Proinflammatory cytokine TNF-α increases the stability of hepatitis B virus X protein through NF-κB signaling

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Hepatitis B virus (HBV) X protein (HBx) is a key player in HBV-induced hepatocellular carcinoma (HCC). HBx interacts with several cell signaling molecules, leading to activation of various transcription factors including nuclear factor-kappaB (NF-κB). Activated NF-κB signaling is implicated in many human cancers including HCC. Here, we present evidence that the NF-κB signaling pathway, highlighting the interplay between HBx protein and NF-κB signaling, may account for HBV-mediated liver carcinogenesis.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third-leading cause of cancer death in the world, with ~1 million deaths/year worldwide (1). The main etiological factors, hepatitis B virus (HBV) and hepatitis C virus are implicated in ~75% of HCC cases worldwide; however, the exact mechanisms by which transformation of hepatocytes occurs remain uncertain (2,3). Experimental evidence has demonstrated the carcinogenic effects of HBV on hepatocytes through both direct and indirect mechanisms (4). HBV is a small DNA virus with 3.2 kb partially double-stranded genome and contains four major genes: pol (reverse transcriptase/ DNA polymerase), core (capsid protein), env (envelope/surface protein) and X (HBx protein) (5). The HBx gene appears to play a more important causal role in HBV-related HCC because it is the most commonly integrated and actively transcribed viral gene in liver cancer cells (6). Moreover, HBx plays an important role in liver cancer development in some transgenic mice models (7,8). HBx is a multifunctional regulator that modulates a variety of host processes, such as transcription (9), cell cycle progress (10), protein degradation pathways (11), apoptosis (12), cell migration (13) and genetic stability (14), by directly or indirectly interacting with host factors (15). These HBx-induced alterations appear to play a critical role in the initiation and development of HCC.

Because of its potential role in HBV-induced HCC development, HBx has been a focus of research in many studies. A well-documented HBx function is its ability to act as a transcription coactivator modulating the activity of several transcription factors (16). HBx interacts directly with some of these, leading to their repression, i.e. p53 (17,18), or activation. Translation factors activated by HBx include C/EBP (19), AP-1 (20), AP-2 (21), Smad4 (22) and STAT3 (23). In addition, several studies have reported that HBx can activate nuclear factor-kappaB (NF-κB) (23–27), and this may account for its role in HCC development.

NF-κB is a crucial transcription factor that plays an important role in various physiological and pathological conditions including immune regulation, inflammation and carcinogenesis (28). NF-κB signaling has been found to be activated in cancerous tissues in experimental animals as well as in many human cancers, including HBV-induced liver cancer (27,29–32). NF-κB consists of five proteins of the Rel family, p50, p52, RelA (p65), c-Rel and RelB, that form homo- and hetero-dimers among themselves and are differently involved in two distinct pathways, referred to as the canonical and non-canonical NF-κB pathways. In the canonical pathway, exposure of cells to stimuli such as pro-inflammatory cytokines leads to the activation of the IκB kinase (IKK) complex, comprising two catalytic subunits (IKKα and IKKβ) and a scaffold subunit (IKKγ). Activated IKK phosphorylates the inhibitor IκB that sequesters NF-κB in an inactive form in the cytoplasm. Phosphorylated IκBα then becomes susceptible to ubiquitination and degradation by the proteasome system. Free NF-κB then translocates into the nucleus to activate the expression of cognate genes (29,33). In the non-canonical signaling pathway, NF-κB activation induced by lymphotoxin β or CD40 family members is largely mediated by the NF-κB-inducing kinase and IKKα homodimers. In this pathway, activated IKKα phosphorylates p100, leading to its maturation into p52, which complexes with RelB, followed by nuclear entry and activation of selected genes (33).

