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Hepatitis C Virus Core Protein Enhances NF-κB Signal Pathway Triggering by Lymphotoxin-β Receptor Ligand and Tumor Necrosis Factor Alpha

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Our previous study indicated that the core protein of hepatitis C virus (HCV) can associate with tumor necrosis factor receptor (TNFR)-related lymphotoxin-β receptor (LT-βR) and that this protein-protein interaction plays a modulatory effect on the cytolytic activity of recombinant form LT-βR ligand (LT-α1β2) but not tumor necrosis factor alpha (TNF-α) in certain cell types. Since both TNF-α/TNFR and LT-α1β2/LT-βR are also engaged in transcriptional activator NF-κB activation or c-Jun N-terminal kinase (JNK) activation, the biological effects of the HCV core protein on these regards were elucidated in this study. As demonstrated by the electrophoretic mobility shift assay, the expression of HCV core protein prolonged or enhanced the TNF-α or LT-α1β2-induced NF-κB DNA-binding activity in HuH-7 and HeLa cells. The presence of HCV core protein in HeLa or HuH-7 cells with or without cytokine treatment also enhanced the NF-κB-dependent reporter plasmid activity, and this effect was more strongly seen with HuH-7 cells than with HeLa cells. Western blot analysis suggested that this modulation of the NF-κB activity by the HCV core protein was in part due to elevated or prolonged nuclear retention of p50 or p65 species of NF-κB in core protein-producing cells with or without cytokine treatment. Furthermore, the HCV core protein enhanced or prolonged the IκB-β degradation triggering by TNF-α or LT-α1β2 both in HeLa and HuH-7 cells. In contrast to that of IκB-β, the increased degradation of IκB-α occurred only in LT-α1β2-treated core-producing HeLa cells and not in TNF-α-treated cells. Therefore, the HCV core protein plays a modulatory effect on NF-κB activation triggering by both cytokines, though the mechanism of NF-κB activation, in particular the regulation of IκB degradation, is rather cell line and cytokine specific. Studies also suggested that the HCV core protein had no effect on TNF-α-stimulated JNK activity in both HeLa and HuH-7 cells. These findings, together with our previous study, strongly suggest that among three signaling pathways triggered by the TNF-α-related cytokines, the HCV core protein potentiates NF-κB activation in most cell types, which in turn may contribute to the chronically activated, persistent state of HCV-infected cells.

Hepatitis C virus (HCV) is a positive-strand RNA virus that has been identified as the major causative agent of posttransfection non-A, non-B hepatitis (25, 51). Its persistent infection may result in chronic active hepatitis, cirrhosis, and hepatocellular carcinoma (19, 83). Intriguingly, HCV persists despite the presence of virus-specific cytotoxic T-lymphocytes (11, 20, 50, 80). The reason for the failure of host immune response to resolve HCV infection is not known. This could be due in part to the effect of viral gene products on the host immune system, as had been noted for several viruses (36). Of at least 10 viral proteins encoded by the HCV genome (10, 37, 59, 100), its nucleocapsid core protein may have such a feature.

Several studies suggested that the core protein of HCV possesses several distinguishing properties. It is phosphorylated (87) and has both cytoplasmic and nuclear localization (61, 86, 88). Additionally, the core protein has regulatory roles in viral and cellular genes and also has effects on cell growth and proliferation (21, 65, 73–77, 87, 88, 113). Recently, studies from several laboratories, including ours, have identified several cellular factors that can associate with the HCV core protein. For example, the core protein forms the complex with apolipoprotein AII of the lipid droplet, which in turn may contribute to the liver steatosis in HCV-infected chimpanzee or humans (11). The interaction between the HCV core protein and the tumor necrosis factor receptor (TNFR)-related lymphotoxin-β receptor (LT-βR) (3, 15, 28) was also demonstrated by two different groups (21, 65). This interaction modulates one of the biological activities, i.e., cytolytic activity, of LT-βR triggering by its recombinant ligand (LT-α1β2) (17, 18, 108) in HeLa cells but not in HuH-7 cells (21). Moreover, the HCV core protein also interacts with TNFR 1 (TNFRI) (113), although its effect on TNF-induced cytolytic activity still remains controversial (21, 76, 113). Like the TNF ligand receptor family (reviewed in references 1, 39, 97, and 103), the LT-βR is also engaged in activation of the transcriptional factor NF-κB and c-Jun in some cell types (22, 62, 71). Conceivably, the interaction of HCV core protein and LT-βR or TNFRI may potentiate their NF-κB or c-Jun N-terminal kinase (JNK) signaling pathways.

The NF-κB signaling pathway is a key component of the cellular response to a variety of extracellular stimuli, including TNF-α, interleukin-1 (IL-1) and phorbol ester (reviewed in references 4, 8, 98, and 105). This transcriptional factor, known to regulate a large number of genes involved in inflammatory response, cell proliferation, and apoptosis, is composed of homo- and heterodimers of Rel family proteins (reviewed in references 4, 5, and 10). These family proteins include at least the following five distinct members: C-Rel, p50, p52, p65 (RelA), and RelB; of these, the p50/p65 heterodimer is the most abundant and ubiquitous (reviewed in references 4 and 10). In the uninduced cells, NF-κB is sequestered in the cytoplasm by binding to a labile IκB family protein with a regula-

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tory and inhibitory function, of which IκB-α and IκB-β appear to be the key members (106). Upon induction by several agents, including virus, inflammatory cytokines, and stresses, the intracellular signaling pathways that generally converge on IκB rapid phosphorylation and/or modification and subsequent degradation in the proteasome are activated (reviewed in references 4 and 8), thus allowing NF-κB complexes to enter the nucleus and activate the target genes. After degradation, the IκB-α is rapidly replenished by NF-κB-mediated transcription of IκB-α gene (57, 93), which then constitutes the autoregulatory loop of NF-κB–IκB activation. Of note, unlike IκB-α, which elicits only transient NF-κB activation, the IκB-β degradation causes a sustained activation of NF-κB due to a large lag period of IκB-β resynthesis (99). Recent studies have identified two cytokine-inducible IκB kinases (IKK), termed IKKα and IKKβ, which appear to form heterodimers in the large multiple complex (700 kDa) and catalyze IκB site-specific phosphorylation (reviewed in references 64, 90, and 105). In spite of this tight regulatory loop for NF-κB activation, this transcriptional factor is activated by different viral proteins with oncogenic potential, such as human T-cell leukemia virus type 1 Tax (35, 43, 54, 92), Epstein-Barr virus latent membrane protein 1 (LMP-1) (40), the X protein of hepatitis B virus (24, 91).

