Hepatitis B virus X protein enhances NFκB activity through cooperating with VBP1

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Hepatitis B virus X protein (HBx) is essential for hepatitis B virus infection and exerts a pleiotropic effect on various cellular machineries. HBx has been also demonstrated as an indirect transcriptional transactivator of various different viral and cellular promoters. In addition, HBx is involved in the development of various liver diseases including hepatocellular carcinoma. However the mechanism of HBx in hepatocellular carcinogenesis remains largely unknown. In this study, to identify possible new cellular proteins interacting with HBx, we carried out yeast two-hybrid assay. We obtained several possible cellular partners including VBP1, a binding factor for VHL tumor suppressor protein. The direct physical interaction between HBx and VBP1 *in vitro* and *in vivo* was confirmed by immunoprecipation assay. In addition, we found that VBP1 facilitates HBx-induced NFκB activation and cell proliferation. These results implicate the important role of HBx in the development of hepatocellular carcinoma through its interaction with VBP1. [BMB reports 2008; 41(2): 158-163]

INTRODUCTION

Human hepatitis B virus (HBV) is one of the causative pathogens of acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (1). HBV has been reported to induce HCC indirectly through chronic liver inflammation resulting from host immune responses (2). HBV also induces HCC directly through chromosomal instability caused by integration of HBV DNA into the host genome (3, 4). In addition, HBV has a trans-acting viral X protein, HBx, associated with the development of HCC (5, 6). HBx is a pleiotropic protein involved in viral replication, signal transduction pathways (7, 8), and apoptosis (9). HBx activates the signal transduction pathways including protein kinase C (PKC) and Ras-Raf-mitogen activated protein kinase (MAPK) which affect several cellular processes

including proliferation and differentiation (10-13). HBx also transactivates various cellular and viral promoters via protein–protein interaction with cellular factors that bind to recognition sequences (14). HBx has been reported to be implicated in HCC formation by deregulating the cell cycle and activating the expression of some oncogenes and inflammatory cytokines (15). Actually, HCC was observed more frequently in transgenic mice expressing HBx than wild type mice (16-18). Also, recent investigations using siRNA targeting HBx in HCC cell line expressing HBx constitutively exhibited the tumorigenicity of HBx (19, 20).

Among transcription factors regulated by HBx, NFκB has been shown to be associated with tumorigenesis by activating the expression of over 200 genes involved in immune response, inflammation, anti-apoptosis, and cell proliferation (21, 22). The constitutive activation of NFκB has been reported in several human cancers (23), HCC cell lines expressing HBx (24), and HCC in transgenic mice (25). The dominant negative mutant of IκBα reducing the level of colony formation in HBx-expressing liver cells exhibited the gravity of NFκB in liver cell proliferation (26). Though HBx activates NFκB in several ways by acting with various proteins (27), the regulatory mechanism of HBx in NFκB activation remains to be elucidated.

To understand the role of HBx in the development of HCC, we attempted to find cellular proteins interacting with HBx by yeast two-hybrid screening analysis. Interestingly, VHL binding protein (VBP1) which is interacting with von Hippel-Lindau protein (VHL) was identified that binds to HBx. Recently it has been reported that VHL is associated with the regulation of NFκB (28). Therefore, we could easily speculate that VBP1 might be involved in the regulatory mechanism of HBx in the activation of NFκB. VBP1 is widely expressed in various cells including skeletal muscle, heart, brain, kidney, spleen, lung, and liver (29). VBP1 is reported to be localized in the cytoplasm, especially in the perinuclear region. VBP1 contains the consensus sequences for tyrosine phosphorylation suggesting the possible regulations by kinases (29). However, the function of VBP1 associated with VHL remains largely unknown, except that VBP1 was localized in the nucleus when VBP1 and VHL were present together (30).

In the present study, we report that HBx binds to VBP1 directly. The physical interaction between HBx and VBP1 *in vitro* and *in vivo* was confirmed by co-immunoprecipitation

analysis. In addition, HBx and VBP1 synergistically activated NFκB and increased cell proliferation. Our data suggest that the interaction between HBx and VBP1 provides a mechanism for the enhancement of NFkB activation by HBx.

