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Molecules and Cells

Hepatitis Delta Virus Large Antigen Sensitizes to TNF-α-Induced NF-κB Signaling

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Hepatitis delta virus (HDV) infection causes fulminant hepatitis and liver cirrhosis. To elucidate the molecular mechanism of HDV pathogenesis, we examined the effects of HDV viral proteins, the small hepatitis delta antigen (SHDAg) and the large hepatitis delta antigen (LHDAg), on NF-κB signaling pathway. In this study, we demonstrated that TNF-α-induced NF-κB transcriptional activation was increased by LHDAg but not by SHDAg in both HEK293 and Huh7 cells. Furthermore, LHDAg promoted TRAF2-induced NF-κB activation. Using coimmunoprecipitation assays, we demonstrated that both SHDAg and LHDAg interacted with TRAF2 protein. We showed that isoprenylation of LHDAg was not required for the increase of NF-κB activity. We further showed that only LHDAg but not SHDAg increased the TNF-α-mediated nuclear translocation of p65. This was accomplished by activation of IκBα degradation by LHDAg. Finally, we demonstrated that LHDAg augmented the COX-2 expression level in Huh7 cells. These data suggest that LHDAg modulates NF-κB signaling pathway and may contribute to HDV pathogenesis.

INTRODUCTION

Hepatitis delta virus (HDV) is a small RNA virus containing a 1.7 kb single-stranded circular RNA genome (Kos et al., 1986; Makino et al., 1987; Wang et al., 1986). HDV is always associated with hepatitis B virus (HBV) infection and often causes fulminant hepatitis and progressive chronic liver cirrhosis in human (Niro et al., 2005). Superinfection of HDV increases the risk of fulminant hepatitis greatly and causes more rapidly progressing liver damage than HBV infection alone in chronic hepatitis patients (Farci, 2003). This virus requires HBV as a helper to supply the envelope proteins for viral particle assembly and infection (Fernholz et al., 1993; Lai, 1995; Rizzetto et al., 1980). HDV genome encodes the only known HDV protein, hepatitis delta antigen (HDAg). HDAg has two isoforms, the small HDAg (SHDAg; 195 amino acids, 24 kDa) and the large HDAg (LHDAg; 214 amino acids, 27 kDa) (Wang et al., 1986; Weiner et al., 1998). These two HDAgs are translated from the same initiation codon of a single open reading frame and share the identical functional domains except for an additional 19-amino-acid at the C terminus of the LHDAg (Lai, 1995; Luo et al., 1990; Weiner et al., 1998). The SHDAg is essential for HDV RNA replication (Chao et al., 1990; Kuo et al., 1989). The LHDAg suppresses viral replication and is necessary for virion assembly (Chang et al., 1991; Chen et al., 1992; Ryu et al., 1992). The LHDAg is synthesized by an RNA editing event that alters the termination codon of the open reading frame (ORF) of SHDAg (Casey and Gerin, 1995; Luo et al., 1990). The LHDAg contains a CaXX box at its C terminus, in which the cysteine residue is isoprenylated (Glenn et al., 1992; Hwang et al., 1992). Isoprenylation of LHDAg is required for virion assembly (Lee et al., 1994).

NF-κB is a dimeric transcription factor that has crucial roles in inflammation, immunity, cell proliferation and apoptosis (Aggarwal, 2004; Ghosh and Karin, 2002; Karin and Ben-Neriah, 2000; Silverman and Maniatis, 2001). The NF-κB complex is composed of homodimers or heterodimers of NF-κB proteins, including REL-A (also known as p65), c-REL, REL-B, p50 and p52. p50 and p52 are derived from the larger precursors p105 and p100, respectively, through proteolytic processing by the proteasome (Baeuerle and Baltimore, 1996; Ghosh and Karin, 2002). All NF-κB proteins contain a highly conserved REL-homology domain (RHD) that is responsible for DNA binding, dimerization, nuclear translocation, and interaction with the IκB proteins (Ghosh et al., 1998). In the canonical pathway, which is the predominant NF-κB signaling pathway, stimulating cells with an agonist such as tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β), activates the IKK complex (Thanos and Maniatis, 1995). The IKK complex includes the scaffold protein NF-κB essential modulator (NEMO; also named IKKγ), IKKα and IKKβ kinases (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Rothwarf et al., 1998; Woronicz et al., 1997; Yamaoka et al., 1998; Zandi et al., 1997). Both IKKα and IKKβ phosphorylate IκBα on Ser^{32} and Ser^{36}, and the resulting phosphorylated IκBα is subsequently ubiquitinated and degraded through the proteosomal pathway, thus exposing the nuclear localization signals on the p50-p65 heterodimer (Karin and Ben-Neriah, 2000; Zandi et al., 1998).

