Hepatitis C Virus Core Protein Activates Nuclear Factor κB-dependent Signaling through Tumor Necrosis Factor Receptor-associated Factor*

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duced by this hepatotropic virus.

Hepatitis C virus (HCV), a member of the Flaviviridae family, is one of the major causes of chronic hepatitis, which can result in cirrhosis and finally hepatocellular carcinoma (1). Its genome consists of a linear, positive-strand RNA molecule of ~9,500 nucleotides (nt) encoding a single polyprotein precursor of ~3,000 amino acids (aa) that is processed into three to four structural proteins at the amino terminus (Core, E1, and E2/p7) and six nonstructural proteins at the carboxyl terminus (nonstructural proteins 2, 3, 4a, 4b, 5a, and 5b) (2, 3). The genomic region of the putative core protein encodes 191 aa and has an apparent molecular mass of 21 kDa (2). The core protein, relatively conserved among all identified HCV isolates (4), may be the fundamental unit for the encapsidation of genomic RNA to help in virus morphogenesis. In addition, previous studies suggested that HCV core protein has various biological properties, one of which is its effect on the nuclear factor κB (NF-κB) pathway (5–9).

NF-κB belongs to a highly conserved Rel-related protein family, which includes RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). Of these, the p50/p65 heterodimer, commonly referred to as NF-κB, is the most abundant and ubiquitous. One of the most intensively studied signals to the NF-κB is induced by tumor necrosis factor (TNF), a proinflammatory cytokine associated with inflammation, immune response, and apoptosis. Currently, this signal transduction pathway is understood as follows (10–15): when TNF binds and activates the TNF receptor 1 (TNFR1), TNFR--associated death domain (TRADD), TNFR-associated factor 2 (TRAF2), and receptor interacting protein (RIP) form a complex with TNFR1. Subsequently NF-κB inducing kinase (NIK) and/or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEKK1) are activated. Activated NIK and/or MEKK1 phosphorylate and activate both IκB kinase (IKK) α and IKKβ. Activated IKKα/β phosphorylate IκBα, which associates with and sequesters NF-κB in the cytoplasm. Phosphorylated IκBα is ubiquitinated and degraded, and then NF-κB translocates into the nucleus and binds to DNA to initiate the transcription of various genes associated with inflammation, the immune response, cell growth, and survival.

There have, however, been conflicting reports up until now about the effect of HCV core protein on this NF-κB pathway. Recently, it was shown that HCV core protein activated the NF-κB pathway (7–9). On the contrary, it was previously shown that core protein suppressed TNF-induced NF-κB activation (5, 6). At present, the mechanism for core protein’s effect on the NF-κB pathway remains unclear; therefore, we focused our attention on the effect HCV core protein has on the NF-κB pathway and tried to determine how core protein affects NF-κB signaling.

EXPERIMENTAL PROCEDURES

Cell Lines—Human cervical carcinoma cells (HeLa), human hepatoma cells (HepG2), and monkey kidney cells (COS-7) were obtained from the RIKEN cell bank (Tsukuba Science City, Japan). HeLa Tet-Off cells, which constitutively express the tetracycline-controlled transactivator, were purchased from CLONTECH (Palo Alto, CA). Cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.)
supplemented with 10% heat-inactivated fetal bovine serum.

**HCV Core Protein-expressing Plasmids—**Type 1b HCV core region (nt 1–575 and aa 1–191 of the prototype HCV type 1b, HCV-J; Ref. 16) was amplified by reverse transcription-polymerase chain reaction (PCR) using the HCV RNA extracted from the sera of a patient with chronic hepatitis C, designated 2-172 (Japanese strain), as a template. For construction of fragments were amplified by PCR using pCXN2-core as a template and pCXN2-core. Control plasmid that expresses a gene, La Jolla, CA) was utilized. pFC-MEKK (Stratagene), which expresses constitutively active MEKK1 (amino acids 360–672) driven by a cytomegalovirus promoter, was used as a positive control plasmid for expression of the core protein upon withdrawal of doxycycline by Western blotting using mouse anti-HCV core antigen IgG fraction, as described previously (18). Positive clones were expanded and rescreened by Western blotting of cells grown in the presence and absence of doxycycline.