The positive impact of HBx on NF-κB signaling is well established, as mentioned above. However, it is not known whether this phenomenon may in turn influence the level of HBx. HBx has a very short half-life and is generally weakly expressed (11). HBx levels can be modulated by a variety of signals that further affect progression of carcinogenesis (34,35), but the possible involvement of NF-κB in HBx function is its ability to act as a transcription coactivator modulating the activity of several transcription factors (16). HBx interacts directly with some of these, leading to their repression, i.e. p53 (17,18), or activation. Translation factors activated by HBx include C/EBP (19), AP-1 (20), AP-2 (21), Smad4 (22) and STAT3 (23). In addition, several studies have reported that HBx can activate nuclear factor-kappaB (NF-κB) (23–27), and this may account for its role in HCC development.

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Materials and methods

Plasmids

Plasmid-containing full-length HBx was obtained from Dr Kim (Yonesi University, Korea). pDNA3-Flag-HBx was obtained by cloning the amplified

Abbreviations: HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; IKK, IκB kinase; IL, interleukin; NF-κB, nuclear factor-kappaB; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; TNF, tumor necrosis factor.

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polymerase chain reaction (PCR) coding sequence of HBs in frame with Flag epitope coding sequence in pcDNA3-Flag vector. The generated recombinant plasmid was verified by sequencing pRK5-Flag-IKKβ, pRK5-Flag-IKKβ KD, the dominant-negative kinase dead (KD) mutants myc-IKKβKD and Flag-IKKβKD were obtained from D.Goeddel (Tulalik, South San Francisco, CA). The mouse p65 plasmid pcDNA3-p65 was obtained from T.Gilmore (Boston University, Boston, MA). The IKK non-phosphorylable mutant pcDNA3-p65-S534A (equivalent of human p65 S536A; [36]) and constitutively phosphorylated mimic pcDNA3-p65-S534E were generated from pcDNA3-p65 by using the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutants were verified by DNA sequencing. enhanced green fluorescence protein (peGFP-C1), NF-κB-luciferase reporter and Renilla constructs were obtained from BD Clontech (Palo Alto, CA).

Antibodies and reagents

Antibodies were used the following: mouse anti-Flag (M2) and rabbit anti-Flag (Sigma, St Louis, MO); rabbit anti-green fluorescence protein and mouse anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-p65 and rabbit anti-phospho-p65 Ser536 (Cell Signaling Technology, Danvers, MA). Human recombinant TNF-α was obtained from R&D Systems (Minneapolis, MN), human recombinant IL-6 from Stem Cell Technologies (Vancouver, Canada), actinomycin D from Euromedex (Souffelweyersheim, France) and cyclohexamide and MG132 from Sigma.

Cells and transfections

Human embryonic kidney cell line (HEK) 293 was maintained in Dulbecco’s modified Eagle’s medium (Gibco/Invitrogen, Scotland, UK) supplemented with 10% fetal calf serum (PAA Laboratories, Pasching, Austria) and antibiotics. The HBV-negative HCC cell line, Huh7, was maintained in Dulbecco’s modified Eagle’s medium-Ham’s F12 (1:1) containing 10% fetal calf serum, 1× Non Essential Amino Acids, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid 10 mM and antibiotics. Cells were maintained at 37°C with 5% CO2. For transfection, 4.5 × 105 cells per well were seeded in six-well plates for 24 h and then transfected by different plasmid constructs using Fugene6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. pcDNA empty vector was used as a control and to equalize the total amount of transfected DNA in each set. pEGFP was included as an internal control for normalization of the transfection efficiency. Cells were harvested 48 h after transfection for further analysis.

Immunoblotting Cells were washed with phosphate-buffered saline (PBS) and lysed in western lysis buffer containing 50 mM Tris–Cl pH 7.4, 0.5% Nonidet P-40, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 3% glycerol and HALT protease and phosphatase inhibitors cocktail (Pierce Biotechnology, Rockford, IL) for 30 min on ice. Insoluble material was removed by centrifugation, and protein was estimated by BCA Assay (Interchim, Montluçon, France). Equivalent amounts of protein extracts were loaded on 10–13% sodium dodecyl sulfate–polyacrylamide gel. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Perkin-Elmer, Zaventem, Belgium). The membranes were used for immuno blotting with specified primary antibodies and peroxidase-conjugated secondary anti-mouse or rabbit antibody (Promega, Madison, WI) and visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK).