In the present study, we demonstrated that TNF-α-induced NF-κB activation was increased by LHDAg. This was accomplished by activating IκBα degradation by LHDAg. These results suggest that LHDAg may play an important role in HDV pathogenesis.

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MATERIALS AND METHODS

Plasmid constructions

cDNA encoding either SHDAg or LHDAg of HDV was amplified by polymerase chain reaction (PCR) by using American isolate (Hwang et al., 1998) as a template and was subcloned into pcDNA3 (Invitrogen) or pEF6B/His-Myc vector (Invitrogen). TNFR1, Flag-TRAF2, Flag-IKKβ, and p65 expression vectors were described previously (Park et al., 2001; 2002; Zhu et al., 1998).

Cell culture and DNA transfection

HEK293 cells, HEK293T cells, Huh7 cells and Hela cells were cultured in Dulbecco's modified Eagle's medium. All these cell lines were maintained at 37°C with 5% CO_2 and supplemented with 10% fetal bovine serum (Hyclone) and 100 IU/ml of penicillin-streptomycin. For transfection, ~5×10^5 cells per plate on 60-mm dishes were transfected with plasmid DNA by using polyethyleneimine (PEI, Aldrich) reagent as we previously described (Park et al., 2009).

Luciferase reporter gene assay

Either Huh7 or HEK293 cells were transfected with 2 μg of expression plasmid, 0.25 μg of NF-κB luciferase reporter (Lee et al., 1997), and 0.5 μg of pCH110 reference plasmid (Amersham Biosciences) containing the *Escherichia coli lac*Z gene under the control of the simian virus 40 promoter. The total DNA concentration in each transfection mixture was kept constant by adjusting with an empty vector. At 36 h after transfection, the cells were stimulated with TNF-α (20 ng/ml, BD science) for 6 h. Data were normalized by measuring β-galactosidase activity. Luciferase and β-galactosidase assays were performed as described previously (Park et al., 2002).

Coimmunoprecipitation assay

Cells were transfected with 4 μg of the corresponding plasmids. Following incubation at 37°C for 24 h, cells were harvested, washed twice in cold phosphate-buffered saline (PBS), and incubated in 400 μl of cell lysis buffer [50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1% Triton X-100, 1.5 mM $MgCl_2$, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The cell lysates were centrifuged at 15,000 rpm for 10 min, and then supernatant was incubated at 4°C for 2 h with the corresponding antibodies. The samples were further incubated with protein-A sepharose 4B beads (Zymed) for 2 h. The beads were washed 5 times with the cell lysis buffer, and the bound proteins were detected by immunoblot analysis.

IκBα degradation

HEK293 cells were transfected with either an empty vector or HDAg expression plasmids. At 24 h after transfection, cells were treated with TNF-α (5 ng/ml) for various times. The cell extracts were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and detected by immunoblotting with anti-IκBα polyclonal antibody (Santa Cruz Biotechnology).

Preparation of cytoplasmic and nuclear fractions

Huh7 cells transfected with either empty vector or HDAg expression plasmids were stimulated with TNF-α (10 ng/ml) for 30 min. At 48 h after transfection, cells were washed twice in PBS and incubated in buffer A [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40] supplemented with 1 mM dithiothreitol (DTT) and 1 mM PMSF. After lysis on ice for 2 min, the cytoplasmic fraction was prepared by centrifugation at 15,000 rpm for 2 min. The pellet was resuspended in buffer B [20 mM HEPES-KOH (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 420 mM KCl, 0.2 mM EDTA] supplemented with 1 mM DTT and 1 mM PMSF and incubated for 30 min by rotating in a cold chamber. The sample was further centrifugation at 15,000 rpm for 10 min and the nuclear fraction was collected from the supernatant.