**Concentration of HeLa Cells Induced to Express Core Protein—**HeLa cells induced to express HCV core protein were generated with use of a tetracycline-regulated gene expression system (Tet-Off gene expression system, CLONTECH). HeLa Tet-Off cells were cotransfected with pTRE2-core and pTK-Hyg (CLONTECH), a selection vector that confers hygromycin resistance, followed by selection in culture medium containing 200 µg/ml hygromycin (CLONTECH) and 1 µg/ml doxycycline (Sigma). Hygromycin-resistant clones, termed HeTOC, were examined for expression of the core protein upon withdrawal of doxycycline by Western blotting using mouse anti-HCV core antigen IgG fraction, as described previously. Positive clones were expanded and rescreened by Western blotting of cells grown in the presence and absence of doxycycline.

**Transfection—**We used the Effectene transfection reagent (Qiagen) for all transfection experiments. Approximately 4 × 10⁵ HeLa cells were plated into the well of a six-well tissue culture plate (Iwaki Glass, Chiba, Japan) 24 h before transfection. To examine the effect of HCV core protein on the NF-κB pathway, HeLa cells were transfected with a total of 0.4 µg of plasmids consisting of 0.19 µg of pNF-κB-Luc, 0.01 µg of pRL-TK, and 0.2 µg of pCXN2 or pCXN2-core. As a positive control, pFC-MEKK was added to the transfection complexes containing pCXN2, or human TNFα (Strathmann Biotech GmbH, Hamburg, Germany) was added to the medium of transfected HeLa cells at a concentration of 20 ng/ml 6 h before harvest. The effect of HCV core protein on the NF-κB pathway in HeLa cells was examined using HepG2 cells with the same protocol as that used for HeLa cells. To examine the doxycycline-dependent effect of core protein on the NF-κB pathway, HeLa cells transfected with a total of 1.2 µg of plasmids consisting of 0.38 µg of pNF-κB-Luc, 0.02 µg of pRL-TK, 0.0–0.8 µg of pCXN2-core, and 0–0.8 µg of pCXN2 adjusted to total 1.2 µg.

In addition to the HeLa cells, which express core protein transiently, HeLa cells stably transfected to express core protein under the control of doxycycline, were used to examine the effect of HCV core protein on the NF-κB pathway. Approximately 4 × 10⁵ HeTOC cells were plated into the well of a six-well tissue culture plate containing 1 µg/ml doxycycline 24 h before transfection. Cells were transfected with 0.39 µg of pNF-κB-Luc and 0.01 µg of pRL-TK and cultured in medium with or without doxycycline. To examine how HCV core protein affects the NF-κB pathway, dominant negative forms of IKKα, IKKβ, TRAF2, and TAK1, and an IKKβ-specific inhibitor were used. To initially examine the effect of the dominant negative forms of components of the NF-κB pathway, HeLa cells were transfected with a total of 1.2 µg of plasmids consisting of 0.38 µg of pNF-κB-Luc, 0.02 µg of pRL-TK, 0.4 µg of pCXN2-core, and 0.4 µg of one of the following plasmids: pIKKα(K44A), pIKKβ(K44A), pTRAF2(87–501), or pTRAF6(289–522). Similarly, HeLa cells were transfected with a total of 1.6 µg of plasmids consisting of 0.38 µg of pNF-κB-Luc, 0.02 µg of pRL-TK, 0.4 µg of pCXN2-core, 0.4 µg of pCMV-TAK1(K63W), and 0.4 µg of pCMV-TAB1. Second, we added 5 mM acetyl salicylic acid (Sigma), which inhibits cyclooxygenase (24) and IKKβ (25), to the medium of HeLa cells transfected with 0.19 µg of pNF-κB-Luc, 0.01 µg of pRL-TK, and 0.2 µg of pCXN2 or pCXN2-core. Acetyl salicylic acid was added 1 h after the transfection of plasmids. Instead of acetyl salicylic acid, 25 mM indomethacin (Sigma), a non-steroidal anti-inflammatory drug, which inhibits cyclooxygenase but not IKKβ (24, 25), was added to the medium as a control.