Immunoprecipitation Cells were washed with PBS and lysed by rapid freeze–thaw at −80°C and then lysed in PBS containing a 0.1% NP-40 and HALT protease and phosphatase inhibitors cocktail (Pierce Biotechnology) and incubated on ice for 15 min. Insoluble material was removed by centrifugation and protein was estimated by BCA Assay (Interchim). Equivalent amounts of protein extracts were then used for immunoprecipitation. For preclariing, cell lysates were incubated with sepharose beads (Sigma) for 30 min on a rotating platform at 4°C. Cleared lysates were then incubated with anti-Flag M2 beads (Sigma) for 2 h on a rotating platform at 4°C. The beads were then washed three times with the lysis buffer and then three times more with modified lysis buffer (PBS containing 0.5% NP-40 and 300 mM NaCl). Finally, the beads were resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis loading dye and boiled at 95°C for 5 min before loading.

Gene silencing A pool of four synthetic oligos (sip65; Pool no. M-003533-00; Dharmacon RNA Technologies, Lafayette, CO) was used for silencing NF-κB p65. Small interfering RNA specific for luciferase (siluc; antisense 3'-
R.Shukla et al. system following the manufacturer’s instructions (Promega). Quantitation of determined in the same cell lysate using the Dual-Luciferase reporter assay efficiency. The enzyme activities of firefly and Renilla luciferase were construct was included as an internal standard for normalization of transfection transfected in HEK-293 cells along with other experimental plasmids. Renilla j To monitor NF- Luciferase reporter assay ImageJ 1.41o software. by LAS AF Lite 2.0.0 software, and colocalization analysis was done using scan microscope (TCS SP5 ABOS; LEICA confocal systems, Wetzlar, Germany). Random fields were then photographed. Images were analyzed (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Immunofluorescence and confocal microscopy
Hub-7 cells were seeded on sterilized coverslips, 18 h before transfection. Forty-eight hours after transfection, cells were fixed with 4% formaldehyde for 20 min at room temperature. Fixed cells were permeabilized by Triton-X-100 0.5% at room temperature for 30 min and blocked with 5% bovine serum albumin at room temperature for 1 h. Cells were then probed with the indicated primary antibodies overnight at 4°C and then Alexa dye tagged secondary antibody (1:150; Molecular Probes, Oregon, OR) as mentioned for 1 h at room temperature. Coverslips were mounted using 4’,-6-diamidino-2-phenylindole-containing mounting media (Vectashield; VECTOR Laboratories, Burlingame, CA) and observed under confocal laser tag secondary antibody (1:150; Molecular Probes, Oregon, OR) as microscope (TCS SP5 ABOS; LEICA confocal systems, Wetzlar, Germany). Random fields were then photographed. Images were analyzed using LAS AF Lite 2.0.0 software, and colocalization analysis was done using ImageJ 1.41o software.

Luciferase reporter assay
To monitor NF-κB activation, NF-κB-luciferase reporter construct was transfected in HEK-293 cells along with other experimental plasmids. Renilla construct was included as an internal standard for normalization of transfection efficiency. The enzyme activities of firefly and Renilla luciferase were determined in the same cell lysate using the Dual-Luciferase reporter assay system following the manufacturer’s instructions (Promega). Quantitation of luminescent signal was done by luminometre (Mgm Instruments Optocomp I, Hamden, CT).

Reverse transcription–PCR and qRT–PCR
Total RNA was extracted using TRIZOL reagent (Life Technologies, Paisley, UK). One microgram of RNA and random primers were used to perform reverse transcription (RT) according to the manufacturer’s protocol for the RevertAide H Minus First Strand cDNA Synthesis kit (Fermentas, Maryland, MD). PCRs were performed with the RT samples using gene-specific primers from Ambion, Austin, TX) was used as a negative control. The small interfering RNAs were transfected using oligofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Fig. 2. TNF-α induces HBx stabilization through IKKα and IKKβ. (A) Huh-7 cells were cotransfected with F-HBx and green fluorescence protein (GFP) plasmids along with increasing concentration of F-IKKα or F-IKKβ for 48 h, and protein expression was monitored by immunoblotting. Data are representative of three to four independent experiments. (B) HEK-293 cells were transfected with indicated plasmids for 48 h, and HBx transcript level was determined by RT–PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Data are representative of three independent experiments. (C) HEK-293 cells were cotransfected with F-HBx and GFP along with the IKKα and IKKβ kinase-dead plasmids (myc-IKKα-KD, F-IKKβ-KD) for 48 h, then stimulated with TNF-α (10 ng/mL) for 1 h as indicated. Immunoblotting was used to determine the protein expression. Data are representative of three independent experiments.