RNA isolation and RT-PCR

Total RNAs were isolated from Huh7 cells transfected with either empty vector or HDAg expression plasmids using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The cDNAs were synthesized by avian myeloblastosis virus (AMV) reverse transcriptase (Promega) from 1 μg of total RNAs with random hexamer primers using the SuperScriptIII kit (Invitrogen). One-tenth aliquot of cDNA was subjected to PCR amplification using the forward primer 5′-TGG ACT TCG AGC AAG AGA TGG-3′ and reverse primer 5′-GGA AGG AAG GCT GGA AGA GTG-3′ for β-actin, the forward primer 5′-CCC TTG GGT GTC AAA GGT AA-3' and reverse primer 5′-AAC TGA TGC GTG AAG TGC TG-3′ for COX-2. Amplified DNA was analyzed by agarose gel electrophoresis.

RESULTS

HDV LHDAg increases TNF-α-induced NF-κB activation

TNF-α has a wide range of actions in inflammation, infection, and immunity. HDV infection often leads to fulminant hepatitis and hepatocellular carcinoma (HCC). Since HDAg is the only protein encoded by HDV genome, we examined the roles of two isoform of HDAg, SHDAg and LHDAg (Fig. 1A), in the TNF-α-induced NF-κB activation using a luciferase reporter gene assay. Huh7 cells were transiently cotransfected with NF-κB luciferase reporter plasmid and either the empty vector or HDAg expression plasmid. At 36 h after transfection, cells were either left untreated or treated with human TNF-α (20 ng/ml) for 6 h, and then luciferase activity was determined. As shown in Fig. 1B, TNF-α induced ~5-fold transactivation of the reporter in vector transfected Huh7 cells. Overexpression of LHDAg elevated TNF-α-induced transcriptional activation 17-fold of the vector control. However, overexpression of SHDAg had no effect on TNF-α-induced transcriptional activation. It is noteworthy that LHDAg in the absence of TNF-α stimulation activated ~6-fold of the reporter gene activity as compared to the control vector. We further investigated the dosage effect of HDAgs on NF-κB activation. Huh7 cells were transfected with NF-κB luciferase reporter plasmid and pCH110 reference plasmid with increased amounts (0.1 to 1.5 μg) of either SHDAg or LHDAg. Indeed, LHDAg but not SHDAg activated NF-κB luciferase reporter activity in a dose dependent manner in the absence of TNF-α stimulation (Fig. 1C). We found that overexpression of green fluorescence protein (GFP) as a control showed no effect on NF-κB reporter activation (data not shown). These results suggest that LHDAg specifically activates the NF-κB signal transduction in Huh7 cells.

LHDAg activates TNF-α-stimulated IκBα degradation

IκBα is rapidly phosphorylated on Ser^{32} and Ser^{36} by TNF-α stimulation and then degraded by the 26S proteasome (Whiteside and Israel, 1997). To investigate the effect of LHDAg on TNF-α-stimulated IκBα degradation, Huh7 cells were transfected with either empty vector or HDAg expression plasmids. At 24 h after transfection, cells were treated with TNF-α (5 ng/ml) for the indicated times. Equal amounts of cell lysates

