Hepatitis B Virus Core Protein Sensitizes Hepatocytes to Tumor Necrosis Factor-Induced Apoptosis by Suppression of the Phosphorylation of Mitogen-Activated Protein Kinase Kinase 7

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ABSTRACT

Hepatitis B, which caused by hepatitis B virus (HBV) infection, remains a major health threat worldwide. Hepatic injury and regeneration from chronic inflammation are the main driving factors of liver fibrosis and cirrhosis in chronic hepatitis B. Proinflammatory tumor necrosis factor alpha (TNF- α) has been implicated as a major inducer of liver cell death during viral hepatitis. Here, we report that in hepatoma cell lines and in primary mouse and human hepatocytes, expression of hepatitis B virus core (HBc) protein made cells susceptible to TNF- α -induced apoptosis. We found by tandem affinity purification and mass spectrometry that receptor of activated protein kinase C 1 (RACK1) interacted with HBc. RACK1 was recently reported as a scaffold protein that facilitates the phosphorylation of mitogen-activated protein kinase kinase 7 (MKK7) by its upstream activators. Our study showed that HBc abrogated the interaction between MKK7 and RACK1 by competitively binding to RACK1, thereby downregulating TNF- α -induced phosphorylation of MKK7 and the activation of c-Jun N-terminal kinase (JNK). In line with this finding, specific knockdown of MKK7 increased the sensitivity of hepatocytes to TNF- α -induced apoptosis, while overexpression of RACK1 counteracted the proapoptotic activity of HBc. Capsid particle formation was not obligatory for HBc proapoptotic activity, as analyzed using an assembly-defective HBc mutant. In conclusion, the expression of HBc sensitized hepatocytes to TNF- α -induced apoptosis by disrupting the interaction between MKK7 and RACK1. Our study is thus the first indication of the pathogenic effects of HBc in liver injury during hepatitis B.

IMPORTANCE

Our study revealed a previously unappreciated role of HBc in TNF- α -mediated apoptosis. The proapoptotic activity of HBc is important for understanding hepatitis B pathogenesis. In particular, HBV variants associated with severe hepatitis may upregulate apoptosis of hepatocytes through enhanced HBc expression. Our study also found that MKK7 is centrally involved in TNF- α -induced hepatocyte apoptosis and revealed a multifaceted role for JNK signaling in this process.

Hepatitis B virus (HBV) infection remains a major public health threat, affecting an estimated 400 million individuals worldwide, with high risk of these individuals developing severe liver diseases, including cirrhosis and hepatocellular carcinoma. The host protective immune response is also responsible for liver pathogenesis during HBV infection.

Proinflammatory tumor necrosis factor alpha (TNF- α) is critical for controlling HBV in clinical settings and in model systems, possibly by destabilizing cytoplasmic viral nucleocapsids and reducing nuclear viral DNA (1, 2). Recently, TNF- α was reported to reduce host cell susceptibility to HBV entry via activation-induced cytidine deaminase (3). On the other hand, TNF- α has also been implicated as a mechanism of hepatic injury through induction of cellular apoptosis during viral hepatitis (4–9). Apoptosis is organized, genetically controlled programmed cell death. Clearance of apoptotic debris stimulates transforming growth factor β expression and induces collagen I secretion by hepatic stellate cells. These processes have been suggested as the core machinery for liver fibrogenesis and cirrhosis (10, 11).

Overexpressing HBV core (HBc), precore, X, and small and middle envelope proteins (S2S), as well as viral replication, are not associated with liver disease in transgenic mouse models (12). Hence, HBV itself is not considered to be directly cytopathic for infected hepatocytes. This notion has to be reexamined, however,

in a pathophysiological setting. Of particular interest, naturally occurring mutations within the basic core promoter of HBV are implicated in severe liver diseases, including fulminant hepatitis, which results in up to 15-fold-increased HBc synthesis (13–17). The fulminant-hepatitis-associated mutant induces apoptosis in primary hepatocytes, and this effect is not reversed by inhibition with nucleoside analogs (15). This finding suggests that HBc

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FIG 1 Schema of TNF- α signaling pathway that induces apoptosis. TNF- α is capable of inducing apoptosis when NF- κ B signaling is blocked (24–26, 34, 46). Upon binding to TNF receptor I, a protein complex, complex I, forms rapidly; this complex activates NF- κ B and JNK signaling pathways. Complex I undergoes ligand-dissociated internalization with formation of complex II. Complex II is composed of FADD and procaspase 8 and triggers apoptosis if NF- κ B signaling fails to induce expression of antiapoptotic genes. JNK is an important regulator of TNF- α -mediated apoptosis (24–28) and is activated via the sequential activation of protein kinases, including two MAP kinase kinases (MKK4 and MKK7) and multiple MAP kinase kinase kinases (MAP3Ks). MKK7 is an essential component of the JNK signaling pathway activated by TNF- α . RACK1 is a scaffold protein that facilitates phosphorylation of MKK7 by upstream MAP3Ks (19). It is widely reported that sustained JNK activation mediates TNF- α -induced cell death, although in hepatocytes JNK has both proapoptotic and antiapoptotic aspects (25, 26). We propose that HBC (red) disrupts interactions between MKK7 and RACK1, rendering cells susceptible to TNF- α -mediated apoptosis.

might be directly responsible for virus-induced hepatocyte death, but the underlying mechanism remains undefined.

