core protein was coprecipitated by the GST-TNF-R1-TRADD-TRAF2 complex (Fig. 4). These results show that HCV core protein is able to bind TNF-R1 signaling complex involving TRADD and TRAF2, possibly due to nonoverlapping binding sites in the cytoplasmic tail of TNF-R1.

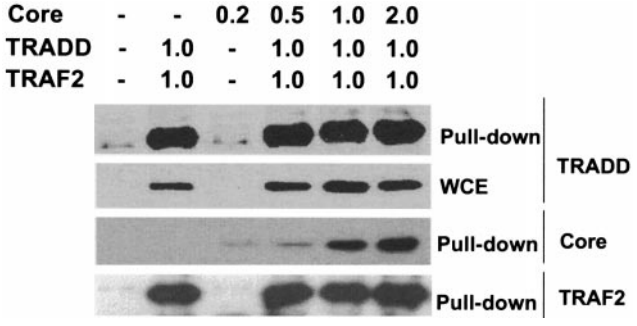

FIG. 4. Overexpression of HCV core protein interacts with the TNF-R1-TRADD-TRAF2 signaling complex. 293 cells were transiently transfected with TRADD, TRAF2, and the increasing amounts of HCV core expression vectors as indicated at the top. Cell lysates were incubated with GST-TNF-R1 fusion protein bound to glutathione-Sepharose beads as described under Materials and Methods. Coprecipitating TRADD, TRAF2, and HCV core were detected by immunoblotting with anti-TRADD, anti-TRAF2, and anti-core Ab.

Our findings imply that TNF-α-induced NF-κB activation is potentiated by HCV core protein through a signaling complex involving TNF-R1-HCV core-TRADD-TRAF2. We further show that the synergistic effects of HCV core protein on NF-κB activation were mediated through TRAF2-IKKβ-dependent pathway. Since NF-κB activation plays an important role in immune response to proinflammatory cytokines including TNF-α and IL-1, it is possible that HCV core protein may accelerate an inflammatory response, resulting in contribute to the chronically activated, persistent state of HCV-infected cells.

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