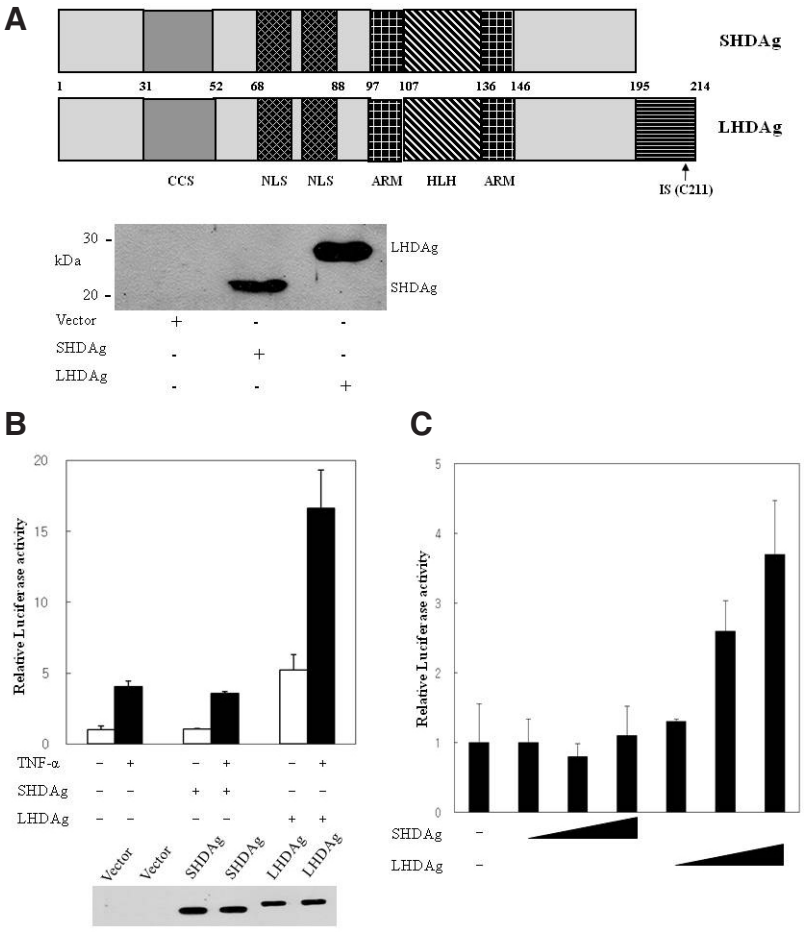

Fig. 1. LHDAg of HDV increases TNF-α-induced NF-κB activation. (A) (upper panel) Schematic diagram of functional domains in SHDAg and LHDAg. CCS, coil-coiled sequence; NLS, nuclear localization signal; ARM, arginine-rich motif; HLH, helix-loop-helix; IS, isoprenylation site. (lower panel) Expression of both SHDAg and LHDAg in Huh7 cells. Cells were transfected with plasmid expressing either SHDAg or LHDAg. One day after transfection, total cell lysates were immunoblotted with rabbit anti-HDAg antibody. (B) Effect of HDAg on TNF-α-stimulated NF-κB activation by reporter gene assay. Huh7 cells were cotransfected with NF-κB luciferase reporter plasmid and pCH110 reference plasmid with empty vector or HDAg expression plasmids. At 36 h after transfection, cells were either left untreated or treated with human TNF-α (20 ng/ml) for 6 h, and then luciferase activity was determined. The results shown are representative of two independent experiments. Protein expressions of both SHDAg and LHDAg in Huh7 cells were verified by rabbit anti-HDAg antibody (bottom panel). (C) LHDAg increases NF-κB activity in a dose dependent manner. Reporter plasmids were cotransfected with selected amounts (0.1, 0.5, and 1.5 μg) of either SHDAg or LHDAg expression plasmid. The total DNA amount was held constant by addition of an empty vector. The results shown are a representative of two independent experiments.

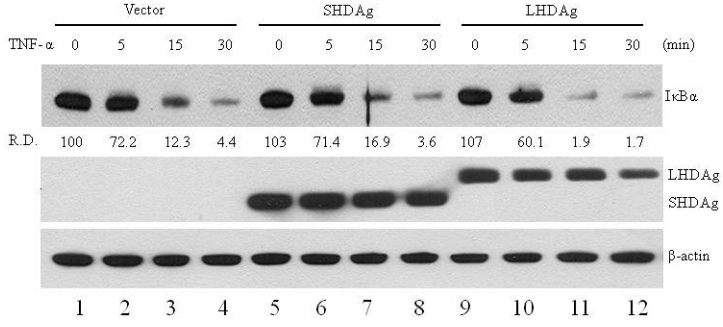

Fig. 2. LHDAg promotes TNF-α-stimulated IκBα degradation. Huh7 cells transfected with either empty vector or HDAg expression plasmids were stimulated with human TNF-α (5 ng/ml) for the indicated times. Equal amounts of cell lysates were analyzed by immunoblotting with anti-IκBα antibody (top panel). The results shown are representative of three independent experiments. Protein levels of HDAgs (middle panel) and β-actin (bottom panel) in the same cell lysates were determined by immunoblot analysis. R.D., relative density.

were analyzed by immunoblotting with anti-IκBα antibody. As shown in Fig. 2, IκBα was immediately degraded by TNF-α stimulation in vector transfected cells. Similar pattern of IκBα degradation was observed in cells expressing SHDAg. Surprisingly, TNF-α-induced IκBα degradation was significantly activated by LHDAg (Lanes 3 and 7 versus lane 11 in Fig. 2). These results indicate that LHDAg activates TNF-α-induced IκBα degradation.