HBC is a 21-kDa viral structural protein with an N-terminal domain that is responsible for viral capsid assembly and a C-terminal arginine-rich polypeptide that interacts with HBV pre-genomic RNA. HBC is found to be distributed in both the nucleus and the cytoplasm in HBV-producing hepatocytes and transgenic mice. Clinically, a nucleus-dominant distribution of HBC is associated with minor hepatitis activity, while a cytoplasmic distribution of HBC is associated with chronic active liver disease (18). Here, we report that expression of HBC increased susceptibility of hepatocytes to TNF- α -induced apoptosis by disrupting the interaction between receptor of activated protein kinase C 1 (RACK1) and mitogen-activated protein kinase kinase 7 (MKK7) (19), leading to downregulation of TNF- α -induced phosphorylation of MKK7 and c-Jun N-terminal kinase (JNK) (Fig. 1). Our results therefore revealed a previously unappreciated role for HBC in liver pathogenesis during viral hepatitis.

MATERIALS AND METHODS

Plasmid DNA and viral vectors. Plasmids encoding HBV polymerase, X protein, S2S, and HBC, as well as hemagglutinin (HA)-tagged RACK1 and Flag-tagged MKK7, were constructed by inserting the appropriate genes into the multiple-cloning site (MCS) of pcDNA3.1(–) (Invitrogen). Genes encoding His-tagged RACK1 or His-tagged HBC were subcloned into the MCS of pET-30a(+) (Novagen) for prokaryotic protein expression. For GST pulldown assay, plasmids encoding GST-tagged HBC truncations and GST-tagged RACK1 truncations were constructed by inserting the appropriate genes into the MCS of pGEX-4T-1 (Promega).

Recombinant adenovirus (rAd) expressing HBC (rAd-HBC/GFP, with a green fluorescent protein [GFP] gene separated by an internal ribosome

entry site [IRES] sequence) was constructed by Obio Technology (Shanghai), using human rAd vector of serotype 5 in which E1 had been deleted. The lentiviral vector expressing HA-tagged RACK1 was constructed by inserting the sequence into the MCS of pCDH-CMV-MCS-EF1-Puro (SBI) and packaging the expression constructs into pseudoviral particles.

siRNAs. Small interfering RNA (siRNA) oligonucleotides against human and mouse MKK7 were synthesized by GenePharma: siMKK7-1, 5'-CAGGACAGUUCCCUACAATT-3', and siMKK7-2, 5'-GCAAGAU GACAGUGGCGAUTT-3'. siRNAs were transfected into cells by Lipofectamine 2000 (Invitrogen). Subsequent treatments were performed 48 h after transfection.

Cells. Huh-7, HepG2, HepG2.2.15, and 293T cells were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Primary mouse hepatocytes (PMH) were isolated from male C57BL/6 mice as described below. Primary human hepatocytes (PHH) were from Mucyte Biotech (Nanjing). PMH and PHH were cultured in Williams' medium E containing 10% FBS, 2 mM L-glutamine, 1 $\times$ nonessential amino acids, and 1% penicillin-streptomycin. HepG2 or Huh-7 cells constitutively expressing HBC were constructed by transfection of cells with pcDNA3.1-HBC and selection with 50 μ g/ml of Geneticin.

Stimulation with TNF- α . To induce massive apoptosis, Huh-7 or HepG2 cells were plated into 12-well plates at 0.25 to 0.5 million cells per well. The next day, cells were pretreated with 0.5 μ g/ml of actinomycin D (Sigma) for 30 min and cultured for 14 h with 100 ng/ml of human TNF- α (hTNF- α ; PeproTech) in the presence of 12 μ M (for Huh-7 cells) or 20 μ M (for HepG2 cells) Bay11-7082 (Sigma). For cotreatment with pyrrolidine dithiocarbamic acid (PDTC; Beyotime), 50 μ M PDTC and 10 ng/ml of hTNF- α were used (for HepG2 cells).

Stimulation with TNF- α in PMH and PHH was done as described previously (20, 21). In brief, cells were pretreated with 0.5 μ g/ml of actinomycin D for 30 min and cultured with 2 ng/ml of murine TNF- α (mTNF- α ; PeproTech) (for PMH) or 100 ng/ml of hTNF- α (for PHH) in the presence of 0.5 μ g/ml of actinomycin D for 14 h.