**Luciferase Assay—**The entire cell lysate was collected 36 h after transfection. A luciferase assay was performed with the PikkaGene luciferase assay system (Toyo Ink, Tokyo, Japan) and a luminometer (Lumat LB9507, EG&G Berthold, Bad Wildbad, Germany). Assays were conducted at least in triplicate. Firefly luciferase activity and sea panay luciferase activity were measured as a relative light unit. Firefly luciferase activity was then normalized for transfection efficiency based on sea panay luciferase activity.

**Indirect Immunofluorescence Staining of HCV Core Proteins—**Indi-
Detection of Transiently Expressed HCV Core Proteins by Western Blotting—Expression of full-length and three deleted core proteins was examined in the soluble protein extracts of transiently transfected COS-7 cells by Western blotting (Fig. 1, A and B). Full-length (pCXN2-core) and aa 1–173 core (pCXN2-core173) were detected by mouse anti-HCV core antigen IgG fraction (Fig. 1A). HA-tagged core aa 1–151 (pCXN2-HAcore151) and aa 92–191 (pCXN2-HAcore92–191) were detected by anti-HA polyclonal antibody (Fig. 1B). The size of each core protein was consistent with the expected size.

Doxycycline-regulated HCV Core Protein Expression in HeTOC Cells—Expression of HCV core protein was detected by Western blotting in HeTOC cells, a clone termed HeTOC-22, which can be induced to express core protein under the control of doxycycline, 48 h after the removal of doxycycline. The induction ratio was greater than 1,000 when the relative amount of expressed HCV core protein with or without doxycycline was analyzed using a LAS-1000 image analyzer (photo film from Fuji, Tokyo, Japan) (data not shown).

Activation of the NF-κB Pathway by the HCV Core Protein—In HeLa cells, HCV core protein (0.2 μg of pCXN2-core) significantly activated the NF-κB pathway at a value 6.2 ± 3.4 (mean ± S.D.) times higher than that of the control, whereas TNF-α (20 ng/ml) activated the pathway at a value 8.6 ± 2.0 times higher (Fig. 2A). Activation of the NF-κB pathway increased in relation to the amount of plasmid utilized for pCXN2-core (Fig. 3). In HepG2 cells, HCV core protein activated the pathway at a value 4.3 ± 0.9 times higher than the control, whereas TNF-α activated the pathway at a value 4.5 ± 2.5 (Fig. 2A).

HeTOC cells, which can be induced to express core protein under the control of doxycycline, were used to examine activation of the NF-κB pathway. Core-expressing HeTOC-22 cells cultured in a medium without doxycycline showed a pathway activation value 5.2 ± 1.5 times higher than that found with HeTOC-22 cells cultured in a medium with doxycycline. Addition of TNF-α to core-expressing HeTOC-22 cells did not affect significantly the activation of NF-κB pathway by core protein (Fig. 2B).

Core Protein Enhances NF-κB-DNA Binding Activity—To examine whether core protein enhances NF-κB-DNA binding, EMSA was performed using the nuclear extracts of HeTOC-22 cells, which were induced to express core protein. As shown in Fig. 4, NF-κB-DNA binding activity was enhanced in He-
Mapping the Region of HCV Core Protein Responsible for Activation of the NF-κB Pathway by HCV Core Protein—To examine whether activation of the NF-κB pathway by HCV core protein is transduced through IKKα or IKKβ, HeLa cells were cotransfected with pCXN2/pCXN2-core, pNF-κB-Luc, and pIKKα(K44A)/pIKKβ(K44A). Expression of IKKβ(K44A), catalytically inactive IKKβ, significantly reduced HCV core induced NF-κB activation to about one-tenth, whereas expression of IKKα(K44A), catalytically inactive IKKα, reduced the activation to about two-fifths (Fig. 7). To confirm the participation of IKKβ in activation of the NF-κB pathway by HCV core protein, we added 5 mM acetyl salicylic acid, an IKKβ-specific inhibitor (28), to the HeLa cells transfected with pCXN2/pCXN2-core and pNF-κB-Luc. Activation of the pathway by core protein was significantly inhibited by acetyl salicylic acid but not by indomethacin, a cyclooxygenase inhibitor (Fig. 8). These results suggest that HCV core protein activates the NF-κB pathway through IKKβ, especially IKKβb.