LHDAg enhances TRAF2-mediated NF-κB activation

To determine whether the activation of NF-κB activity by LHDAg is mediated through TNFR1 signaling cascades, we investigated the effects of LHDAg on NF-κB activation induced by TNFR1 signaling transducers, including TNFR1, TRAF2, IKKβ, and p65. Figure 3 showed that LHDAg activated TRAF2-, IKKβ- and p65-mediated NF-κB activation. However, TNFR1-mediated NF-κB activation was not affected by the LHDAg. These results suggest that LHDAg acts as an activator of NF-κB activation in downstream transducers of TNFR1.

Both LHDAg and SHDAg interact with TRAF2

Because LHDAg but not SHDAg activated NF-κB signal transduction pathway, we speculated that LHDAg might regulate NF-κB signaling through specific protein interplay with TNFR1 signal transducers. Many viruses are known to modulate TNF-α signaling through interaction with signal transducers. Be-

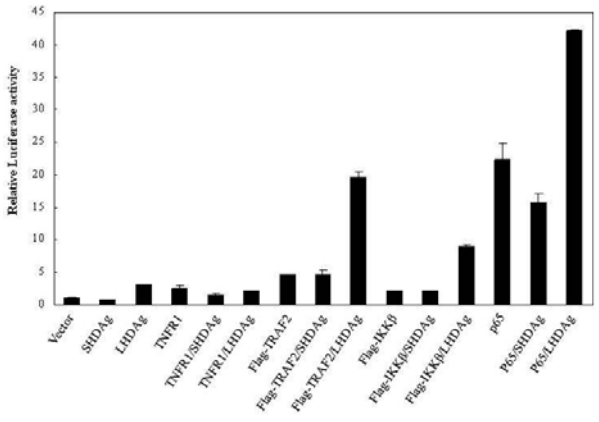

Fig. 3. LHDAg enhances TRAF2-induced NF-κB activation. The effects of HDAgs on NF-κB activity in TNF-α signal transducers were determined. Huh7 cells were cotransfected with reporter plasmids and TNF signaling molecules (TNFRI, TRAF2, IKKβ, and p65) either alone or together with HDAg plasmids, and then NF-κB reporter gene activity was determined. The results shown are representative of three independent experiments.

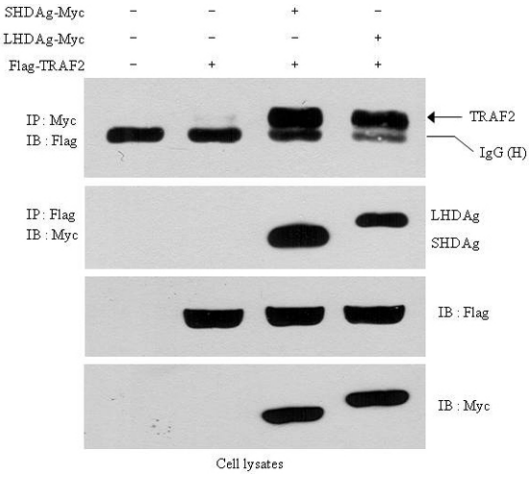

Fig. 4. Both SHDAg and LHDAg interact with TRAF2. HEK293 cells were transfected with the indicated combination of expression plasmids. Total cell lysates were immunoprecipitated with anti-Myc antibody, and then bound proteins were detected by immunoblotting with anti-Flag antibody (1st panel). Reciprocally, total cell lysates were immunoprecipitated with anti-Flag antibody, and then bound proteins were detected by anti-Myc antibody (2nd panel). Protein expressions of TRAF2 (3rd panel) and HDAgs (4th panel) in the same cell lysates were verified by immunoblotting with anti-Flag and anti-Myc antibodies, respectively. IP, immunoprecipitation; IB, Immunoblot.

cause LHDAg increased the TRAF2-induced NF-κB reporter activity but not TNFR1-induced NF-κB reporter activity, we examined the possible interaction between LHDAg and TRAF2 protein by immunoprecipitation assay. TRAF2 protein was co-expressed with either SHDAg or LHDAg in HEK293 cells. At 48 h after transfection, total cell lysates were immunoprecipitated with anti-Myc antibody and the coprecipitated proteins were detected by immunoblotting with anti-Flag antibody. Unexpectedly, both SHDAg and LHDAg interacted with TRAF2 protein *in*