Isolation of PMH. PMH were isolated from C57BL/6 male mice by a two-step collagenase perfusion technique (22). The study was approved by the Animal Ethics Committee of Institut Pasteur of Shanghai (no. A2012008-2). Living hepatic parenchymal cells were separated by 35% Percoll. After seeding for 4 to 6 h, cells were refreshed with medium and cultured overnight. The next day, cells were transduced with rAd-HBC/GFP or a control rAd vector (rAd-GFP) at a multiplicity of infection (MOI) of 20. After culture for 2 days, cells were treated with mTNF- α as described above.

Apoptosis analysis. Apoptosis was assessed 14 h after stimulation with TNF- α , using PE Annexin V Apoptosis Detection Kit I (BD; catalog no. 559763) and the PE Active Caspase-3 Apoptosis Kit (BD; 550914) according to the manufacturer's protocol.

Immunoblotting. Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% Triton X-100) containing 1 $\times$ protease inhibitor cocktail (Roche) and 1 $\times$ PhosSTOP phosphatase inhibitor cocktail (Roche). Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting. Antibodies against phospho-JNK (Thr183/Tyr185), JNK, and MKK7 were from Cell Signaling Technology (9251, 9252, and 4172, respectively). Anti-phospho-MKK7 (Thr275) was from Sigma (SAB4504624). Anti-RACK1 was from BD (610178). Anti-HBC was from Dako (B0586). Antibodies against cleaved caspase 3 and poly(ADP-ribose) polymerase (PARP) were from Cell Signaling Technology (9661 and 9542, respectively).

Tandem affinity purification. HepG2 cells stably expressing HBC fused with C-terminal Strep and Flag tags were lysed in RIPA buffer containing 1 $\times$ protease inhibitor cocktail. Tandem affinity purification (TAP) of Strep- and Flag-tagged HBC was performed by two rounds of affinity purification as previously described (23). Eluates were analyzed by

SDS-PAGE and mass spectrometry (Shanghai Applied Protein Technology).

Coimmunoprecipitation (co-IP) and GST pulldown. Cells were lysed in RIPA buffer containing 1× protease inhibitor cocktail for 1 h on ice, with brief vortexing every 10 min. Lysates were incubated with 1 μg of antibody at 4°C overnight, with gentle rotation. After centrifugation, supernatants were incubated with protein A/G beads (Life Technologies) at 4°C for 1 h. The immunoprecipitates were separated by SDS-PAGE for immunoblotting.

Glutathione S-transferase (GST) fusion proteins or His-tagged proteins were expressed in *Escherichia coli* BL21 and purified using glutathione-Sepharose 4B (GE Healthcare) or nickel affinity resins (Qiagen) according to the manufacturer's instructions. For the pulldown assays, GST fusion proteins were immobilized on glutathione beads and incubated with purified His-tagged proteins in RIPA buffer containing 0.5% bovine serum albumin (BSA) at 4°C overnight, with gentle rotation. Bound proteins were analyzed by SDS-PAGE and immunoblotting.

Immunofluorescence assay. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 15 min, followed by permeabilization with 0.1% Triton X-100. After blocking with 5% goat serum, cells were incubated with 1:250 anti-RACK1 (BD; 610178) and 1:200 anti-HBc (Dako; B0586) in PBS containing 1% goat serum and 0.05% Triton X-100 at 4°C overnight. Alexa Fluor 555 goat anti-mouse antibody and Alexa Fluor 488 goat anti-rabbit antibody (Life Technologies) were used as secondary antibodies for anti-HBc and anti-RACK1 staining, respectively.

Statistics. Data are expressed as means ± standard errors of the means (SEM). Student's unpaired *t* tests were performed with GraphPad Prism software.

RESULTS

HBc increases TNF-α-induced apoptosis in hepatoma cells and primary hepatocytes. TNF-α is implicated in hepatic injury from a variety of liver diseases. In the presence of the NF-κB inhibitor Bay11-7082 (see Fig. S1 in the supplemental material), stimulation with TNF-α significantly increased the percentage of annexin V-binding HepG2.2.15 cells compared with HBV-free HepG2 cells. Treatment with lamivudine did not significantly alter annexin V binding to HepG2.2.15 cells, suggesting that the viral replication had little effect on TNF-induced apoptosis (Fig. 2A and B).

Upon TNF-α/Bay11-7082 treatment, annexin V binding increased in Huh-7 cells that were constitutively expressing HBc but not in cells with expression of the other viral proteins (Fig. 2C). Expression of all these viral proteins could be readily detected by specific antibodies (data not shown), although they were likely to be at different levels. Mutant assays on the HBV genome also revealed that HBc might be directly responsible for increased apoptosis in HBV-producing cells (Fig. 2D). In either HepG2 cells (Fig. 2E) or Huh-7 cells (data not shown), ectopic expression of HBc increased the production of active caspase 3, a typical marker of apoptosis.

rAd vectors were constructed for transduction assays in primary mouse or human hepatocytes. In the presence of actinomycin D (20, 21), an inhibitor of *de novo* gene transcription, TNF-α treatment upregulated active caspase 3 and increased apoptosis in the hepatocytes transduced with rAd-HBc/GFP, compared with that in rAd-GFP transduced cells (Fig. 2F and G). Collectively, these data indicated that HBc promoted TNF-α-induced apoptosis in cultured hepatoma cells and primary hepatocytes.