Fig. 3. p65 is a key player in TNF-α–induced accumulation of HBx. (A) HEK-293 cells were cotransfected with indicated plasmids for 48 h, then harvested for immunoblotting. Data are representative of three independent experiments. (B) HEK-293 cells were transfected with p65 and control luciferase small interfering RNAs for 24 h, then transfected with the indicated plasmids for another 48 h and treated or not with TNF-α (10 ng/mL) for 1 h before harvesting the cells. Immunoblotting was used to determine the protein expression. The data are representative of at least three independent experiments. (C) HEK-293 cells were cotransfected with F-HBx, green fluorescence protein (GFP) and p65-wild type (wt) or a phosphodefective p65-S534A mutant for 48 h, then treated with cyclohexamide (CHX, 20 μg/ml) for different times before immunoblotting analysis. Data are representative of three to four independent experiments.

UUUGCAUGCGCCUUAGAAGCU-5’ and sense 5’-CGUACCGGAAUA CUUCAUU-3’ from Ambion, Austin, TX) was used as a negative control.

Reverse transcription–PCR and qRT–PCR Total RNA was extracted using TRIZOL reagent (Life Technologies, Paisley, UK). One microgram of RNA and random primers were used to perform reverse transcription (RT) according to the manufacturer’s protocol for the RevertAide H Minus First Strand cDNA Synthesis kit (Fermentas, Maryland, MD). PCRs were performed with the RT samples using gene-specific primers for HBx (forward 5’-ATGGCCTGTTAGTGGCTGCT-3’ and reverse 5’-TTAGGCAGAGGTGAAAAATGC-3’) and glyceraldehyde-3-phosphate dehydrogenase (forward 5’-GCCAAAAGGGTCATCATC-3’ and reverse 5’-TGGCCAGTGGCTTCCCGTTC-3’). Real-time PCR was performed using the Express SYBR GreenER quantitative PCR Supermix Universal (Invitrogen). The following primers were used: cIAP2: forward 5’-CTGGGAACCAAAGGATGATG-3’ and reverse 5’-ATTGGAGTCGTGCTGCTG-3’; cIAP2: forward 5’-CCTAAGTAGTTTCCAAAGGTG-3’ and reverse 5’-TGGGCTGTCTGATG-TGGATA-3’; SURVIVIN: forward 5’-GTCTGGACAGAGCAGGT-3’ and reverse 5’-GGAAGGACCCCG-GATGATAC-3’; 18S forward 5’-GGAACAGGACAGATTGACA-3’ and reverse 5’-ACCCACGGAATTGGAAAG-3’. 18S was used for normalization. Relative expression was calculated using the comparative Ct method.
where in first delta-CT values for each sample were obtained by subtracting the values for 18S Ct from the Ct of each tested gene. Then, one difference in delta-CT was calculated as a 2-fold difference in the level of messenger RNA (2−ΔΔCt).

Statistical analysis
Statistical analysis of the data was performed using Student’s t-test, and the results are given as the mean ± standard deviation.