## RESULTS AND DISCUSSION

VBP1 binds to HBx directly

To search for novel cellular proteins that interact with HBx, yeast two-hybrid analysis was performed by screening the human cDNA library with the full length of HBx as bait. From 1 × 10^6 independent transformants, 14 cDNA clones fulfilled the criteria for interaction with HBx. These clones were sequenced and verified with the EMBL/GeneBank database. One of them was VBP1 gene located on chromosome X. Although VBP1 has been reported to interact with VHL, the effect of VBP1 on VHL is not clear. Of course, there are speculations that VBP1 may affect the functions of VHL based on the reports exhibiting that VBP1 has consensus sequences for tyrosine phosphorylation, translocates to the nucleus by VHL, and requires the C-terminal end of VHL for binding similar to VHL cofactor, Elongin C (29, 30).

The interaction between HBx and VBP1 was confirmed by transformation of yeast with bait HBx plasmid and prey VBP1 plasmid (data not shown). To reconfirm the interaction between VBP1 and HBx, *in vitro* binding experiments were performed. E.coli-expressed GST-VBP1 was purified and mixed to the extracts of 293T cells expressing FLAG-HBx followed by GST pull down and Western blot analysis with anti-FLAG antibody (Fig. 1A). These results indicated that HBx directly interacts with VBP1. This specific interaction was further tested by

analyzing 293T cells cotransfected with pFLAG-HBx and pGST-VBP1. The physical binding of GST-VBP1 and FLAG-HBx was confirmed by GST pull down assay of cell extracts followed by Western blot analysis (Fig. 1B). In addition, the reciprocal co-immunoprecipitation analysis was carried out to demonstrate the interaction between HBx and VBP1 *in vivo*. After 293T cells were cotransfected with pFLAG-HBx and pGST-VBP1, cell extracts were prepared and immunoprecipitated with anti-FLAG antibody. Immunocomplexes were analyzed by Western blot analysis with anti-GST antibody. These data confirmed that HBx directly interacts with VBP1 *in vivo* (Fig. 1C). Now it is very clear that HBx and VBP1 have direct physical interactions with each other. HBx is a promiscuous protein containing various functions by interacting with a multitude of cellular proteins. Since VBP1 is a bona fide cellular protein interacting with HBx, the reciprocal effects of each protein in the regulation of other protein needs to be tested.

### VBP1 facilitates cell proliferation by cooperating with HBx
Other groups previously reported that HBx promotes the proliferation of cells (31, 32). To investigate the effect of VBP1 on cell proliferation, we preformed MTT assay in 293T cells transfected with plasmids expressing HBx and VBP1. As seen in Fig. 2, HBx promoted the proliferation of cells as previously reported (26). In contrast, cell proliferation was increased slightly by VBP1. However, cell proliferation was synergistically increased in 293T cells when HBx and VBP1 were expressed together. These results suggest that VBP1 enhances cell proliferation by cooperating with HBx.
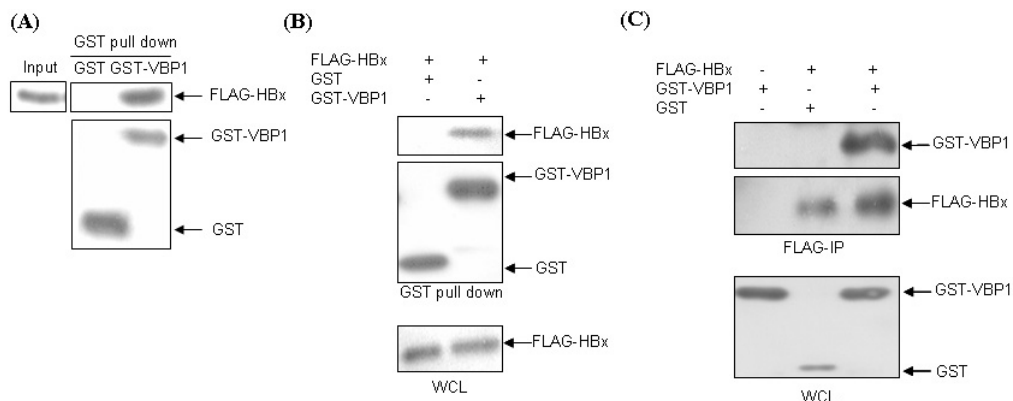
Fig. 1. Physical interaction between HBx and VBP1. (A) The interaction between HBx and VBP1 was confirmed *in vitro*. E. coli-expressed GST-VBP1 was mixed with the lysates of 293T cells transfected with pFLAG-HBx. The reaction mixtures were applied to glutathione-Sepharose beads. Protein complex conjugated with glutathione-Sepharose beads were pulled down and separated by SDS-PAGE followed by immunoblotting with anti-FLAG antibody. (B) The interaction between HBx and VBP1 was confirmed by GST pull down assay *in vivo*. 293T cells were transfected with pFLAG-HBx and pGST-VBP1. At 36 h after transfection, whole cell lysates (WCL) were prepared, incubated with glutathione-Sepharose 4B, pulled down by centrifugation, and subjected to SDS-PAGE followed by immunoblotting with anti-FLAG antibody. (C) The interaction between HBx and VBP1 was confirmed by immunoprecipation assay *in vivo*. The extracts of 293T cells transfected with pFLAG-HBx and pGST-VBP1 were immunoprecipitated with anti-FLAG antibody. The immunocomplexes were separated by SDS-PAGE followed by immunoblotting with anti-GST antibody.