Dominant Negative Forms of TRAF2/6 Reduced the Activation of the NF-κB Pathway by HCV Core Protein—To examine whether activation of the NF-κB pathway by HCV core protein was transduced through TRAF2/6, HeLa cells were cotransfected with pCXN2/pCXN2-core, pNF-κB-Luc, and pTRAF2-(87–501)/pTRAF6-(289–522). Expression of the dominant negative form of TRAF2 (aa 87–501) significantly reduced core-induced NF-κB activation to about two-fifths in HeLa cells (Fig. 9). Expression of the dominant negative form of TRAF6 (aa 289–522) also reduced core-induced NF-κB activation (Fig. 9). These results suggest that HCV core protein activates the
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In this study, HCV core protein clearly activated the NF-κB pathway not only in a dose-dependent manner in mammalian cells transiently transfected with core protein expression plasmid, but also in HeLa cells inducibly expressing core protein. In fact, activation of NF-κB signaling by transfection of 0.4 μg of pCXN2-core corresponded to activation by 20 ng/ml TNFα (Fig. 2; Ref. 7–9). This concentration of TNFα, which is widely used to study responses to TNF, is enough to induce cytolysis in murine fibrosarcoma L929 cells (29). However, there are contradicting data regarding the effect core protein has on the NF-κB pathway; two studies demonstrated that core protein enhanced NF-κB signaling in cells stably expressing the core protein when using an EMSA (8) or in cells transiently expressing the core protein when using a reporter assay (7, 9), while other studies showed that HCV core protein suppressed TNFα-induced NF-κB activation in cells stably expressing HCV core protein with use of an EMSA (5, 6). These contradicting results may be due to differences in the type of cells stably or transiently expressing core protein and the method for detecting NF-κB activation. To solve this problem, we adopted a tightly regulated, high level core protein expression system responsive to doxycycline (30). This system allowed us to overcome the problem of low transfection efficiency in the transient transfection assay. Thus, we could analyze the effect of HCV core protein on NF-κB signaling in the same cells with or without doxycycline by use of both a reporter assay and an EMSA.

This study also demonstrated that HCV core protein activates the NF-κB pathway through IKKα/β (Fig. 7). Core protein may predominantly modulate the activity of one of the two IKKα/β kinases. The pathway may be upstream of NIK in the IL-1-activated signaling pathway and associate with TRAF6 during IL-1 signaling (23). To examine whether activation of the NF-κB pathway by HCV core protein was transduced through TAK1, HeLa cells were cotransfected with pCXN2/pCXN2-core, pNFκB-Luc, pCMV-TAB1, and pCMV-TRAF2/6. Although expression of catalytically inactive TAK-1 and its activator, TAB1, efficiently suppressed IL-1-induced activation of the NF-κB pathway, they had no effect against core-induced activation of the pathway (Fig. 10), suggesting that HCV core protein activates the NF-κB pathway independent of TAK1.

The Effect of HCV Core Protein on IL-1β or TNFα Promoter—HCV core protein activated the IL-1β promoter 2.5 ± 0.6 times higher than the control. However, core protein did not have significant influence on the TNFα promoter. This activation of IL-1β promoter was cancelled by catalytically inactive IKKβα or dominant negative TRAF2/6 (Fig. 11), suggesting HCV core protein activates IL-1β promoter mainly through NF-κB signaling.

DISCUSSION

NF-κB pathway through TRAF2/6.

Catalytically Inactive TAK1 Has No Effect on Activation of the NF-κB Pathway by HCV Core Protein—The kinase TAK1 was shown to act upstream of NIK in the IL-1-activated signaling pathway and associate with TRAF6 during IL-1 signaling (23). To examine whether activation of the NF-κB pathway by HCV core protein was transduced through TAK1, HeLa cells were cotransfected with pCXN2/pCXN2-core, pNFκB-Luc, pCMV-TAB1, and pCMV-TRAF2/6. Acetyl salicylic acid reduced NF-κB activation by HCV core protein. Acetyl salicylic acid, an IKKβ-specific inhibitor, was added to the medium of HeLa cells transfected with pCXN2/pCXN2-core and pNFκB-Luc at a concentration of 5 mM. Luciferase activities were measured and expressed as described in the legend for Fig. 2. Activation of the pathway by HCV core protein was significantly inhibited by acetyl salicylic acid but not indomethacin, a cyclooxygenase inhibitor.