Results
Proinflammatory cytokine TNF-α augments levels of HBx protein by increasing its stability
NF-κB-activating proinflammatory cytokines are commonly secreted during liver carcinogenesis and appear to play a key role in HCC development (37). Based on these findings, we evaluated whether TNF-α could influence the levels of HBx protein. We exposed HEK-293 cells expressing HBx to TNF-α and monitored the expression of HBx both at protein and messenger RNA levels. We found that TNF-α significantly increased the levels of HBx protein (Figure 1A). The amount of HBx accumulated in the cells raised with the increased level of TNF-α, suggesting a direct role of the cytokine in this event (Figure 1A). The increased level of HBx induced by TNF-α is specific, as the level of green fluorescence protein used as a control remains constant. Indeed, >10-fold relative increase of HBx level was obtained in the presence of TNF-α (Figure 1A). Furthermore, kinetics of HBx accumulation in HEK-293 cells was determined by exposing the cells to TNF-α (10 ng/ml), and a significant increase in HBX protein level is observed within 30 min of TNF-α stimulation. Hence, accumulation of HBx upon TNF-α stimulation is an immediate early event (supplementary Figure S1 is available at Carcinogenesis Online). RT–PCR analysis revealed that the level of HBx transcript remained unaffected after TNF-α stimulation (Figure 1B). To rule out the possibility that the observed phenomenon is restricted to a non-hepatic cell line, we repeated similar experiment in human HCC cell line, Huh-7. As observed in HEK-293 cells, TNF-α exposure increased the level of HBx protein in Huh-7 cells as well (Figure 1C). Interestingly, IL-6, another cytokine inducing NF-κB that is upregulated in HCC (38), also increased HBx level in Huh-7 cells (Figure 1C).

Accumulation of protein within the cell is often associated with the increased stability of the protein. To corroborate this assumption, cells expressing HBx were treated with the protein synthesis inhibitor cyclohexamide, in absence or presence of TNF-α and HBx protein levels were monitored at specific intervals. TNF-α stimulation significantly increased the half-life of HBx (Figure 1D). Since HBx is degraded mainly through a proteasome-mediated pathway (11), we asked whether TNF-α prevents this event in HBx stabilization. Treatment with the specific proteasome inhibitor MG132 resulted, as expected, in a greatly increased level of HBx in unstimulated cells (Figure 1E; compare lane 2 with 4). A moderate increase in HBx occurred in cells exposed to TNF-α and treated with MG132 (Figure 1E, compare lane 3 with 5), suggesting a significant decrease in proteasomal degradation of HBx protein upon TNF-α exposure. Thus, TNF-α-induced accumulation of HBx could be partially attributed to an increased stability of the protein due to a decrease in its proteasomal degradation.

TNF-α-induced HBx stabilization requires active IKKα and IKKβ
TNF-α stimulates NF-κB signaling via activation of NF-κB effector kinases IKKα and IKKβ. Therefore, we determined whether TNF-α-induced HBx stabilization was mediated by IKKα or IKKβ. HBx protein was expressed in Huh-7 cells together with IKKα or IKKβ, and the level of HBx was determined by immunoblotting. As shown in Figure 2A, overexpression of both IKKα and IKKβ increased HBx protein levels. Similar results were obtained in HEK-293 cells as well (data not shown). Overexpression of IKKα and IKKβ did not affect the level of HBx transcript (Figure 2B), indicating that as with TNF-α, the increase in HBx protein level is due to increased protein stability. In addition, dominant-negative kinase-dead mutants of IKKα and IKKβ can abrogate HBx accumulation induced by TNF-α (Figure 2C). Moreover, the chemical inhibitor of the IKK complex (Bay-11) also nullified the effect of TNF-α on HBx level (data not shown). Together, these data show that TNF-α-mediated HBx stabilization requires activated IKKα and IKKβ.

NF-κB p65 is required in TNF-α-induced HBx stabilization
The data presented above suggest the role of IKKα and IKKβ in the accumulation of HBx within cells upon TNF-α exposure, and the involvement of downstream NF-κB signaling molecules remains plausible. Thus, we assessed whether activated p65 could play a role in HBx stabilization. As shown in Figure 3A, the overexpression of p65 increased HBx protein levels. Moreover, similar to TNF-α stimulation, p65 overexpression decreased the proteasome-mediated degradation of HBx (data not shown). To further demonstrate the direct role of p65 in HBx stabilization, we used an RNA interference approach to downregulate the expression of endogenous p65 and monitored HBx accumulation induced by TNF-α or IKKs. Knocking down p65 substantially diminished the HBx stabilization induced by TNF-α stimulation (Figure 3B). Similarly, the accumulation of HBx mediated by IKKα or IKKβ is significantly compromised with the reduced expression of p65 (Figure 3B). Taken together, these data strongly suggest that p65 plays a key role in TNF-α-induced HBx accumulation, and this process requires activated IKK complex.