HBc downregulates TNF-α-induced MKK7 and JNK phosphorylation. TNF-α is a conditional death ligand. Its apoptotic

capability is largely modulated by the NF-κB and JNK signaling cascades (Fig. 1). HBc did not affect NF-κB signaling pathway activity, as measured by reporter assays (Fig. 3A). However, TNF-α-induced phosphorylation of MKK7 and downstream JNK activation were substantially suppressed in HepG2 (Fig. 3B) and Huh-7 (Fig. 3C) cells that constitutively expressed HBc compared to those in HBc-negative cells. TNF-α also induced phosphorylation of MKK4 in Huh-7 cells, but this was not affected by HBc (Fig. 3C). The inhibitory effect of HBc on TNF-α-induced MKK7 and JNK activation occurred also in the context of an intact HBV genome. TNF-α-stimulated MKK7 and JNK activation was reduced in HepG2 cells expressing the 1.2-mer overlength HBV genome, but not in cells with an HBc-null mutant genome (Fig. 3D). These data clearly indicated that expression of HBc blocked TNF-α-stimulated activation of MKK7 and JNK.

Proapoptotic activity of HBc is mediated by blockage of MKK7 phosphorylation. TNF-α alone stimulated rapid, transient activation of JNK (within 1 h) (Fig. 3) and does not cause apoptosis (data not shown). In the presence of the NF-κB inhibitor, however, TNF-α stimulation induced prolonged phosphorylation of MKK7 and JNK (Fig. 4A). This prolonged JNK activation is thought to be essential in regulating TNF-induced apoptosis, although its precise role in this process remains controversial (24–28). While the activation of MKK7 and JNK decreased from hours 8 to 12, cleaved caspase 3 and PARP were detected as early as 4 h after stimulation, indicating the onset of programmed cell death (Fig. 4A). Of particular interest, the prolonged MKK7 and JNK activation was remarkably suppressed (mainly limited to 1 to 4 h after stimulation) in HBc-expressing HepG2 cells that underwent significantly enhanced apoptosis (Fig. 4A).

We then tested whether inactivation of MKK7 and JNK was directly responsible for TNF-α-induced apoptosis. This was achieved by knockdown of MKK7 expression through RNA interference. HepG2 cells transfected with MKK7-specific siRNAs had increased apoptosis upon stimulation with TNF-α (Fig. 4B). Similar results were obtained by knockdown of MKK7 expression in mouse primary hepatocytes (Fig. 4C). Collectively, these data suggested that the proapoptotic activity of HBc was achieved by blocking MKK7 activation.

RACK1 is a cellular protein that interacts with HBc. To understand the mechanism of the proapoptotic activity of HBc, we performed tandem affinity purification to identify potential cellular interacting proteins. RACK1, a multifaceted scaffold protein, was identified as a candidate for HBc interaction by mass spectrometry. In 293T cells, HA-tagged RACK1 coimmunoprecipitated with Flag-tagged HBc, and vice versa (Fig. 5A and B). In HepG2.2.15 cells that stably produce HBV, endogenous RACK1 coimmunoprecipitated with HBc (Fig. 5C). Consistent with their physical interaction, both HBc and endogenous RACK1 were distributed throughout the cytoplasm of HepG2.2.15 cells (Fig. 5D).

HBc has an N-terminal domain (amino acids [aa] 1 to 149) and a C-terminal arginine-rich polypeptide (aa 150 to 183) (29), while RACK1 contains 7 Trp-Asp (WD) repeats (30). Interaction between HBc and RACK1 was further verified by GST pulldown assay. In particular, His-tagged RACK1 coprecipitated with the GST-fused HBc N-terminal domain but not the HBc C-terminal polypeptide (Fig. 5E), while His-tagged HBc coprecipitated only with GST-fused RACK1 WD6-7 and not WD1-3 or WD1-5 (Fig. 5F and G). Neither GST-fused RACK1 WD6 nor WD7 pulled down His-tagged HBc (Fig. 5G). These data indicated that RACK1