FNκB pathway through TRAF2/6.

Acetyl salicylic acid reduced NF-κB activation by HCV core protein. Acetyl salicylic acid, an IKKβ-specific inhibitor, was added to the medium of HeLa cells transfected with pCXN2/pCXN2-core and pNFκB-Luc at a concentration of 5 mM. Luciferase activities were measured and expressed as described in the legend for Fig. 2. Activation of the pathway by HCV core protein was significantly inhibited by acetyl salicylic acid but not indomethacin, a cyclooxygenase inhibitor.

Fig. 7. Catalytically inactive IKK reduced the NF-κB activation by HCV core protein. HeLa cells were transfected with pCXN2-core, pIKKα(K44A)/pIKKβ(K44A), and pNFκB-Luc. Luciferase activities were measured and expressed as described in the legend for Fig. 2. Catalytically inactive IKKβ blocked activation of the NF-κB pathway by HCV core protein more significantly than catalytically inactive IKKα. TNFα (20 ng/ml) was added 6 h before harvest to function as an inducer of the pathway (positive control).

Fig. 8. Acetyl salicylic acid reduced NF-κB activation by HCV core protein. Acetyl salicylic acid, an IKKβ-specific inhibitor, was added to the medium of HeLa cells transfected with pCXN2/pCXN2-core and pNFκB-Luc at a concentration of 5 mM. Luciferase activities were measured and expressed as described in the legend for Fig. 2. Activation of the pathway by HCV core protein was significantly inhibited by acetyl salicylic acid but not indomethacin, a cyclooxygenase inhibitor.

Fig. 9. Dominant negative TRAF2/6 reduced NF-κB activation by HCV core protein. HeLa cells were transfected with pCXN2-core/pCXN2, pTAR2-(87–501)/pTAR6-(289–522), and pNFκB-Luc. Luciferase activities were measured and expressed as described in the legend for Fig. 2. Dominant negative TRAF2 blocked activation of the NF-κB pathway by HCV core protein. TNFα (20 ng/ml) was added 6 h before harvest to function as an inducer of the pathway (positive control).
IκB kinases, IKKβ. The dominant negative mutant of IKKβ completely abrogated activation of the NF-κB pathway by HCV core protein, in contrast to the minor role played by the comparable mutant IKKα. Moreover, acetyl salicylic acid, an IKKβ-specific inhibitor (25), significantly blocked the action of HCV core protein on NF-κB. Recently, it was shown that only IKKβ phosphorylation contributes to IKK activation by proinflammatory cytokines or by cotransfected NIK and MEKK1 (31). This result is consistent with the genetic analysis of IKK function; whereas disruption of the IKKα locus has no effect on IKK activation and IκB degradation in response to proinflammatory stimuli, disruption of the IKKβ locus results in a major defect in both events (32, 33). In fact, the dominant negative mutant of IKKβ blocked activation of the pathway by HCV core protein and TNF more efficiently than IKKα (Fig. 7). These results suggest that HCV core protein mimics proinflammatory cytokine activation of the NF-κB pathway.

Furthermore, the dominant negative forms of TRAF2/6 significantly blocked activation of the NF-κB pathway by HCV core protein. TRAF proteins are known to function as signal transducers for distinct receptor families. TRAF2 is thought to be a common mediator of TNFR and CD40 signaling (21), whereas TRAF6 is thought to be a signal transducer for IL-1 (22). Because CD40 is not expressed and CD40 signaling is not activated by signal-activating anti-CD40 antibody (34) monoclonal antibody 89, Immunotech, Marseille, France) in HeLa and HepG2 cells (data not shown), TNFR or IL-1 signaling is the temporary candidate for the target of HCV core protein.