The second branch of the stress response is the JNK pathway, which targets to the activation of transcriptional factor AP-1, ATF-2, and E1K-1 (29, 38, 49, 52, 53, 109). The signal transduction cascade of JNK activation is well defined and involves small GTP-binding proteins (Cdc42 and Rac), p21-activated protein kinase, and mitogen-activated protein kinase kinase kinase members (MEKK1 and MKK4) (27, 30, 56, 68, 84, 111). Many stimuli that induce NF-κB, such as TNF-α, UV irradiation, and lipopolysaccharide, also activate the JNK cascade (29, 79), thereby raising the possibility that the two pathways share common signal transduction components. Supporting this notion is the fact that TRAF2 and MEKK1 are two critical components of both the JNK and NF-κB stress response pathways (44, 58, 67, 79, 89), although contradictory findings were also reported (60). Despite these discrepancies, these two signal pathways diverge at a discrete level. For example, while JNK and its target c-Jun are critical mediators of apoptosis induced by TNF-α or ceramide (104, 110), the NF-κB in general has an anti-apoptotic effect (12, 60, 101, 107).

In this study, we examined the effects of HCV core protein on the NF-κB and JNK signaling pathways induced by LT-α1β2 or TNF-α cytokine. Our results as shown here indicated that HCV core protein significantly potentiates NF-κB activation pathway triggering by LT-α1β2 or TNF-α in HuH-7 cells and to a lesser extent in HeLa cells. These modulation effects are mediated by the differential sensitivity of IκB-α and IκB-β degradation in the HCV core protein-producing cells, which in turn increases the nuclear retention of NF-κB subunits and potentiates both basal and cytokine-treated NF-κB activities. However, no significant modulation effect on JNK activation was detected in those two HCV core protein-producing cells when stimulated by LT-α1β2 or TNF-α, suggesting that these two stress responses are differentially regulated by the HCV core protein.

HCV core protein enhances the NF-κB DNA-binding activity triggered by LT-$\alpha_1\beta_2$ or TNF-α in HeLa and HuH-7 cell lines. To elucidate whether the HCV core protein can modulate the NF-κB activation stimulated by LT-$\alpha_1\beta_2$ or TNF-α, the levels of induction of NF-κB DNA-binding activity in the nuclear extracts of both HCV core protein-expressing HeLa and HuH-7 cells (HeLa/C190 and HuH-7/C190) relative to that of their parental cells were compared at different times (30 min to 2 h) after the cytokine treatment. The NF-κB DNA-binding activity was examined by the electrophoretic mobility shift assay (EMSA) with a ^{32}P-labeled 45-mer oligonucleotide probe (5′-TTGTTACAAGGGACTTTCCGCTGGGGACTT TCCAGGGAGGCGTGG-3′) from the human immunodeficiency virus type 1 long terminal repeat containing two κB-binding sites. A mutated oligonucleotide with a single mutated κB site (5′-AGTTGAGGCGACTTTCCCAGGC-3′) (22 mer; Santa Cruz) was also used to examine the binding specificity of NF-κB by EMSA. Results suggested that addition of the LT-$\alpha_1\beta_2$ ligand (500 ng/ml for 30 min to 1 h) greatly enhanced the NF-κB DNA-binding activity in HCV core protein-producing HuH-7/C190 and HeLa/C190 cells compared to that in their parental cells without the core protein (Fig. 1). The induction of NF-κB DNA-binding activity in LT-$\alpha_1\beta_2$-treated HeLa/C190 cells peaked at 30 min and slightly declined at 60 min (Fig. 1A), while in LT-$\alpha_1\beta_2$-treated HuH-7/C190 cells the induced NF-κB activity was still sustained at 60 min (Fig. 1B). A similar finding was obtained with TNF-α-treated HeLa/C190 cells. Treatment of TNF-α (20 ng/ml) in this cell line induced NF-κB activity significantly at 30 min, reached plateau at 60 min, and even remained elevated at 2 h. This NF-κB induction profile of HeLa/C190 cells is distinct from that observed for HeLa cells, as the NF-κB activity in the HeLa cells within the same period was only slightly induced by TNF-α (Fig. 2A). A distinct NF-κB activation kinetics was also observed for the TNF-α-treated HuH-7 and HuH-7/C190 cells (Fig. 2B). Although the NF-κB DNA-binding activities of these two cell lines were similar at the initial phase (30 min to 1 h) of induction, the activity of HuH-7/C190 at late phase (2 h) of treatment remained stronger than that of HuH-7 cells, suggesting a prolonged NF-κB activation in core-producing cells (Fig. 2B). Interestingly, a slight enhancement of NF-κB DNA-binding activity was also observed for both core-producing cells without the cytokine treatment (compare lanes 2 and 7 in Fig. 1A and B, and lanes 2 and 8 in Fig. 2A and B), implicating a constitutive activation of NF-κB in core-producing cells. It should be noted that the cytokine-induced NF-κB DNA-binding activity observed in these EMSAs is specific, since it was ablated by an excess of unlabeled wild-type competitor but not by the mutated one (see lanes 5, 6, 10, and 11 in Fig. 1 and lanes 6, 7, 12, and 13 in Fig. 2). Additionally, the invariant fast-migrating band was a nonspecific complex observed in NF-κB EMSA studies.