FIG 2 HBc confers susceptibility to TNF- α -induced apoptosis in hepatoma cells and primary hepatocytes. (A) (Left) Annexin V staining of HepG2 or HepG2.2.15 cells 14 h after cotreatment with TNF- α and Bay11-7082. Cells treated with Bay11-7082 alone, or mock treated, were used as controls. To block HBV replication, HepG2.2.15 cells were pretreated with 20 μ g/ml of lamivudine (LAM) for 48 h. (Right) Representative flow cytometry analysis of cells treated with TNF- α and Bay11-7082. Cells were doubly stained with 7-amino actinomycin D (7-AAD) and annexin V. Annexin V-positive populations were calculated. (B) The top blot is a Southern blot of HBV replication in HepG2, HepG2.2.15, and LAM-treated HepG2.2.15 cells. Replication intermediates of HBV DNA are relaxed circular (RC), double-stranded linear (DSL), and single strand (SS). The bottom blot is an immunoblot for HBc detection. (C) Annexin V staining of Huh-7 cells stably transfected with a control plasmid or the plasmid encoding HBc, polymerase (Pol), X protein (HBx), or S2S proteins of HBV. Cells were treated with TNF- α and Bay11-7082 as described for panel A. (D) Annexin V staining of HepG2 cells stably transfected with a 1.2-mer genome of HBV (payw1.2) or mutant HBV genome deficient in HBc and HBc (payw1.2-e/C-null). Mock-transfected cells were used as controls. (E) (Left) flow cytometry of active caspase 3 expression after TNF- α and Bay11-7082 treatment in HepG2 cells stably transfected with a control plasmid or the plasmid encoding HBc (pcDNA3.1-HBc). (Right) representative flow cytometry analysis of cells treated with TNF- α and Bay11-7082. Cotreatment with TNF- α and the NF- κ B inhibitor PDTC was also tested, with similar results (see Fig. S2 in the supplemental material). (F and G) PMH (F) or PHH (G) were transduced with rAd-HBc/GFP, or rAd-GFP as a control, for 48 h at an MOI of 20:1. Cells were then treated with mouse or human-derived TNF- α and actinomycin D (ActD), with active caspase 3 expression analyzed by flow cytometry. The error bars indicate SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ns, no significance.

FIG 3 HBc suppresses TNF- α -stimulated MKK7 and JNK phosphorylation. (A) HepG2 cells were cotransfected with pcDNA3.1-HBc, or a control plasmid, together with a NF- κ B luciferase reporter vector. Luciferase activity was measured after stimulation with TNF- α for 12 h. (B and C) Effects of HBc on TNF- α -induced JNK, MKK7, and MKK4 phosphorylation in HepG2 (B) and Huh-7 (C) cells at indicated time points. Cells were mock transfected or stably transfected with pcDNA3.1-HBc. JNK protein has two alternatively spliced forms. p-JNK, phosphorylated JNK; p-MKK7, phosphorylated MKK7. (D) Immunoblot analysis of TNF- α -induced MKK7 and JNK phosphorylation in HepG2 cells stably transfected with payw1.2 or payw1.2-e/C-null. Mock-transfected cells were used as controls. The error bars indicate SEM. ns, no significance. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

bound the HBc N-terminal domain directly through the WD6-7 region, probably through the RACK1 knob structure between the WD6 and WD7 domains (30).

HBc proapoptotic activity is independent of capsid particle formation. HBc is a structural protein of HBV and assembles into

icosahedral capsid particles. We thus investigated if free monomeric or dimeric protein or the capsid particle accounted for HBc proapoptotic activity.

We used an HBc mutant with a tyrosine-to-alanine substitution at aa 132 (Y132A). This mutation prevents HBc from self-

FIG 4 Blocking MKK7 phosphorylation sensitizes hepatocytes to TNF- α -induced apoptosis. (A) (Top) Immunoblot showing HbC effects on sustained activation of MKK7 and JNK induced by TNF- α /Bay11-7082 cotreatment of HepG2 cells at indicated time points. Cells were mock transfected or stably transfected with the HbC-expressing plasmid. Apoptosis markers cleaved caspase 3 and cleaved PARP were detected. Nonspecific signal in the lowest blot was from the prestained protein ladder (Thermo Scientific; 26616). (Bottom) Signal intensities of p-JNK, p-MKK7, cleaved caspase 3, and cleaved PARP at the indicated time points were measured by densitometry. Data were first normalized to GAPDH and then compared with those from HbC-negative HepG2 cells (circles) at hour 1 (for p-JNK and p-MKK7), or with those from HbC-expressing cells (squares) at hour 12 (for cleaved caspase 3 and cleaved PARP) to calculate the relative densities. The error bars indicate the relative densities of protein species from three independent experiments (Fig. S3 in the supplemental material shows results of a repeated experiment with similar results). (B) Flow cytometry of percentage of annexin V-binding HepG2 cells 14 h after TNF- α and Bay11-7082 treatment. Expression of MKK7 was knocked down by specific siRNAs (siMKK7-1 and siMKK7-2) for 48 h before TNF- α stimulation. Scrambled siRNA was the control. (C) An experiment performed with PMH treated with mTNF- α and actinomycin D. Scrambled siRNA or siMKK7-1 was used. The error bars indicate SEM. ***, $P < 0.001$.

assembly into capsid particles but retains the ability to form homodimers (31, 32). As revealed by native gel electrophoresis assays, ectopically expressed wild-type HbC formed capsid particles that were absent in cells transfected with the HbC mutant (Fig. 6A). In contrast to previous reports (1, 2), TNF- α treatment, with or without NF- κ B inhibitor, did not significantly reduce capsid particles in the cytosol. This result probably reflected a difference between the genuine nucleocapsids and the empty capsids that lack HBV DNA/RNA (33). Expression of HbC Y132A was detected by SDS-PAGE with immunoblotting, albeit with a relatively lower level than that of wild-type HbC. In the presence of TNF- α and Bay11-7082, expression of the assembly-defective HbC mutant displayed cytotoxicity similar to that of wild-type HbC (Fig. 6B). In this regard, the HbC mutant engaged in a direct interaction with

RACK1 (Fig. 6C). These data clearly suggested that the proapoptotic activity of HbC was independent of capsid formation.