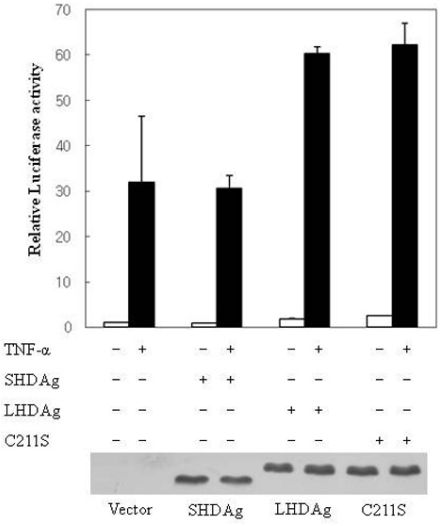

Fig. 5. Isoprenylation is not involved in LHDAg-mediated NK-κB activation. Huh7 cells were cotransfected with NF-κB luciferase reporter plasmid and pCH110 reference plasmid with the indicated plasmids. At 36 h after transfection, cells were either left untreated or treated with human TNF-α (20 ng/ml) for 6 h, and then luciferase activity was measured. The results shown are representative of three independent experiments.

vivo (top panel in Fig. 4). We further confirmed this result by a reciprocal experiment using anti-Flag antibody for immunoprecipitation and anti-Myc antibody to detect coprecipitated protein (2nd panel in Fig. 4). These results show that both SHDAg and LHDAg specifically interact with TRAF2 protein but this interaction is not involved in enhancement of TRAF2-mediated NF-κB activation by LHDAg.

Isoprenylation of LHDAg is not required for the enhancement of NK-κB activity

Both SHDAg and LHDAg are identical in sequence except an additional 19 aa at the C-terminus of LHDAg. There is only one cysteine residue in aa 211 of LHDAg, which is isoprenylated at cysteine residue by posttranslational modification. Hence isoprenylation is the major difference between two isoforms because SHDAg is not isoprenylated. We therefore asked whether isoprenylation of LHDAg was involved in TNF-α-induced signal transduction. To address this question, we performed luciferase reporter gene assay using isoprenylation mutant of LHDAg, in which cysteine residue of the CaXX box was changed to serine (C211S). C211S is an isoprenylation-deficient mutant as we previously reported (Hwang and Lai, 1993). HEK293 cells were cotransfected with NF-κB luciferase reporter plasmid and pCH110 reference plasmid together with SHDAg, LHDAg, and C211S, respectively. At 36 h after transfection, cells were either left untreated or treated with human TNF-α (20 ng/ml) for 6 h, and then luciferase activity was determined. As previously shown in Fig. 1B, LHDAg but not SHDAg enhanced TNF-α-induced transcriptional activation. However, LHDAg-mediated NF-κB signal activation was not altered by C211S (Fig. 5), indicating that isoprenylation of LHDAg was not required for the up-regulation of NF-κB activity.