VBP1 enhances HBx-mediated NFκB activation

Cell proliferation is associated with the activation of NFκB (21). HBx is deeply involved in NFκB activation (26, 27, 33). Since VBP1 enhanced cell proliferation, we investigated the
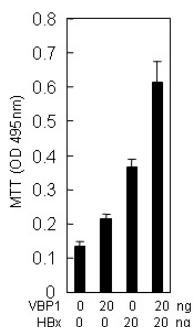
Fig. 2. Effects of VBP1 on cell proliferation. 293T cells were transfected with plasmids expressing HBx and VBP1. At 36 h after transfection, MTT was added into each culture well and incubated for 1 h. The levels of cell viability were determined by the activities of mitochondrial dehydrogenases which were measured as the absorbances at 495nm. Cell viability data were obtained from three independent experiments.

regulatory effect of VBP1 on HBx-mediated NFκB activation. First, we tested the effect of VBP1 on NFκB activation. Though slightly, VBP1 increased the NFκB activity in a dose-dependent manner (Fig. 3A). Because VBP1 has been reported to interact with VHL directly, we speculated that the regulatory effect of VBP1 on the NFκB activity might be related to VHL-mediated NFκB regulation (28). As previously reported (28), we confirmed that the NFκB activity was repressed by VHL (Fig. 3B). Then, we tested whether VBP1 could recover the NFκB activity repressed by VHL (Fig. 3C). We found that VBP1 inhibited the repression of NFκB by VHL suggesting that VBP1 might be involved in VHL-mediated NFκB regulation as a repressor of VHL. Since VBP1 has a regulatory function in NFκB repression induced by VHL which is a binding protein of VBP1, we can also simply speculate that VBP1 may have a regulatory function in NFκB activation induced by HBx which is another binding protein of VBP1. As we expected, HBx certainly induced NFκB activation in a dose-dependent manner (Fig. 3D). Interestingly, VBP1 significantly enhanced the level of NFκB activation induced by HBx suggesting that VBP1 was synergistically involved in NFκB regulation of HBx (Fig. 3E). This result suggests that VBP1 may be a strong coactivator of HBx in NFκB regulation. To further investigate the effect of VBP1 on HBx-mediated NFκB regulation, we tested the effect of VBP1 on NFκB DNA-binding activity (Fig. 3F). After 293T
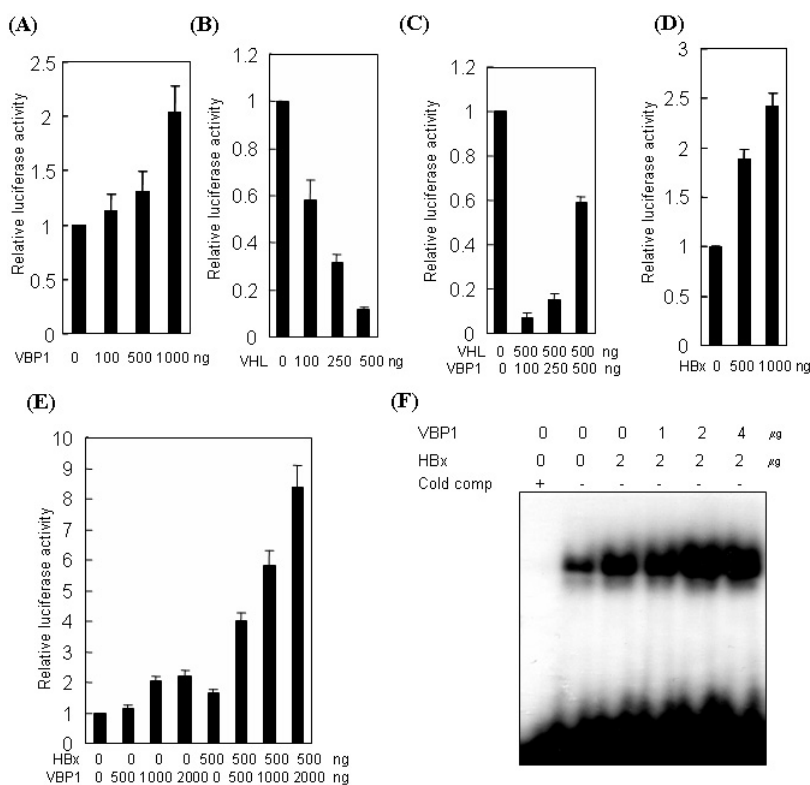