HCV core protein differentially enhances the NF-κB-dependent transcriptional activity triggered by LT-$\alpha_1\beta_2$ or TNF-α in HeLa and HuH-7 cells. We next assessed whether this enhancement or persistent induction of NF-κB DNA-binding activity in the HCV core protein-producing cells also could reflect on the NF-κB-dependent transcriptional activity. To this end, we examined the activity of luciferase reporter plasmid (NF-κB-fosp-1783:3.2Luc) (kindly provided by H. Wajant, University of Stuttgart, Stuttgart, Germany) under the control of the κB response element by transient transfection to HuH-7 or HeLa cells with or without the expression of core protein. Additionally, to provide a stringent control and to ensure the measured luciferase reporter plasmid activity mainly reflecting the NF-κB-specific transcriptional activity, an NF-κB-independent control plasmid (pCH110) (Pharmacia) containing β-galactosidase reporter gene under simian virus 40 early promoter control was also cotransfected into cells, and its reporter activity was used for normalization. As shown in Fig. 3, relative to the level in the parental HeLa cells, about twofold enhancement of luciferase activity for both basal and cytokine-treated HeLa/C190 cells was noted (Fig. 3A). Likewise, the presence

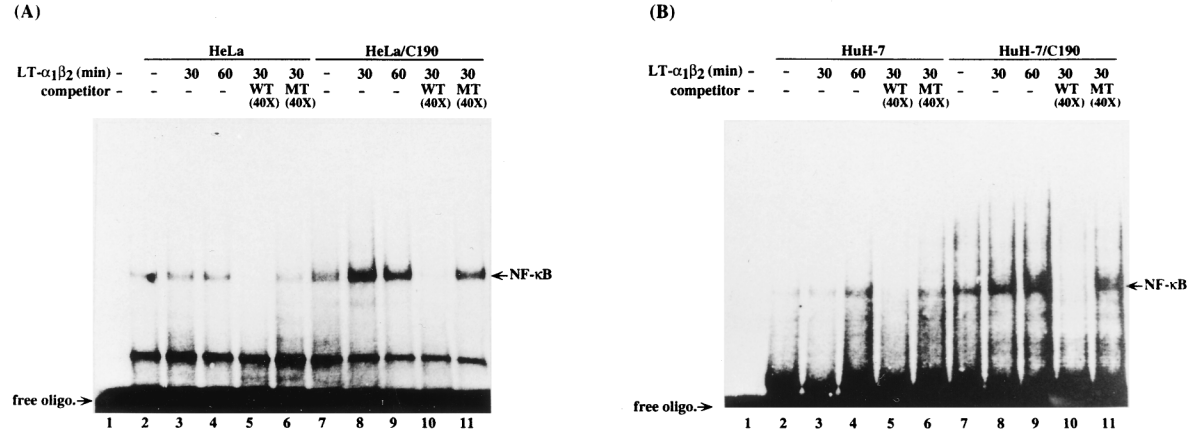

FIG. 1. EMSA of LT-$\alpha_1\beta_2$-stimulated NF-κB activation in various HCV core protein-producing cell lines. (A) NF-κB DNA-binding assays with nuclear extracts from untreated (lanes 2 and 7) or LT-$\alpha_1\beta_2$-treated HeLa and HeLa/C190 cells (lanes 3 to 6 and 8 to 11) were performed. The nuclear extracts were prepared as described by Mackay et al. (62) with some modification. Briefly, 2×10^6 cells after being pretreated with recombinant ligand LT-α1β2 (500 ng/ml) (17) (kindly provided by J. L. Browning [Biogen]) for the proper time (30 min to 2 h) were harvested, washed, and suspended in a hypotonic buffer (buffer A) (20 mM HEPES [pH 7.4], 1 mM $MgCl_2$, 10 mM KCl, 0.3% Nonidet P-40, 0.5 mM dithiothreitol [DTT], 0.1 mM EDTA) at 4°C for 30 min. Cell nuclei were collected by centrifugation, and the nuclear proteins were extracted with high-salt buffer (buffer B) (20 mM HEPES [pH 7.4], 20% glycerol, 0.42 M NaCl, 1 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT) for 1 h on ice. The supernatants recovered from centrifugation were stored at −70°C and used for an EMSA. For the EMSA, 5 μg of nuclear extracts was incubated with 50 fmol of ^{32}P-end-labeled 45-mer synthetic double-stranded NF-κB oligonucleotide in a binding buffer (10 mM HEPES [pH 7.8], 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, 10% glycerol) containing 1 μg of poly(dI-dC) and 30 μg of bovine serum albumin. After incubation at room temperature for 45 min, the DNA-protein complex formed was separated from free oligonucleotide on a 4% native polyacrylamide gel using buffer containing 0.25× TBE (22.5 mM Tris-borate, 0.5 mM EDTA [pH 8.0]). After electrophoresis, the gel was dried and visualized with a PhosphorImager. Competition experiments were carried out by including unlabeled oligonucleotides containing either mutated (MT) (40-fold excess) (lanes 6 and 11) or wild-type (WT) (40-fold excess) (lanes 5 and 10) NF-κB binding sites. The main NF-κB-specific band shift induced is indicated. Lane 1, ^{32}P-labeled free oligonucleotide. (B) Binding assays were identical to that described for panel A except that the nuclear extracts were prepared from HuH-7 and HuH-7/C190 cells.

of core protein in HuH-7 cells exerted about 2.4-fold increase of basal NF-κB-dependent luciferase activity (Fig. 3B). However, treatment with either cytokine elicited a more than five-fold increase of luciferase activity in HuH-7/C190 cells relative to that of HuH cells, indicating a stronger potentiation of core-mediated NF-κB transcriptional activity in cytokine-stimulated HuH-7 cells than in cells without cytokine treatment (Fig. 3B). Therefore, coupled with the data from the EMSA (Fig. 1 and 2), our results clearly indicated that the HCV core protein can enhance the basal and cytokine-stimulated NF-κB transcriptional activities in both HeLa and HuH-7 cells, although the strength or kinetics of NF-κB induction may vary between cell lines.

HCV core protein does not alter the expression levels of NF-κB but affects their nuclear translocation. To understand the molecular basis of the enhancement of NF-κB activation in HCV core protein-producing cells, the total expression levels of NF-κB in different cell lines were examined by immunoblotting using antibodies specific for the subunits of NF-κB. Figures 4 reveals that although the expression levels of NF-κB family proteins p50, p52, and p65 in HuH-7 cells were more abundant than those in HeLa cells, the core protein did not affect their expression levels. Furthermore, following stimulation with TNF-α or LT-α1β2 for 30 min or 1 h, there was little difference in the total expression levels of NF-κB family proteins in core-producing cells of HeLa and HuH-7 compared to those of their parental cells (data not shown). However, in response to 1 h of cytokine treatment, a marked enhancement of nuclear retention of p50 and p65, but not p52, was noted in HuH-7/C190 cells relative to that in parental HuH-7 cells (Fig.