LHDAg increases the nuclear translocation of NF-κB and expression of target gene

To investigate the molecular mechanism of NF-κB activation by

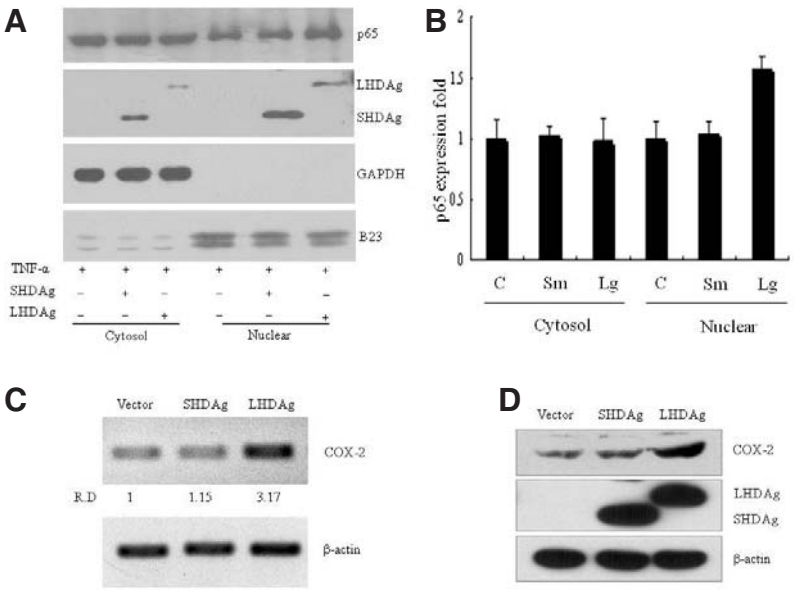

Fig. 6. LHDAg increases the nuclear translocation of NF-κB and up-regulates COX-2 expression. (A) Huh7 cells were transfected with either empty vector or HDAgs expression plasmids. At 48 h after transfection, cells were stimulated with TNF-α (10 ng/ml) for 30 min. Both cytoplasmic and nuclear fractions were prepared as described in "Materials and Methods" and immunoblotted with anti-p65 antibody to reveal the localization of NF-κB subunit (1st panel). Protein expressions of both SHDAg and LHDAg were verified by immunoblot analysis using rabbit anti-HDAg antibody (2nd panel). Subcellular fractions were verified by using anti-GAPDH antibody for cytoplasmic fraction (3rd panel) and anti-B23 antibody for nuclear fraction (4th panel). (B) Triplicate experimental data of Fig. 6A were quantified, and each bar represents the average level of p65. C, control vector; Sm, SHDAg; Lg, LHDAg. (C) LHDAg up-regulates RNA level of COX-2 gene. Total RNAs were isolated from Huh7 cells transfected with either empty vector or HDAg expression plasmids. The cDNAs were synthesized by avian myeloblastosis virus reverse transcriptase from 1 μg of total RNAs. cDNA was amplified by COX-2 specific primers and analyzed by an agarose gel electrophoresis. β-actin was used as a control. The COX-2 mRNA level was measured by using a gel analysis program and normalized by calculating the ratio of mRNA to β-actin. (D) LHDAg increases protein expression level of COX-2 gene. Huh7 cells were transfected with either vector or HDAg expression plasmids. Total cell lysates harvested at 24 h after transfection were immunoblotted with anti-COX-2 antibody.

LHDAg, the cytoplasmic and nuclear fractions in cells expressing LHDAg were compared for the level of NF-κB. Huh7 cells transfected with either empty vector or HDAg expression plasmids were stimulated with TNF-α and then both cytoplasmic and nuclear fractions were immunoblotted with p65 antibody. The nuclear fraction was verified using anti-B23 antibody and cytoplasmic fraction was identified by using anti-GAPDH antibody. As shown in Fig. 6A, P65 level in nucleus was not altered by SHDAg as compared with vector control cells. However, p65 level in nucleus was increased in cells expressing LHDAg, demonstrating that LHDAg promoted the translocation of p65 to the nucleus and hence LHDAg up-regulated the TNF-α-induced NF-κB activity. This was confirmed by triplicate experimental data as shown in Fig. 6B.

To further verify the effect of LHDAg on TNF-α-induced NF-κB activation, we examined the COX-2, one of NF-κB target genes, level in cells expressing either empty vector or HDAg. Huh7 cells were transfected with either vector or HDAg expressing plasmids and treated with TNF-α for 6 h. Total cellular RNAs were then extracted and COX-2 mRNA level was determined by RT-PCR. The Cox-2 mRNA level was quantitated by using a gel analysis program (Image J. ver. 1.41) and normalized by calculating the ratio of mRNA to β-actin. Figure 6B showed that COX-2 mRNA level was increased in cells expressing LHDAg but not SHDAg. We then compared the COX-2 protein expression level in cells expressing vector, SHDAg, and LHDAg, respectively. As shown in Fig. 6C, COX-2 protein expression level was also significantly increased by LHDAg. However, COX-2 protein level was not altered by SHDAg as in vector control. Because protein expression levels of both SHDAg and LHDAg were not affected by TNF-α, these data further confirmed that LHDAg promoted TNF-α-induced NF-κB activation.