Fig. 3. Synergistic effects of VBP1 on NFkB activation induced by HBx. 293T cells were cotransfected with pNFkB-Luc, pSV2-β-gal, and combinations of various plasmids expressing VBP1, VHL, and HBx. At 36 h after transfection, luciferase assays were performed. All luciferase activities were normalized to the activities of β-galactosidase. The results are the means of three separate experiments. (A) VBP1 increased NFkB activities in a dose dependent manner. (B) VHL repressed NFkB activities. (C) VBP1 recovered NFkB activities repressed by VHL. (D) HBx induced NFkB activation. (E) VBP1 enhanced NFkB activation induced by HBx synergistically. (F) 293T cells were transfected with HBx expression plasmid and increasing amounts of VBP1 expression plasmid. At 36 h after transfection, nuclear extracts were prepared and NFkB DNA binding was analyzed by EMSA. Specific NFkB DNA binding was shown by adding NFkB cold competitor DNA into binding reaction mixture (lane 1).

cells were co-transfected with plasmids expressing VBP1 and HBx, cells were harvested and nuclear extracts were prepared. NFκB DNA-binding probes were mixed with nuclear extracts and EMSA assays were carried out to determine the specific DNA binding affinity of NFκB. Competitive reaction using cold probe showed the specific binding of NFκB to DNA probes (Fig. 3F, lanes 1-2). HBx increased NFκB DNA binding activity as we expected (Fig. 3F, lane 3). When VBP1 was present with HBx, NFκB DNA binding activity was dramatically elevated (Fig. 3F, lanes 4-6). These data also suggest that VBP1 is a coactivator of HBx in the activation of NFκB DNA-binding.

Knock-down of VBP1 inhibits HBx-mediated NFκB activation
To determine whether VBP1 is cooperative for NFκB activation induced by HBx, we designed small-interfering RNA (siRNA) to reduce the expression of endogenous VBP1. First, we tested whether VBP1-siRNA was able to knock-down the gene expression of VBP1 specifically. As expected, VBP1-siRNA reduced the levels of endogenous VBP1 mRNA and FLAG-VBP1 protein (Fig. 4A and B). Then, we investigated whether the specific knock-down of VBP1 by siRNA affected HBx-mediated NFκB activation. While NFκB activation by HBx was evident in cells transfected with control siRNA, the enhancement of NFκB activities by HBx was markedly reduced when VBP1 was repressed by siRNA (Fig. 4C). These results suggested that VBP1 has a key role in HBx-mediated NFκB activation. Certainly, VBP1 plays as a coactivator of HBx in the enhancement of NFκB activity.