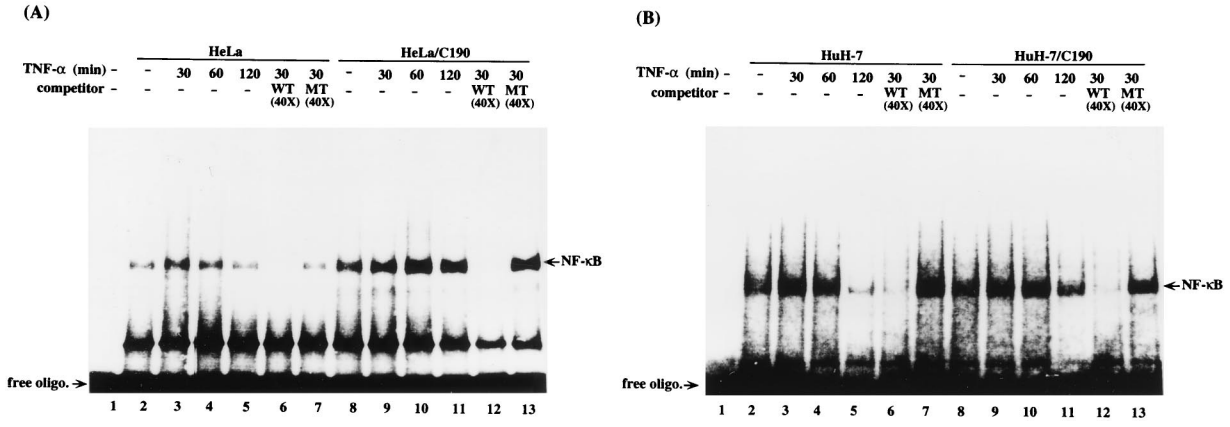

FIG. 2. EMSA of TNF-α-stimulated NF-κB activation in various HCV core protein-producing cell lines. All experimental conditions were as described in the legend to Fig. 1 except that cells were stimulated with 20 ng of TNF-α/ml for 30, 60, or 120 min, respectively.

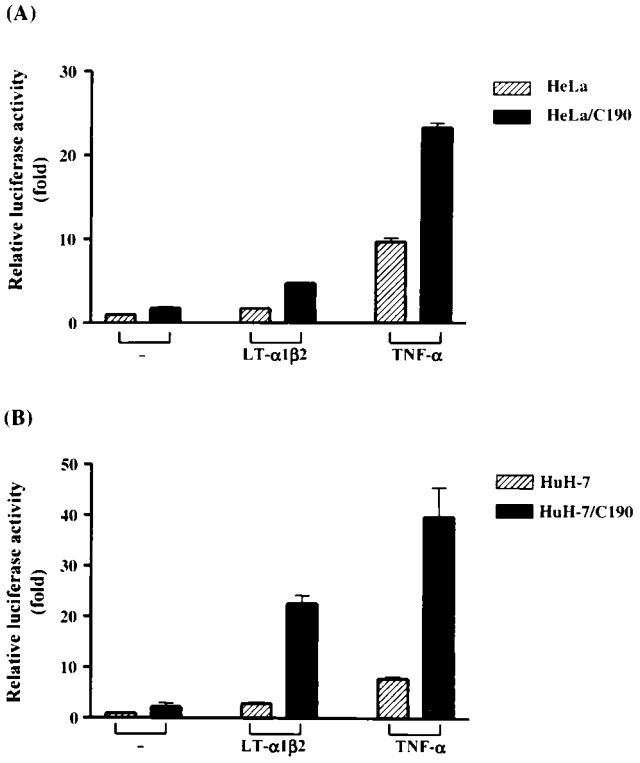

FIG. 3. Analysis of cytokine-stimulated NF-κB-dependent transcriptional activity in HCV core protein-producing cells. (A) HeLa or HeLa/C190 cells seeded at 1.5×10^5 cells/well density were cotransfected with equal amounts (0.4 μg each) of NF-κB-dependent luciferase reporter plasmid and an internal control plasmid carrying the β-galactosidase gene as a reporter with the SuperFect transfection reagent (Qiagen, Hilden, Germany). At 18 h posttransfection, cells were either left untreated (marked with −) or treated with TNF-α (20 ng/ml) or LT-$\alpha_1\beta_2$ (500 ng/ml) for 6 h prior to harvest. After three cycles of freezing and thawing, cells were lysed in 150 μl of lysis buffer (25 mM Tris-HCl [pH 7.8], 70 mM potassium phosphate buffer [pH 7.8], 2.1 mM $MgCl_2$, 0.7 mM DTT, 0.1% Nonidet P-40, and protease inhibitor cocktail [Complete; Boehringer]). Eighty microliters of cell extracts recovered from the centrifugation was then mixed with 250 μl of luciferase assay buffer (43.2 mM glycylglycin [pH 7.8], 22 mM $MgSO_4$, 2.4 mM EDTA, 7.4 mM ATP, 1 mM DTT, and 0.4 mg of bovine serum albumin/ml), and the resulting mixtures were assayed for luciferase activity by using 100 μl of 0.5 mM luciferin (Sigma) as the substrate and measured with AutoLumat LB953 (Berthold, Bad Wildbad, Germany). The β-galactosidase activity in the cell extracts of cotransfected cells was determined essentially as described previously (21). The luciferase activities were normalized on the basis of β-galactosidase expression. The NF-κB-dependent luciferase activity is represented as fold induction relative to that of HeLa cells without treatment. (B) All experimental conditions were similar to those described for panel A except that HuH-7 and HuH-7/C190 cells were used for study. Values shown in all panels are averages (means ± standard deviations) of one representative experiment in which each transfection was performed in triplicate.

