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Hepatitis B virus X protein increases the IL-1β-induced NF-κB activation *via* interaction with evolutionarily conserved signaling intermediate in Toll pathways (ECSIT)

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ABSTRACT

Hepatitis B virus X protein (HBx) transactivates multiple transcription factors including nuclear factor-kappa B (NF-κB) that regulates inflammatory-related genes. However, the regulatory mechanism of HBx in NF-κB activation remains largely unknown. This study reports that HBx augments the interleukin-1β (IL-1β)-induced NF-κB activation *via* interaction with a Toll-like receptor (TLR) adapter protein, ECSIT (evolutionarily conserved signaling intermediate in Toll pathways). GST pull-down and co-immunoprecipitation analyses showed that HBx interacted with ECSIT. Deletion analysis of HBx in a CytoTrap two-hybrid system revealed that the interaction region of HBx for ECSIT was attributed to aa 51–80. Co-transfection of HBx and ECSIT in IL-1β-stimulated cells appeared to activate IKK and IκB signaling pathway as phosphorylation of both IKK α/β and IκBα was increased whereas knockdown of ECSIT or HBxΔ51–80 mutant attenuated the phosphorylation. As a consequence of IκBα degradation, NF-κB was activated as evidenced by increases in NF-κB transcriptional activity and the nuclear translocation of p65 and p50 that resulted in the induction of IL-10. In contrast, knockdown of ECSIT by siRNA or treatment with an NF-κB selective inhibitor (helenalin) abolished the NF-κB activation and IL-10 expression. We conclude that ECSIT appears to be a novel HBx-interacting signal molecule and their interaction is mechanistically important in IL-1β induction of NF-κB activation.

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1. Introduction

Chronic infection of Hepatitis B virus (HBV) is the major etiological factor for a wide range of liver diseases including chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (Kew, 2010; Lok et al., 2001). While epidemiological evidence has suggested a strong link between HBV infection and chronic liver diseases (CLD), due to extremely complex and diverse nature of HBV-associated pathogenesis, the underlying mechanisms remain to be fully elucidated. However, intensive molecular studies has witnessed the most carcinogenic potential of HBx among the 7 HBV viral proteins as it can intersect with a wide variety of signaling pathways in the cytoplasm (Bouchard and Schneider, 2004; Bouchard et al., 2006; Lian et al., 2001) and activate several nuclear transcription factors, in particular nuclear factor κB (NF-κB) (Chan et al., 2004; Guo et al., 2001; Jiao et al., 2011; Lucito and Schneider, 1992; Tai et al., 2000; Yun et al., 2002). The link between the expression of HBV proteins and activation of the

Abbreviations: AP-1, activating protein-1; ECSIT, evolutionarily conserved signaling intermediate in Toll pathways; HB, xhepatitis B virus X protein; I, κBinhibitor of NF-κB; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; MEKK-1, mitogen-activated protein kinase/ERK kinase kinase-1; MKK, MAPK kinase; NF-κB, nuclear factor κB; TAK1, transforming growth factor-β-activated protein kinase 1; TLR, Toll-like receptor; TRAF6, tumor necrosis factor receptor-associated factor 6.

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NF-κB pro-survival pathway was first enforced from seminal work by Schneider and colleagues(Lucito and Schneider, 1992) demonstrating that ectopic expression of HBx in hepatocytes enhances transcriptional activation of NF-κB. Subsequent mechanistic studies ascribed the activation to the ability of ectopic HBx to induce the degradation of two NF-κB cytoplasmic inhibitors, IκBα and p105 (Chirillo et al., 1996; Su and Schneider, 1996), or to interact with IκBα then transport it to the nucleus thereby preventing its re-association with DNA-bound NF-κB (Weil et al., 1999).

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that has an important role in autoimmunity and inflammatory responses associated with arthritis (van den Berg, 1999), sepsis (Walley et al., 1996), and cancer(Pantschenko et al., 2003). It exerts a pro-inflammatory effect on cells through engagement with the cell surface Type I IL-1 receptor (IL-1R1), also termed IL-1R/Toll-like receptor (TLR) based on a significant homology in its cytosolic region to the *Drosophila melanogaster* protein Toll (Gay and Keith, 1991). This initiates the IL-1β signaling cascade by inducing conformational changes in the receptor that dock cytoplasmic adaptor and effector proteins IL-1RacP, MyD88, and Tollip onto the receptor tail (Burns et al., 2000; Huang et al., 1997; Muzio et al., 1997; O'Neill and Greene, 1998; Wesche et al., 1997) leading to the recruitment of interleukin-1 receptor-associated kinase (IRAK) family members into the receptor complex (Li et al., 2002; Muzio et al., 1997). IRAKs subsequently recruit TRAF6, a member of the TNF receptor-associated factor family of adaptor proteins (Qian et al., 2001). The IRAK-TRAF6 complex can then activate two distinct pathways, *i.e.*, TAK1-TAB1-TAB2/IKKs/IκB/NF-κB and MEKK1/MKKα/JNK/p38/AP-1 leading to activation of the respective NF-κB and AP-1 that regulates pro-inflammatory genes (Moustakas and Heldin, 2003). Notably, the two branches also modulate each other as TAK1 can activate MKKα (Akira, 2003) and MEKK1 can activate IKKs (Kopp et al., 1999). While individual TLRs interact with different combinations of adapter proteins and activate various transcription factors such as NF-κB, AP-1 and interferon regulatory factors (Kawai and Akira, 2006, 2007), an adapter protein, named ECSIT (evolutionarily conserved signaling intermediate in Toll pathways), has been identified as an intermediate molecule situated between TRAF6 and MEKK1 thereby linking TRAF6 to a kinase that can activate both NF-κB and AP-1 (Kopp et al., 1999; Moustakas and Heldin, 2003).