HbC and MKK7 bind RACK1 competitively. RACK1 is reported to interact with MKK7 through its WD6-7 region, which facilitates MKK7 phosphorylation by upstream mitogen-activated protein kinase kinases (MAP3Ks) (19). This suggested that HbC and MKK7 might bind RACK1 competitively.

RACK1-MKK7 interaction was confirmed in our study by a GST pulldown assay. His-tagged RACK1 coprecipitated with the GST-fused MKK7, which, however, was largely blocked in a dose-dependent manner by coinubation with purified His-tagged HbC (Fig. 7A). In 293T cells expressing Flag-tagged-MKK7 (MKK7-Flag), coexpression of HbC led to a significant decrease in the amount of MKK7-Flag-coprecipitating RACK1 (about 4-fold,

FIG 5 RACK1 binds HBc. (A and B) Interaction between ectopically expressed Flag-tagged HBc (HBc-Flag) and HA-tagged RACK1 (RACK1-HA) in 293T cells by coimmunoprecipitation with anti-Flag (A) or anti-HA (B). (C) Interaction between endogenous HBc and RACK1 in HepG2.2.15 cells by coimmunoprecipitation with anti-HBc. HepG2 cells not expressing HBc were controls. (D) Cellular distribution of HBc and RACK1 in HepG2.2.15 cells by immunofluorescence, DAPI, 4',6-diamidino-2-phenylindole. (E) Mapping of the HBc RACK1-binding region. GST fusions of HBc N-terminal domain (GST/HBc-N), C-terminal polypeptide (GST/HBc-C), and the intact HBc (GST/HBc) were expressed in *E. coli* and assayed by GST pulldown with His-tagged RACK1 (RACK1-His). (F and G) Mapping of the HBc-docking site of RACK1. GST fusions of RACK1 truncations were expressed in *E. coli* and assayed by GST pulldown with His-tagged HBc (HBc-His). Higher-molecular-weight bands in lanes with GST/WD1-3 and GST/WD1-5 (F) might be aggregated truncated proteins. IB, immunoblotting.

Fig. 7B). Moreover, in HepG2 cells with high endogenous expression of MKK7, RACK1 and transforming growth factor β -activated kinase 1 (TAK1, an MAP3K that phosphorylates MKK7 upon TNF- α stimulation) coimmunoprecipitated with MKK7. Those interactions were barely detectable when HBc was expressed (Fig. 7C). The proteins RACK1, TAK1, MKK7, and JNK were all detected in PHH, but at lower levels than in HepG2 or Huh-7 cells (see Fig. S4 in the supplemental material). Our results therefore indicated that HBc disrupted interaction between

MKK7 and RACK1, which might abrogate TNF- α -mediated MKK7 or JNK activation.

Proapoptotic activity of HBc is reversed by RACK1 overexpression. Competitive binding to RACK1 thus appeared to be the means by which HBc affected TNF- α -induced apoptosis. This hypothesis was verified by overexpression of RACK1 to counteract the effect of HBc. As shown in Fig. 8A, expression of HBc in HepG2 cells resulted in reduced MKK7 and JNK phosphorylation upon TNF- α stimulation, which, however, was reversed by ecto-

FIG 6 Capsid particle formation is not obligatory for the proapoptotic activity of HBc. (A) Immunoblot of wild-type HBc or the assembly-defective HBc mutant (Y132A) in cytoplasmic lysates from stably transfected HepG2 cells. Lysates were separated by native agarose gel electrophoresis (top) or SDS-PAGE (middle), followed by immunoblotting of capsid particles or total HBc protein. GAPDH was used as a loading control (bottom). Cells were mock treated, treated with TNF- α and Bay11-7082, or treated with TNF- α only. HBc-negative HepG2 cells were used as controls. (B) Flow cytometry of annexin V-stained HepG2 cells or cells expressing HBc or HBc Y132A, 14 h after treatment with TNF- α and Bay11-7082. The error bars indicate SEM. ***, $P < 0.001$. (C) Interaction between ectopically expressed HBc Y132A and endogenous RACK1 in HepG2 cells by coimmunoprecipitation with anti-HBc.

pic expression of HA-tagged RACK1 in these cells through lentiviral transduction. Moreover, in the presence of NF- κ B inhibitor Bay11-7082 (data not shown) or PDTC (Fig. 8B), overexpression of RACK1 prevented the upregulation of TNF- α -induced apoptosis by HBc in these cells. These data suggested that HBc suppressed TNF- α -mediated MKK7 and JNK activation and promoted apoptosis mainly through RACK1 blocking.