It has been previously reported that IKK complex can phosphorylate p65 at S536 (or S534 in case of mouse p65) (36). Hence, we hypothesized that IKK-phosphorylated p65 induced by TNF-α may in a second step mediate the stabilization of HBx. To test this hypothesis, HBx was expressed in the presence of the wild-type p65 or the non-IKK-phosphorylatable p65-S534A mutant. After cyclohexamide treatment and chase, the level of HBx protein expression was determined. Similar to TNF-α exposure (Figure 1D), p65 wild-type, but
not p65-S534A mutant, significantly increased the stability of HBx after cyclohexamide treatment (Figure 3C). The protein band detected in the p65-S534A mutant line by the phospho-specific antibody (Figure 3C) probably corresponds to the endogenous phospho-p65. From these experiments, we can conclude that the expression of non-phosphorylatable p65-S534A mutant is impaired to prevent the degradation of HBx, in contrast to the wild-type p65. These data indicate that phospho-p65 generated by activated IKK complex is an important downstream effector of HBx stabilization following TNF-α exposure.

Role of NF-κB-regulated genes in TNF-α-induced HBx stabilization

Upon NF-κB activation, NF-κB heterodimers (p65/p50) translocate into the nucleus and activate transcription of a variety of genes, which contains NF-κB-binding sites in their promoters. The stability of HBx observed upon TNF-α stimulation could be mediated directly by p65 or may require transcription of a specific NF-κB-regulated gene(s). To discriminate between these two possibilities, HEK-293 cells were first treated with actinomycin D, a general inhibitor of transcription, and then exposed to TNF-α. The effectivity of actinomycin D was confirmed by RT–PCR analysis of HBx and 1xBx transcripts (data not shown). Thus, there is no absolute requirement of induction of NF-κB-regulated genes for increasing the intracellular level of HBx upon TNF-α stimulation. Hence, p65 appears to play a major role in TNF-α-induced HBx stabilization, with some contribution of NF-κB-regulated genes.

Interaction of HBx with phospho-p65 correlates with its stability

Next, we sought to determine whether p65 forms a complex with HBx, and if the phosphorylation of p65 plays any role in this interaction for HBx stabilization. In agreement with a previous report (39), we showed that p65 indeed interacts with HBx (Figure 5 and supplementary Figure S2 is available at Carcinogenesis Online). The phospho-defective mutant p65-S534A showed lesser affinity to HBx in comparison with wild-type p65. These data indicate that phospho-p65 generated by activated IKK complex is an important downstream effector of HBx stabilization following TNF-α exposure.

Fig. 5. HBx interacts with phospho-p65. (A) and (B) HEK-293 cells were transfected with indicated plasmids for 48 h, then harvested to carry out immunoprecipitation using anti-Flag M2 beads. The input lysates and immunoprecipitated complexes were then analyzed by immunoblotting. The data are representative of at least two to three independent experiments. (C) Huh-7 cells were transfected with indicated plasmids for 48 h and stained for immunofluorescence using mouse-anti-Flag and rabbit-anti-p65 antibodies. (D) Huh-7 cells were stained as in (C) by using rabbit-anti-phospho-p65 (p-p65) antibody. (E) Huh-7 cells were transfected with indicated plasmids for 48 h, then stained as in (C). In all cases, anti-mouse-Alexa-488 (green) and anti-rabbit-alex-555 (red) were used as secondary antibodies to detect p65/p-p65 and F-HBx respectively. Nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI) (blue), and cells were observed under a confocal microscope at ×63 objective. Images in ‘Colocalize (R + G)’ (R for red and G for green) were obtained by using the Image J 1.41 software to better estimate the level of interaction of analyzed proteins. The immunofluorescence images are representative of two to three independent experiments.
TNF-α stabilizes HBx