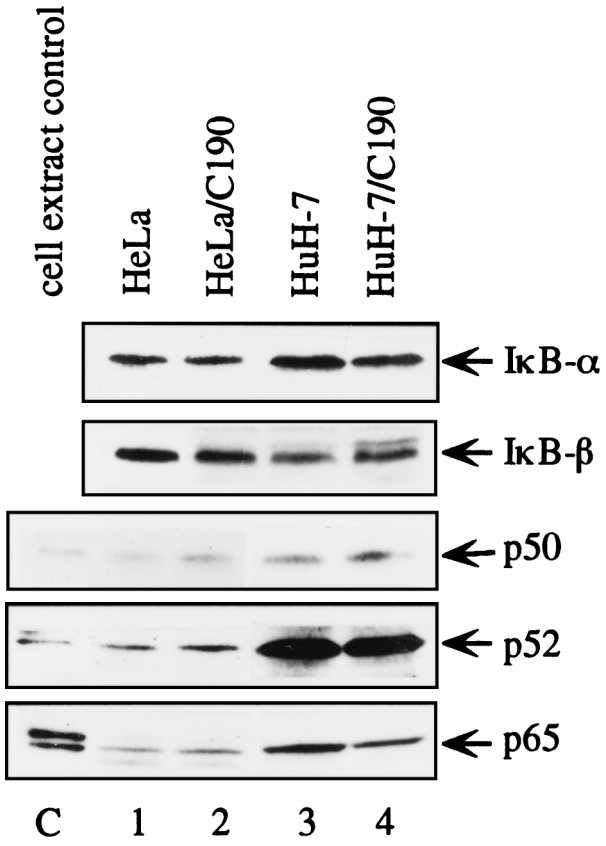

FIG. 4. Western blot analysis of NF-κB/Rel and IκB family proteins in various HCV core protein-producing cell lines. The total cell extracts (60 μg) from various cell lines lysed in 5× sampling buffer (55) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis. Anti-NF-κB p65, p52, and p50 subunit antibodies (Upstate Biotechnology Inc.) and IκB-α and IκB-β antibody (Santa Cruz) were used at the dilutions suggested by the manufacturer. The antigen-antibody reactions were visualized with horseradish peroxidase-coupled goat anti-rabbit immunoglobulin (Transduction) (1:2,000 dilution) using the enhanced chemiluminescence (ECL) detection system (Amersham). The control cell lysates (lane C) provided by the manufacturers are A341 cells for p65 and p50 and Raji cells for p52.

5B and D). This enhancement of p50 or p65 nuclear retention by the core protein was not so evident in cytokine-treated HeLa cells, although a slight enhancement of the nuclear level of p50 was also noted in TNF-α-treated HeLa/C190 cells (compare Fig. 5A and C with Fig. 5B and D). Moreover, following the cytokine treatment a different kinetics of nuclear translocation of p50 and p65 between the LT-$\alpha_1\beta_2$-stimulated HeLa and HeLa/C190 cells was found: while the nuclear level of p50 or p65 in LT-$\alpha_1\beta_2$-treated (1 h) HeLa/C190 cells declined to the steady level, their levels in the nuclear fractions of HeLa cells within the same period remained elevated compared to pretreatment levels (Fig. 5A). Based on these results, it appears that the molecular mechanism for NF-κB activation in core-producing cells relative to that of their parental cells may differ between cell lines or cytokines. Additionally, a slight enhancement of the nuclear level of p50 was noted in both core-producing cells of HeLa or HuH-7 even without the cytokine treatment (Fig. 5). This suggested a constitutive activation of NF-κB by the HCV core protein, which is in accordance with the data from the EMSA (Fig. 1 and 2) and the reporter plasmid assay (Fig. 3).

HCV core protein enhances the degradation of IκB-α and IκB-β in a cell line- and cytokine-specific manner. Since both LT-$\alpha_1\beta_2$ and TNF-α may elicit transient NF-κB activation by affecting the degradation of NF-κB inhibitor IκB, the expression levels of IκB inhibitors in HeLa and HeLa/C190 cell lines before or after the cytokine treatment were examined by immunoblot analysis using the IκB-α- and IκB-β-specific antibodies. The results shown in Fig. 4 indicate that the HCV core protein did not alter the expression of IκB-α and IκB-β. However, LT-$\alpha_1\beta_2$ treatment (500 ng/ml) of HeLa/C190 but not HeLa cells caused proteolytic breakdown of IκB-α within 10- to 60-min time intervals and the amounts then returned to the control level by 1.5 h (Fig. 6A). This enhancement of IκB-α degradation did not occur in HuH-7/C190 cells. In fact, the IκB-α inhibitor of HuH-7 and HuH-7/C190 cells was unre-