Activation of those key transcription factors by the IL-1β signaling cascade modulates the expression of genes involved in immunity and inflammation. In this context, NF-κB has long been linked to the induction of inflammatory-related gene expression and has attracted substantial interest as a target for the treatment of inflammatory diseases (Muriel, 2009).

Despite the fact that HBx has been linked to NF-κB activation, the molecular mechanisms remain poorly defined and explored. In the present study, we have identified ESCIT as an HBx-interacting signal molecule and report here that interaction of HBx with the TLR adapter protein ECSIT is required for the ability of HBx to enhance IL-1β-mediated activation of NF-κB.

2. Materials and methods

2.1. Vector construction

Expression vectors pFLAG-HBx, pSos-HBx deletion mutants including HBxΔ1–25 (deletion of amino acids 1–25), HBxΔ26–50 (deletion of amino acids 26–50), HBxΔ51–80 (deletion of amino acids 51–80), HBxΔ81–120 (deletion of amino acids 81–120), HBxΔ121–154 (deletion of amino acids 121–154) and pFLAG-HBx Δ51–80 were constructed as previously described(Jiao et al., 2011).

The pREP-HBV vector, harboring 1.2 × unit-length of the HBV genome (genotype B, adw subtype, GenBank Accession No. AF100309) and the control vector pREP-Sal I were constructed as described previously (Huang et al., 2009). To generate an HBx non-expressing HBV mutant (designated as pREP-HBV-HBx-mutant), a stop codon was introduced to the eighth amino acid of HBx (Lin et al., 2010) by means of fusion PCR using the pREP-HBV vector as the template. The two pairs of primers used were F1 (5′-AAGTATGTCAACGAATTGT-3′) and R1 (5′-GCAGCACAGCCTAGC-AGCCATGGA-3′), F2 (5′-CATGGCTGCTAGGCTGTGCTGCTAACTGGA-TCCTGCGCGGGA-3′), and R2 (5′-ACGCGTCGACGAGATCTCGAATA-3′). The product of fusion PCR was digested by *Dra* III and *Sal* I, and cloned to replace the counterpart fragment in the pREP-HBV vector.

The full-length cDNA of ECSIT gene (GeneBank Accession No. AF243044) was reverse-transcribed from the total RNA isolated from Huh-7 hepatoma cells. Recombinant vector pCMVTNT-ECSIT for *in vitro* translation of ECSIT and pcDNA3.1-ECSIT for overexpression of ECSIT in eukaryotic cells was constructed by inserting of ECSIT gene into the *Xho* I/*Kpn* I site of pCMVTNT (Promega, Madison, WI) and *Xho* I/*Hind* III site of pcDNA3.1/myc-His(−)A (Invitrogen, Carlsbad, CA).

2.2. Cell lines and transfection

Human embryonic kidney 293A cells (Invitrogen) and the human hepatoma Huh-7 cell line (Health Science Research Resources Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

2.3. Chemicals and antibodies

Interleukin-1β (IL-1β) was purchased from PeproTech (London, UK). A selective NF-κB inhibitor, helenalin, was purchased from Enzo Life Sciences (Farmingdale). The specific antibodies used in this study included anti-Flag (1:1000 dilution; Sigma-Aldrich, St. Louis, MO), anti-myc (1:1000 dilution; Invitrogen), anti-HBx (1:500 dilution; Millipore, Billerica, MA), anti-ECSIT (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Sos1 (1:250 dilution; BD Biosciences, San Jose, CA), anti-IKK α/β, anti-pIKK α/β (1:500 dilution; Santa Cruz Biotechnology), anti-IκBα, anti-pIκBα (1:1000 dilution; Cell Signaling Technology, Beverly, MA), anti-β-actin (1:2000 dilution; Sigma), anti-NF-κB (p65) (1:500 dilution; Santa Cruz Biotechnology), anti-NF-κB (p50) (1:2000 dilution; Abcam, Cambridge, MA), anti-lamin B (1:200 dilution; Santa Cruz Biotechnology), and anti-α-tubulin (1:2000 dilution; Genscript Scotch Plains, NJ).

2.4. GST pull-down assay

Glutathione S-transferase (GST)-fused HBx was expressed and purified as described previously (Jiao et al., 2011). ^{35}S-labeled ECSIT protein was generated by using *in vitro* T7-coupled reticulocyte lysate system (Promega) with the addition of 2 μg of pCMVTNT-ECSIT and 50 μCi of [^{35}S]methionine (Amersham Pharmacia Biotech, Arlington, IL). ^{35}S-labeled ECSIT was added to the GST-HBx immobilized beads and incubated overnight at 4 °C. Beads were washed three times with 1% Triton X-100 in phosphate-buffered saline (PBS), re-suspended in SDS sample buffer, and subjected to 12% SDS-PAGE. The presence of ^{35}S-ECSIT was detected by autoradiography.

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