Positive feedback loop regulation of HBx and NF-κB activation

HBx is reported to activate NF-κB, and this process is known to be important in hepatocellular carcinogenesis. We show here that the main actors in NF-κB signaling, namely the IKK complex and the effector p65 play a key role in the stabilization of HBx. We then checked whether stabilized HBx could enhance the TNF-α or IKK-induced NF-κB activation. To address this issue, NF-κB reporter luciferase assay was carried out in cells singly or cotransfected with HBx and IKKα or IKKβ. As shown in Figure 6A, enhanced NF-κB activation is observed in cells expressing HBx alone with IKKα or IKKβ than individually. Furthermore, NF-κB reporter luciferase assay was also carried out in cells with or without transfected with HBx and treated with TNF-α. As shown in Figure 6B, the presence of HBx enhanced and sustained the effect of TNF-α on NF-κB activation and this correlated with the prolonged accumulation of p65 in the nucleus. Indeed, a substantial amount of p65 is retained activation and this correlated with the prolonged accumulation of HBx after TNF-α removal (supplementary Figure S3A, available at Carcinogenesis Online, compare lane 4 with 8, and quantification in supplementary Figure S3B, available at Carcinogenesis Online). Finally, transcript levels of key NF-κB-regulated genes cIAP1, cIAP2 and SURVIVIN were determined in cells with or without transfected with HBx and treated with TNF-α. Similar to the luciferase reporter assay in Figure 6A, cooperation was observed between HBx and TNF-α for induction of NF-κB-regulated survival genes cIAP1, cIAP2 and SURVIVIN, with a lesser extend for cIAP1 (Figure 6C). Thus, a positive feedback loop exists between HBx and TNF-α and this cooperation may account in progression of HBV-induced HCC.

Discussion

HBx is a multifunctional protein essential for viral replication and a key player in virus-induced HCC (15). However, the mechanisms by which HBx affects cellular signaling in the process of liver carcinogenesis are not clearly elucidated. HBx is a highly unstable protein and is ubiquitinated and degraded by proteasome complex (11). Ubiquitination-independent proteasomal degradation of HBx has also been reported (42). Several studies have documented the effects of cellular proteins on the stability of HBx protein. p53 and Id-1 are thought to facilitate proteasomal turnover of HBx (34,43,44), whereas Pin1 and DDB1 directly interact and increase stability of HBx (35,45). On the other hand, HBx can induce NF-κB activation, and this event could contribute to HBx-mediated tumor development and progression but may have a direct impact on HBx protein and functions as well.
Fig. 7. Proposed model for interplay between HBx and NF-κB signaling pathway. HBV infection results in liver damage as a result of chronic inflammation triggered by the immune system. Integrated viral genome expresses HBx that activates several signaling pathways, including NF-κB, and leads to secretion of proinflammatory cytokotkines. HBx-mediated NF-κB signaling includes activation of IKK complex that phosphorylates p65. Phosphorylated p65 (p-p65) binds to HBx and stabilizes it by protecting it from proteasome-mediated degradation. Other proteins could possibly be recruited to the complex to increase the level of HBx. This increase in HBx level, together with secreted cytokotkines, could further activate NF-κB and this event may accelerate HBV-induced liver tumors.