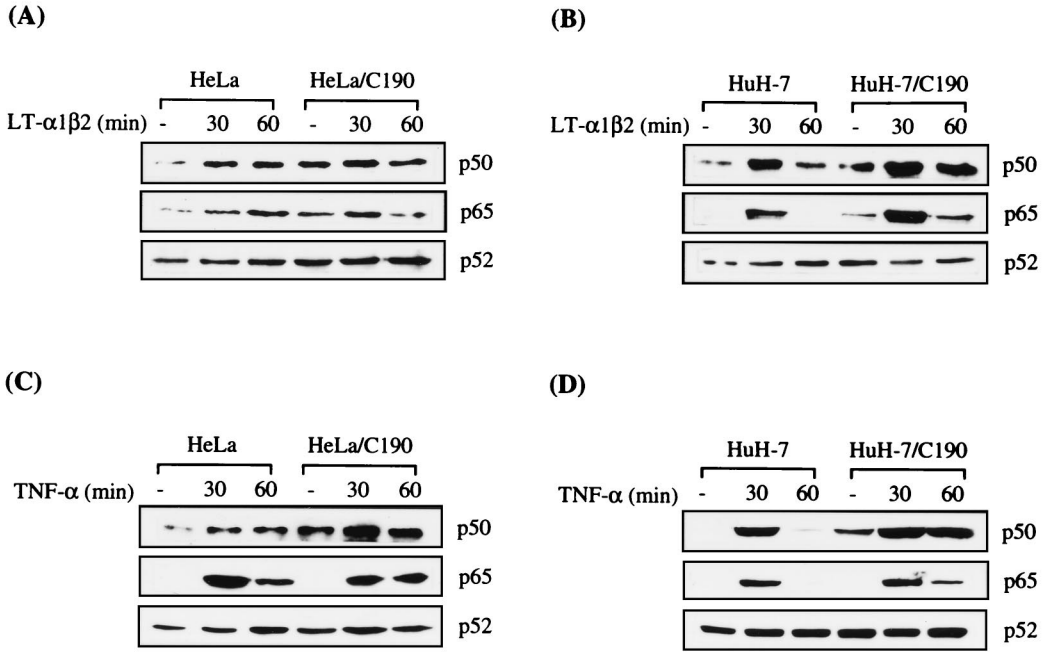

FIG. 5. Subcellular distribution of NF-κB family proteins in HCV core protein-producing cells after LT-$\alpha_1\beta_2$ or TNF-α stimulation. Cells were treated with 500 ng of LT-$\alpha_1\beta_2$ ligand/ml (panels A and B) or 20 ng of TNF-α/ml (panels C and D) for 30 or 60 min. The nuclear extracts (40 μg of protein each) prepared from the cytokine-treated or untreated cells were examined for the expression level of NF-κB family proteins (p65, p50, and p52) by immunoblotting using the ECL detection system.

sponsive to LT-$\alpha_1\beta_2$ stimulation (Fig. 6A). Interestingly, compared to what occurred in their parental cells, the degradation of IκB-β inhibitor was enhanced and/or sustained in both LT-$\alpha_1\beta_2$-stimulated core-producing cells of HeLa and HuH-7 but with different kinetics. For LT-$\alpha_1\beta_2$-treated HeLa/C190 cells, the IκB-β degradation occurred within a 0.5- to 1-h time interval, while for HuH-7/C190 cells, it occurred at a 0.5- to 2-h interval (Fig. 6B). Furthermore, similar to the case of IκB-α, the IκB-β protein in HeLa cells was unresponsive to stimulation with LT-$\alpha_1\beta_2$ (Fig. 6B). However, a slight depletion of IκB-β was observed in LT-$\alpha_1\beta_2$-treated (0.5 to 1 h) HuH-7 cells (Fig. 6B).

In TNF-α-treated cells, as expected, IκB-α had a rapid turnover in all cells examined (Fig. 6C). However, the resynthesis of IκB-α in both core-producing cells after TNF-α treatment was initiated earlier (30 min) than that of parental cells (Fig. 6C). Notably, the degradation of IκB-β was induced in both HuH-7 and HuH-7/C190 cells throughout the period (0.5 to 8 h) of treatment with TNF-α (Fig. 6D). For example, the degradative turnover of IκB-β in HuH-7/C190 prolonged and lasted at least 4 h before the resynthesis of this inhibitor occurred, and the level did not return to the basal one even after 8 h of treatment. In the TNF-α-treated HuH-7 cells, the IκB-β level was also reduced for at least 8 h but reached a minimum level after 1 h of treatment (Fig. 6D). Additionally, relative to HeLa cells, an enhancement of IκB-β breakdown was also observed with TNF-α-treated (1 or 4 h) HeLa/C190 cells (Fig. 6D).

Altogether, our results suggested that the degradation of IκB (and in particular IκB-β), may contribute to the enhancement of the NF-κB activity in cytokine-treated core-producing cells of HeLa and HuH-7. As for the role of IκB-α degradation in core-mediated NF-κB activation, it seems more restricted on certain cytokines and cell lines we examined.

HCV core protein does not potentiate the TNF-α or LT-$\alpha_1\beta_2$-stimulated JNK activity of HeLa and HuH-7 cells. Since in addition to NF-κB activation, both cytokine treatments also lead to JNK activation (21, 41), we carried out experiments to determine whether the triggering of JNK activity by the cytokines was also modulated by the HCV core protein. Cytoplasmic extracts from uninduced or cytokine-treated cells were assayed for JNK activity through the immunocomplex kinase method using glutathione *S*-transferase–C-Jun_{1-79} as the substrate (14). The results indicated that although TNF-α (10 ng/ml) could induce a strong transient response (after 10 to 30 min) of JNK activation (maximum of eight- to ninefold in HeLa or HeLa/C190 cells and three- to fourfold in HuH-7 or HuH-7/C190 cells), the presence of HCV core protein in both HeLa and HuH-7 cells did not show any modulatory effect on JNK activation (data not shown). In the LT-$\alpha_1\beta_2$-treated cells (500 ng/ml), the cytokine-induced JNK activity was weaker than the response induced by TNF-α (maximum of 3- to fivefold in LT-$\alpha_1\beta_2$-treated HeLa and HeLa/C190 cells; less than twofold in LT-$\alpha_1\beta_2$-treated HuH-7 and HuH-7/C190 cells) (data not shown). Moreover, the core protein either had no effect (HuH-7/C190 cells) or slightly downregulated the LT-$\alpha_1\beta_2$-induced JNK activity (HeLa/C190 cells) (data not shown). Therefore, our results suggested that unlike in NF-κB activation, the HCV core protein does not potentiate JNK activation stimulated by both cytokines.

Discussion. In this study, we analyzed the mechanisms and kinetics of LT-α1β2-stimulated NF-κB activation in comparison to those of TNF-α. Additionally, the NF-κB signal pathways of these two stimuli in HCV core protein-producing cells (HeLa/C190 and HuH-7/C190) were also parallel to those of their parental cells. An interesting phenomenon was noted in this study. It appears that varying patterns of NF-κB potentiation (in regard to the kinetics of NF-κB activation, NF-κB