DISCUSSION

HDV often causes fulminant hepatitis and HCC in human. However, the molecular mechanisms underlying HDV pathogenesis are not fully understood. Because TNF-α has a wide range of actions in inflammation, infection, and immunity, we have investigated the potential involvement of HDV HDAg in TNF-α-stimulated NF-κB signal transduction. Nuclear transcription factor NF-κB plays a key role in regulating expression of many cytokines and immunoregulatory proteins (Baeuerle and Baltimore, 1996; Baldwin, 1996; Barnes and Karin, 1997). Using reporter gene assay, we have demonstrated that LHDAg but not SHDAg increased the TNF-α-induced NF-κB activation in hepatic cells. We have further shown that LHDAg increased TRAF2-, IKK-β-, and P65-mediated NF-κB activations and subsequent IκBα degradation in Huh7 cells. However, TNFR1-mediated NF-κB activation was not affected by LHDAg. These results suggest that the increase of TNF-α-stimulated NF-κB activation by HDV LHDAg is mediated through TRAF2 protein. We therefore investigated the potential interaction between TRAF2 and HDAg. Indeed, both SHDAg and LHDAg interacted with TRAF2 protein. Because both SHDAg and LHDAg equally bound to TRAF2, this interaction was not involved in TRAF2-mediated NF-κB activation by LHDAg.

We further explored the potential involvement of isoprenylation in TNF-α-stimulated NF-κB activation by LHDAg. Isoprenylation mediates protein-protein interaction (Hwang and Lai, 1993) and HDV assembly (Lee et al., 1994). In addition, isoprenylation facilitates membrane localization of certain proteins and regulates signal transduction (Mizuno et al., 1991). Therefore, we speculated that isoprenylation might confer the transactivation activity in LHDAg. To investigate this possibility, we performed luciferase reporter gene assay by using isoprenylation mutant of LHDAg (C211S). However, isoprenylation-deficient mutant equally enhanced TNF-α-stimulated NF-κB activation to wild-type LHDAg. This result suggests that isoprenylation is not involved in LHDAg-mediated NF-κB activation. Hence precise mechanism other than isoprenylation needs further studies to verify why only LHDAg increases the TNF-α-

stimulated NF-κB activation. In fact, we previously reported that HDAgs are phosphoprotein and LHDAg is phosphorylated six times more that SHDAg (Hwang et al., 1992). The possible involvement of phosphorylation in LHDAg may be alternative way to determine whether it is involved in NF-κB activation.

We also found that nuclear translocation of p65 was increased in cells expressing LHDAg although total protein level of p65 in cells was not altered by LHDAg. This result indicated that LHDAg increased the TNF-α-stimulated NF-κB activity. We further demonstrated that both mRNA and protein levels of COX-2 were augmented by LHDAg in Huh7 cells. Because COX-2 is one of the NF-κB target genes, this result further confirms that LHDAg increases the TNF-α-stimulated NF-κB activation. Although alteration of NF-κB signaling in cancer cells is not well understood, overactivation of NF-κB and subsequent production of cytokines, inflammatory enzymes, growth factors, and antiapoptotic proteins have been found to be involved in the progression of cancers. Likewise, alteration of NF-κB activity by LHDAg may be involved in HDV pathogenesis.

Some hepatitis viruses are known to evolve their gene products to modulate the NF-κB pathway for viral replication or evasion of immune responses. HCV core protein can either enhance TNF-α-induced NF-κB activation (Chung et al., 2001; Hideo et al., 2001) or suppress TNF-α-induced NF-κB activity (Shrivastava et al., 1998; Zhu et al., 1998). We reported that HCV NS5A and NS5B proteins also modulated TNF-α-induced NF-κB activation through distinct mechanisms (Choi et al., 2006; Park et al., 2002). Furthermore, HBX protein directly interacted with IκBα to prevent the reassociation of IκBα with NF-κB (Weil et al., 1999).

In the present study, we demonstrated that LHDAg promoted the TNF-α-induced NF-κB signaling cascade, which is one of the main mechanisms of inflammation in virus-infected cells. LHDAg acted as a positive regulator of NF-κB activation in TNFR1 signal transduction. Collectively, we demonstrated that LHDAg activated TNF-α-stimulated IκBα degradation, promoted nuclear translocation of NF-κB, and then up-regulated its target gene. Therefore, modulation of TNF-α-induced NF-κB activation by LHDAg may contribute to HDV pathogenesis.

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