We demonstrated in this report that TNF-α, a potent activator of NF-κB signaling, increases the intracellular concentration of HBx by increasing its stability (Figure 1D). Notably, IL6 also potentiates HBx stabilization (Figure 1C). IKK complex seems to play an active role in the TNF-α effects as overexpression of kinase dead mutants of IKKα and IKKβ overcomes the effect of TNF-α on HBx (Figure 2C). NF-κB activation reduces proteasomal degradation of HBx (Figure 1E), which could be due to direct protein–protein interaction between HBx and components of NF-κB signaling pathway molecules or modification of HBx by NF-κB signaling pathway. A potential role of NF-κB responsible genes in this event is also plausible. To elucidate the downstream effector of NF-κB signaling in HBx stability, we showed that p65 induced increased levels of HBx similar to TNF-α and IKK (Figure 3A). Furthermore, inhibiting endogenous p65 expression prevents TNF-α and IKK effects on HBx stabilization, providing evidence for the direct involvement of p65 in this event (Figure 3B). In addition, blocking expression of NF-κB-inducible genes by actinomycin D treatment partially inhibits TNF-α effects on HBx stabilization (Figure 4). Hence, stabilization of HBx by TNF-α is mainly mediated directly by p65, with some contributions of NF-κB-inducible genes. Taken together, these data raise the possibility that TNF-α, through IKK, utilizes p65 for HBx stabilization. Indeed, activated IKK by TNF-α or other NF-κB activation inducers phosphorylate p65, and this phosphorylation is known to enhance the transactivation activity of p65. We showed that in contrast to wild-type p65, the IKK-unphosphorylatable p65 mutant is impaired from inducing HBx stabilization (Figure 5C). Furthermore, non-phospho-p65 mutant has less affinity with HBx in comparison with wild-type or phospho-mimic p65 (Figure 5A and B). Thus, phospho-p65 generated following activation of IKK by TNF-α is an important factor for HBx stabilization. As HBx is prone to proteasomal degradation, binding of phospho-p65 to HBx could provide a protective effect and prevent HBx-induced degradation. Likewise, p65 is also directly involved in the stability of other proteins like cyclin D1 (46) and 1κBz (47). However, it is not known at this point whether this HBx-phospho-p65 dimer also recruits additional proteins to enhance HBx stability, which is not possible via the HBx-non-phospho-p65 dimer and this is an interesting area for further investigation. The increased level of HBx further increases activation of NF-κB in a second step. Our data corroborate with a recent study wherein Bui-Nguyen et al. (39) demonstrated that HBx interacts with p65, and the HBx/p65 complex increases transcriptional activity of p65.

The synergy between HBx and TNF-α or IKK in NF-κB signaling is demonstrated from the luciferase reporter assay for NF-κB activation, wherein HBx works cooperatively with IKKα and IKKβ toward NF-κB activation and prolongs the effect of TNF-α (Figure 6). In accordance with previous reports (39,48), cells expressing HBx exhibit ~2-fold activation in NF-κB level, which is lower compared with activation by TNF-α stimulation (10- to 20-fold). However, the presence of HBx can prolong the effect of TNF-α in the cells upon stabilization as a positive feedback mechanism, and this increased level of HBx could prevent reassociation of 1κBz to NF-κB by directly sequestering the inhibitor (25). This is shown in our study, where cells expressing HBx exhibit longer retention of p65 in the nucleus after TNF-α exposure compared with untransfected cells (supplementary Figure S3 is available at Carcinogenesis Online). This observation is corroborated by an increased expression of survival genes regulated by NF-κB (Figure 6C). Furthermore, HBx also cooperates with ethanol toward activation of hepatic NF-κB and involves TNF-α receptor 1 (49). Thus, it appears that cytokotkines such as TNF-α and IL6 that also induce HBx stabilization could contribute to HCC development in addition to their pro-inflammatory effects by inducing HBx stabilization, which in turn activates NF-κB, another contributing factor of liver cancer progression (Figure 7). It is worth noting that our model is basically generated from experimental conditions using overexpressed HBx protein as HBx is barely detectable in HBV-positive HCC cell lines due to the instability of the protein and exogenous expression of HBx is the most common way of studying the protein. Nevertheless, TNF-α, IKK or p65 significantly increase the steady state level of HBx. Moreover importantly, knocking down the expression of the endogenous p65 affects dramatically the stabilization of HBx by TNF-α or IKK and this account in the physiological role of p65 in HBx stability.

Overall, the present study highlights the cooperation between HBx and NF-κB signaling that may play a pivotal role in initiation and development of HCC, thus underscoring the importance of NF-κB as a therapeutic target for HBV-induced HCC. Anti-TNF-α therapy is now being used to treat a wide range of autoimmune and inflammatory disorders, including chronic HBV infections (50). The present study supports the development of TNF-α antagonists for HBV therapy; however, its safety should be evaluated and proper guidelines for a clinical approach should be set.

Supplementary material
Supplementary Figures S1–S3 can be found at http://carcin.oxfordjournals.org/