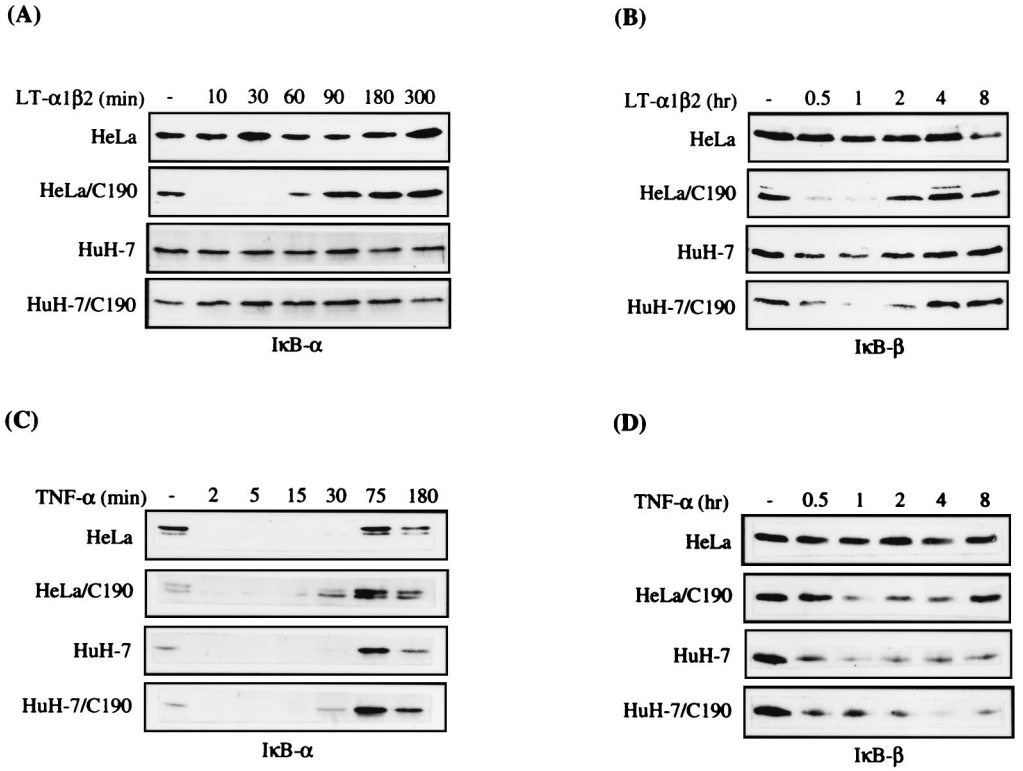

FIG. 6. Degradation of IκB proteins in various LT-$\alpha_1\beta_2$- or TNF-α-stimulated HCV core protein-producing cells. Cells were stimulated with 500 ng of LT-$\alpha_1\beta_2$ ligand/ml (panels A and B) or 20 ng of TNF-α/ml (panels C and D) and at various time intervals (2 min to 8 h), the total cell extracts were prepared and portions of cell lysates (40 μg of protein each) were examined for the expression level of IκB-α or IκB-β protein by using rabbit polyclonal antibody against human IκB-α or IκB-β (Santa Cruz) and ECL detection system.

nuclear translocation, or IκB degradation) between cell lines and stimuli were observed. These results suggest that the complexity of NF-κB signaling pathway triggering by either stimulus and presumably the different cell type-specific pathways are responsible for this phenomenon, as has been previously noted for both the TNF-α and the LT-α1β2 system (4, 62). Despite this, our results clearly demonstrate that following either stimulus, in both cell lines expressing HCV core protein the IκB-β steady-state level substantially declined in parallel with the increase of NF-κB-DNA binding activity and the nuclear translocation of the p65 and p50 NF-κB species (Fig. 1, 2, 5, and 6). However, unlike that observed with IκB-β, the increase in IκB-α turnover appeared only in LT-α1β2-stimulated HeLa/C190 cells; in HuH-7 cells with or without the HCV core protein, the LT-α1β2 ligand failed to stimulate IκB-α turnover, and no apparent enhancement of IκB-α degradation was found in TNF-α-triggering core-producing cells relative to the level in parental cells (Fig. 6). Our results also indicated that unlike the p50 and p65 members, the nuclear translocation of p52 is inert to both cytokine stimuli (Fig. 5) and may be irrelevant to the mechanism of NF-κB activation by these agents. These findings suggest that the enhancement of NF-κB-dependent transcriptional activity following cytokine stimulation in both core-producing cells (Fig. 3) may be mediated by a complex mechanism involving the deregulation of various cytoplasmic inhibitors of NF-κB, which may differ between the stimuli and the cell lines.

IκB-α and IκB-β, encoded by separate genes, contain various numbers of ankyrin repeats, which bind to and inactivate p65 and C-Rel with slightly different affinities (10, 98, 106). In contrast to that of IκB-α, IκB-β degradation occurs with slow kinetics and that degradation occurs only in cells stimulated with certain inducers, such as the bacterial LPS and IL-1 (99), although the degradation of IκB-β in TNF-α-stimulated or TNF-α- and gamma interferon-costimulated endothelial cells was also reported (23, 48). Moreover, since the expression of the IκB-β gene, unlike IκB-α, appears not to be induced by NF-κB, the depleted IκB-β protein cannot be rapidly replenished through de novo protein synthesis (99). Thus, breakdown of IκB-β is generally associated with persistent activation of NF-κB. In our detection system, IκB-β, and to a lesser extent IκB-α, is likely to be the major determinant that mediates the effects of HCV core protein on NF-κB activation. Interestingly, the dual specificity of HCV core protein for IκB-α and IκB-β in NF-κB activation is closely analogous to that described for Tax-induced NF-κB activation (35, 54, 66).

Despite the apparent functional interplay between the HCV core protein and NF-κB, the underlying mechanism by which this viral protein accesses host signaling pathways for IκB-β or IκB-α inactivation has remained elusive. Several possibilities may account for this phenomenon. First, in view of the facts that the HCV core protein physically associates with cytoplasmic domains of TNFRI and LT-βR (21, 65, 113) and that these two receptors signaling NF-κB activation are mediated by receptor-association factors such as TRADD and TRAF family proteins (7, 34, 45, 46, 69, 71, 97, 102), the core protein may modulate the interaction of receptor with its cell-associated factors, accordingly enhancing the process of NF-κB induction. A typical example of this possibility is the LMP1 protein of Epstein-Barr virus, which activates NF-κB through association with TRADD and TRAF molecules (47, 69, 85). Second, the core protein may directly associate with the NF-κB or IκB