DISCUSSION

TNF- α mediates both cell survival and cell death signaling through TNF receptor 1, by activation of NF- κ B and caspase 8, respectively (Fig. 1). Alteration in NF- κ B signaling largely accounts for the change in TNF- α function from antiapoptotic to proapoptotic. However, the underlying mechanism and the physiological relevance of the NF- κ B signaling alteration are poorly understood. Nevertheless, TNF-mediated apoptosis can be experimentally induced in the setting of global transcriptional or translational arrest or selective inactivation of NF- κ B (25, 26, 34). Our study took advantage of these experimental conditions, based on previous studies by others, to investigate the potential role of HBc in TNF- α -induced apoptosis. Bay11-7082 has been widely used as a tool compound that inhibits NF- κ B activation. In our study, NF- κ B inhibitor PDTC was also used in order to avoid possible off-target effects of Bay11-7082 (see Fig. S2 in the supplemental material). However, both Bay11-7082 and PDTC are thought to be multitarget-directed compounds, and the mechanisms whereby these compounds affect NF- κ B signal transduction are not well understood.

The role of TNF- α must be carefully examined in different pathophysiological settings. For example, TNF- α is implicated in HBV clearance via noncytotoxic mechanisms in which TNF- α -stimulated NF- κ B activation is indispensable (1–3). On the other hand, we propose a scenario for the cytotoxic function of TNF- α (8, 9) in which the NF- κ B activation might be coordinately inhibited. For example, multiple apoptotic stimuli, including from TNF- α and professional death ligands such as FasL and Apo2L/TRAIL, might function cooperatively during severe hepatitis. The

professional death ligands directly induce caspase 8 activation but poorly activate NF- κ B (34, 35). It is noted that the apical caspase activation is weak to induce apoptotic death of “type II cells” such as hepatocytes and requires amplification through cross talk to the TNF-triggered JNK activation pathway (25, 26, 34). In particular, active caspase 8 suppresses TNF-triggered NF- κ B signaling, thereby accelerating the programmed cell death (34). We propose that this scenario might occur during severe or fulminant hepatitis, in which HBV mutant-related HBc overexpression is likely to be important in enhancing TNF- α -mediated apoptosis.

JNK is an important regulator of TNF- α signaling and a member of the MAP kinase subfamily. Inhibition of *de novo* protein synthesis or NF- κ B activation sensitizes hepatocytes to TNF- α -induced hepatocyte death as a result of sustained JNK activation (25, 26). Initial JNK activation is thought to be transient and associated with cell survival and proliferation through AP-1. Sustained JNK activation and reactive oxygen species (ROS) accumulation are associated with apoptotic and necrotic cell death (26). Interestingly, we found that the proapoptotic activity of HBc was associated with significant suppression of JNK phosphorylation. Possible explanations are as follows. (i) The precise role of JNK in TNF- α -mediated hepatocyte apoptosis is controversial (26, 27). In a recent study, JNK-1 transduced an antiapoptotic signal in TNF- α -mediated hepatocyte apoptosis through Mcl-1 stabilization (28). (ii) HBc might not have completely abolished the sustained activation of JNK. MKK7 and MKK4 are both upstream activators of JNK. Phosphorylation of MKK7 but not MKK4 was suppressed during HBc coexpression. However, MKK4 is reported to be only slightly activated by proinflammatory cytokines (36). (iii) Inactivation of MKK7 itself might be directly proapoptotic by an unknown mechanism independent of JNK signal transduction.

Direct evidence regarding MKK7 functions in TNF- α -induced hepatocyte death might come from analysis of a mouse model with a specific genetic abrogation. However, mice lacking MKK7 or MKK4 die before birth (37). TAK1 is a direct activator of MKK7 that is critical for the survival of hematopoietic cells and hepato-

FIG 7 HBc disrupts interaction between MKK7 and RACK1 and between MKK7 and TAK1. (A) Competition GST pulldown assay. GST-fused MKK7 (GST/MKK7), His-tagged RACK1 (RACK1-His), and His-tagged HBc (HBc-His) were expressed in *E. coli* and purified with glutathione beads or nickel affinity resins. GST/MKK7 was incubated with RACK1-His alone or coincubated with RACK1-His and increasing amounts of HBc-His. Precipitated RACK1-His was analyzed by immunoblotting with anti-RACK1. HBc-His input was detected by anti-His. (B) Analysis of HBc effects on the interaction between endogenous RACK1 and ectopically expressed MKK7-Flag in 293T cells by coimmunoprecipitation using anti-Flag. Cells were cotransfected with a plasmid expressing MKK7-Flag and the HBc-expressing plasmid at a 1:2 ratio. Total input DNA was balanced with the empty pcDNA3.1 vector. (C) Analysis of HBc effects on the interaction between endogenous MKK7 and RACK1, and the interaction between MKK7 and TAK1, by coimmunoprecipitation using anti-MKK7 of HepG2 cell with or without constitutive HBc expression. Nonspecific rabbit IgG was used as the control.