Although TRAF6 is thought to be involved in IL-1 signaling and not in TNFR signaling, previous (35) and present studies have shown that the dominant negative form of TRAF6 suppresses the activation of TNFα activation of NF-κB (Fig. 9). On the other hand, the dominant negative form of TRAF2 suppresses only TNFR signaling and not IL-1 signaling. Moreover, catalytically inactive TAK1, which links TRAF6 to the NIK-IKK cascade in the IL-1 signaling pathway, has no effect against the core-induced activation of NF-κB. These data may imply that HCV core protein mimics proinflammatory cytokine activation of the NF-κB pathway, especially TNFR signaling through TRAF2/6.

Recently, HCV core protein was shown to interact with the cytoplasmic tail of lymphoptxin-β receptor (36, 37), a member of the TNFR family, and also with the cytoplasmic domain of TNFR1 (6), where TRADD and RIP interact to produce TNF-induced NF-κB activation (11, 12). There may be the possibility that HCV core protein activates the NF-κB pathway through interaction with TNFR1. Our finding that the N-terminal 91-aa deleted core protein did not activate the NF-κB pathway may support this idea because the N-terminal 115 aa of HCV core protein is important for the interaction with TNFR1 (6). However, we could not detect an in vivo interaction between core protein and TNFR1, TRADD, and TRAF2 by coimmunoprecipitation assay (data not shown). Deletion analysis of the core protein showed that the C-terminal 18 aa, a highly hydrophobic region (38), is also important for NF-κB activation. It was suggested that this region is responsible for the association of HCV core protein with intracellular membranes, and the C-terminal deleted core protein translocates into the nucleus (39, 40). As shown in Fig. 6, the C-terminal deleted core proteins are located in the perinuclear region or the nucleus, whereas full-length core protein was diffusely located in the cytoplasm. These data may support our finding that core protein activates NF-κB in the cytoplasm through TRAF2/6.

The NF-κB pathway is known to be activated by oncogenic viral proteins such as X protein of the hepatitis B virus, Tax of human T-cell leukemia virus type 1, and latent membrane protein 1 (LMP-1) of the Epstein-Barr virus (41). Hepatitis B virus X protein interacts directly with IκBα to probably prevent the reassociation of IκBα with NF-κB (42). Tax was shown to interact with components of the IKK complex, such as MEKK1 (28), IKK (43), and the NF-κB essential modulator (44), thereby activating the NF-κB pathway. Tax has been shown to also associate with IκBα, p105, p100, RelA, and c-Rel (41). Thus, more than one molecule may be the target of HCV core protein to activate the NF-κB pathway as well as Tax.

TNFα activates not only NF-κB signaling, but also activator protein (AP)-1 signaling through TRAF2/45. TRAF2 activates germinal center kinase (46) /germinal center kinase-related (47) or MEKK1 (48), which subsequently activate c-Jun N-terminal kinase-AP-1 cascade (49). Since HCV core protein activates both NF-κB and AP-1-associated pathways (9), as well as LMP-1 (51), the shared components between these pathways may be the target molecules for HCV core protein. This also supports our finding that HCV core protein activates NF-κB in the cytoplasm through TRAF2/6.

The NF-κB pathway plays an important role in cellular response to proinflammatory cytokines such as TNF-α and IL-1 and induces an inflammatory response by the up-regulation of many cytokines, including IL-1, -2, -6, -8, and -12, and TNF-α (10). In this study, HCV core protein is shown to mimic proinflammatory cytokine activation of the NF-κB pathway, especially TNFR signaling, and actually activates IL-1β promoter mainly through NF-κB signaling pathway. Recently we have shown that HCV core protein activates also IL-8 promoter...
through NF-κB pathway (9). Therefore, it is quite conceivable that HCV core protein could induce an inflammatory response and cause "hepatitis." In fact, the eradication of HCV by interferon leads to the rapid resolution of acute and chronic hepatitis (52, 53). Moreover, serum or intrahepatic expression of IL-1β, 2, 6, 8 and TNF-α are elevated from 2 to 10 times higher in patients with active chronic hepatitis C than those of a control group, and reduced after eradication of the virus by interferon treatment (54–58). Although the host immune response caused by cytotoxic T lymphocytes is believed to play a pivotal role in the pathogenesis of C-viral hepatitis (50), our findings suggest that HCV core protein directly induces hepatitis through inflammatory cytokine production. Therefore, blockage of the activation of the NF-κB pathway may become an attractive option for the treatment of chronic hepatitis C in the future.

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