inhibitor, which then disrupts its association or affects its stability and phosphorylation, thus contributing to both basal and cytokine-stimulated NF-κB activation. This possibility is reminiscent of recent studies with the Tax protein of HTLV-1, which have revealed that NF-κB activation by Tax acts through its physical interaction with p100, p105, and p50 subunits or p65/p50- and p65/C-Rel-bound DNA complex (13, 16, 42, 43, 94–96). An alternative view is that HCV core protein may physically associate with or activate host kinases that differentially phosphorylate IκB-α or IκB-β and that phosphorylation targets IκB for degradation by proteasome. This suggestion is rather attractive, since recently candidate kinases, such as IκB kinase, IKKα and IKKβ, have been shown to differ in their phosphorylation efficiencies between IκB-α and IκB-β inhibitory proteins (32, 78, 90), and their kinase activities are also differentially regulated by upstream kinases (72), thereby providing variations on the common theme of signal-regulated IκB phosphorylation, which may explain the cell-type- or cytokine-specific IκB inactivation by the HCV core protein. Along this line is the more recent finding (26, 112) that Tax-mediated NF-κB activation results from direct interaction of Tax and MEKK1 or IKK-α/β, components of the IκB kinase complex, leading to predominantly enhanced phosphorylation of IκB-α, which strongly supports this view. Notably, it seems that these possible activation mechanisms of NF-κB are not mutually exclusive, at least in the case of Tax-mediated NF-κB activation. It will thus be interesting to find out whether this feature is also applicable to the core protein of HCV.

In this study, we also found evidence that the core protein of HCV apparently did not have a significant effect on JNK pathway triggering by TNF-α or LT-α1β2 (data not shown). Since both JNK and NF-κB activation share some common signaling molecules, such as MEKK1 or TRAF2 (44, 58, 67, 79, 89), one may argue that these two common modulators may not be the target for core protein-mediated NF-κB activation. However, judging from the complexity or divergence of these two intracellular pathways that are regulated by a network of kinases, it is still too early to formally exclude the involvement of MEKK1 or TRAF2 in core-mediated NF-κB activation.

This work together with our previous study (21) strongly suggests that, with regard to three signaling pathways triggered by TNF-α and LT-α1β2, in HeLa cells the direct association of LT-βR with HCV core protein modulates the NF-κB and cytolytic pathways of LT-βR/LT-α1β2 as opposed to that of TNF-α, where the core protein modulates only its NF-κB activation pathway. However, in HuH-7 cells the core protein potentiates only the NF-κB signal pathway but not the cytolytic activity or JNK pathway of both cytokines. Our results therefore imply that the HCV core protein may deregulate NF-κB activation triggering by TNF-related cytokines in most cell types. Moreover, it appears that in HeLa cells the HCV core protein has a plethoric effect on LT-βR/LT-α1β2 signaling relative to that of TNF-α, even though core protein associates with the cytoplasmic domains of both receptors. This differential effect on the cytokine-induced biological activities exerted by the core protein may reflect the distinct nature of each receptor's signaling pathway. Additionally, a more general effect by the core protein on NF-κB signaling but not on the cytolytic activity of both receptors is in accordance with the effect of their signaling on the cytolytic activity relative to NF-κB activation being probably more diverged downstream following the receptor engagement. Supporting this view are the findings which indicate that in NF-κB activation, both receptors share the same adapter molecule, such as TRAF2 and TRAF5 (2, 7, 22, 45, 71, 81), which may serve as the common target for core protein in eliciting its effect on cytokine-induced NF-κB signaling. On the other hand, these two receptors are differentiated by having a death domain in TNFRI and lacking it in LT-βR, which then identifies the death signaling of TNFRI as the Fas-like pathway (7, 45, 46, 70) and that of LT-βR as TRAF mediated (34, 102). Additionally, emerging evidence shows that there are discrepancies in the core-mediated effect on TNF-α-stimulated cytolytic activity or NF-κB activation, where core protein has been shown to have the opposite effect or no effect, depending on the cell type (21, 76, 113). In light of the pleiotropic nature of HCV core protein and the complication of signaling molecules involved in cell death or NF-κB activation elicited by TNF-α, these discrepancies likely stem from the different intracellular milieus used for examination. Therefore, these findings emphasize the importance of the signaling context in determining the consequence of TNF-α or other cytokine signaling.

In the present study, we demonstrate that like other viruses, HCV adopts its core protein to subvert the NF-κB/IκB autoregulatory pathway. Of particular interest and relevant to the HCV pathogenesis is the outcome of this deregulation. Since the lists of target genes for NF-κB transcriptional factor include both pro-apoptotic and anti-apoptotic genes, the role of NF-κB as a promoter or attenuator of cell death may ultimately depend upon both the cell type and the nature of the stimulus (reviewed in references 4, 6, and 106). In different cell types, NF-κB may perform opposite functions by activating distinct patterns of genes in conjuction with cell-type-specific transcriptional factors. Moreover, different stimuli may elicit distinct signaling pathways in addition to the ones controlling cytolytic activity and NF-κB. Apart from the NF-κB transcriptional factor, additional transcriptional factors may further influence the spectrum of induced genes to determine whether NF-κB can induce or protect against cell death. In view of these considerations, it appears that the role of NF-κB is determined by a set of genes that it can access in a given cell type. Conceivably, the consequences of NF-κB activation vary considerably among cell types and stimuli. Since in human hepatoma HuH-7 cells, NF-κB activation but not cytolytic activity is potentiated by TNF-α or LT-α1β2 (reference 21 and this work), we are inclined to believe that the biological significance of core-mediated upregulation of NF-κB lies in its ability to deliver a survival signal and thus allow persistence of HCV in a long-lived cell compartment, thereby establishing a chronic, activated state of HCV infection. If this conjecture is correct, the feature of HCV core protein then is very similar to that of the Tax protein in establishing the chronic state of viral infection (35, 54, 66, 92). Furthermore, since both TNF-α/TNFRI and LT-α1β2/LT-βR signaling play pivotal roles in a wide range of cellular functions, including immunoregulatory responses, proliferation, differentiation, and immune organ development (1, 4, 5, 31, 33, 63, 106, 108), the association of core protein with these two sets of receptor may have a detrimental effect on these biological functions, which may, at least in part, account for the role of core protein in HCV pathogenesis.

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