cytes (38). Deletion of *TAK1* results in bone marrow and liver failure in mice, due to the massive apoptotic death of hematopoietic cells and hepatocytes (38). Cultures of primary hepatocytes deficient in *TAK1* exhibit spontaneous cell death that is further increased in response to TNF- α (39). Similar to abrogation of MKK7 activation by HBc as described in our study, deletion of *TAK1* in hepatocytes abolishes JNK and NF- κ B activation and confers susceptibility to TNF- α -mediated cell death.

Scaffolding of multicomponent regulatory systems is recognized as a major mechanism for controlling signal transduction pathways (40). Specificity of regulation is achieved by organization of MAPK modules, in part through scaffolding and anchoring proteins. RACK1 is a widely expressed, highly conserved scaffolding protein of 7 WD repeat domains. It interacts with a range of proteins and is involved in many cellular processes (30). A previous study demonstrated that RACK1 is an adaptor for protein kinase C-mediated JNK activation through direct binding to JNK1 and JNK2 (41). More recently, Guo and colleagues reported that RACK1 engages directly with MKK7, enhancing the binding of MKK7 to its upstream MAP3Ks and promoting MKK7 phosphorylation (19). The authors therefore proposed that overexpression of RACK1 augments JNK activity, promoting growth of hepatocellular carcinoma. Our study showed that HBc abrogated TNF- α -induced MKK7 phosphorylation by competitively binding to RACK1. In-depth studies are needed to investigate whether expression of HBc affects development of HBV-related hepatocellular carcinoma.

The proapoptotic activity of HBc might harm HBV infection. In this regard, induction of apoptosis is an effective way to sacrifice virus-infected host cells and restrict pathogen spread (42). Huang et al. recently found that virus-infected cells use the mitochondrial antiviral signaling protein (MAVS)-MKK7-JNK2 signaling pathway to induce apoptosis as an innate immune protection mechanism (43). HBc therefore could be a pathogen-associated molecular pattern that sensitizes hepatocytes to TNF- α -induced apoptosis. Supporting this possibility, Lin et al. (31, 44) recently showed that HBc is a major viral factor for HBV clearance in a hydrodynamics-based mouse model. This effect depends on the self-assembly of HBc into capsid particles, which, however, is not obligatory for the proapoptotic activity of HBc described here.

HBV is believed to establish a stealthy infection without alerting significant host immunity. In a chimpanzee model with acute viral hepatitis, the proportion of apoptotic and mitotic hepatocytes is lower than 0.3% even at the viral clearance phase, although the turnover rate is virtually accelerated (45). Nevertheless, it is the low rate of cell destruction and long-term persistent viral infection that cumulates fibrogenesis and cirrhosis in the liver. Apoptosis is thought to be the nexus of liver injury and fibrosis (11). The proapoptotic activity of HBc described here thus could be critical for understanding the pathogenesis of chronic hepatitis B infection.

It will be interesting to discover whether HBc-related mutants of HBV have particular clinical manifestations. Naturally occurring mutations in the HBV basic core promoter provide evidence on this issue. A prevalent HBV variant is a double mutation of C to T at nucleotide 1768 and a change of T to A at nucleotide 1770, resulting in highly upregulated core promoter activity. This change is associated with a clinical outbreak of fatal fulminant hepatitis. In particular, this HBV strain induces substantial apoptosis in primary *Tupaia* hepatocytes by a mechanism potentially

FIG 8 Downregulation of TNF- α -induced MKK7 and JNK phosphorylation and promotion of apoptosis by HBc is reversed by RACK1 overexpression. (A) Immunoblot showing the effects of RACK1 on TNF- α -induced MKK7 and JNK phosphorylation. HBc-expressing HepG2 cells were transduced with either an empty lentiviral vector or the vector encoding HA-tagged RACK1 (RACK1-HA). HBc-negative HepG2 cells were used as controls. (B) Flow cytometry of RACK1 effect on the proapoptotic activity of HBc in HepG2 cells (as described for panel A) upon TNF- α stimulation. NF- κ B inhibitor PDTC was used for cotreatment. Percentages of annexin V-binding cells were determined. The error bars indicate SEM. ***, $P < 0.001$.

independent of enhanced replication (15), possibly the strongly upregulated expression of proapoptotic HBc.

In conclusion, our study found that HBc protein sensitizes hepatocytes to TNF- α -induced apoptosis by competing with MKK7 for binding to RACK1, abrogating MKK7 phosphorylation. The study is thus the first indication that HBc is pathogenic in liver injury during hepatitis B.

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