Herein, we demonstrated that HBx physically interacts with VBP1 and these interactions play important roles in enhancement of cell proliferation. VBP1 also enhances the activation of NFκB induced by HBx not only in DNA binding but also in transcription. It is quite possible that VBP1 may play a role as a cofactor for various functions of HBx including signal transduction and apoptosis. NFκB activation by HBx is pivotal in hepatocellular carcinogenesis associated with HBV. Therefore, VBP1 is a new cellular protein which is possibly involved in HBx-induced hepatocellular carcinogenesis. Taken together, these results contribute to elucidate the protein network comprised of HBx and NFκB in the development of HCC associated with HBV infection.

MATERIALS AND METHODS

Cell and transfection
A human embryonic kidney cell line 293T was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Transfection was performed with ExGen 500 transfection regents (MBI Fermentas) according to the manufacturer's directions.

Antibodies
Anti-FLAG and anti-GST antibodies were purchased from Sigma. Anti-HA antibody was obtained from Santa Cruz Biotechnology.

Plasmids
FLAG-HBx was constructed as previously described (9). For the expression of GST-VBP1, DNA fragments containing VBP1 gene were cloned into pGex4T-1 vector and pEBG vector to construct plasmids Gex4T-VBP1 and pGST-VBP1 respectively. A full length of VBP1 gene amplified by PCR from pGST-VBP1 was subcloned into pFLAG-CMV2 and confirmed by sequencing analysis. To construct the shRNA plasmid targeting VBP1 gene, double-stranded oligonucleotides prepared by annealing the forward strand DNA (GATCCCCGTATAAGTTTATGGAAC TCTTCAAGAGAGAGTTCCATAAACTTATACTTTTTTGGAAAT TTTTA) and reverse strand DNA (AGCTTAAAAAAGCTTTTCC AAAAAAGTATAAGTTTATGGAACTCTCTCTTGAAGAGTTCC ATAAACTTATACGGGG) were cloned into pSUPER vector. The resultant recombinant plasmid was confirmed by sequencing analysis.
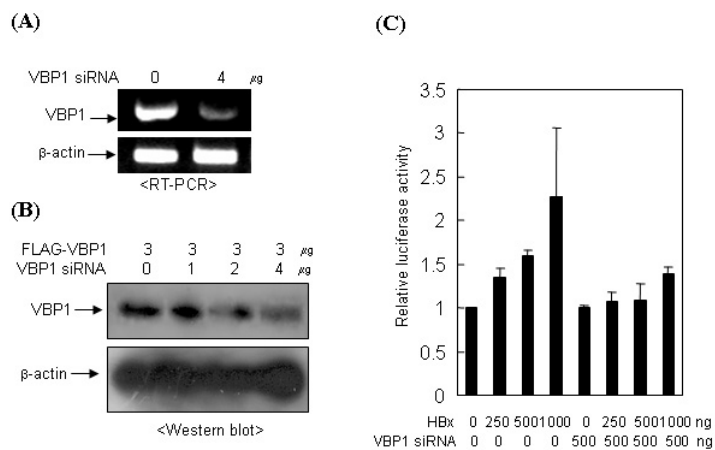
Fig. 4. Effect of specific knock-down of VBP1 on HBx-induced NFκB activation. (A) 293T cells were transfected with pVBP1-siRNA and the level of endogenous VBP1 RNA was analyzed by RT-PCR. (B) 293T cells were transfected with pFLAG-VBP1 and increasing amount of pVBP1-siRNA. Cell extracts were prepared and the protein level of VBP1 was analyzed by Western blot analysis. (C) Cell extracts prepared from 293T cells transfected with pFLAG-HBx and pVBP1-siRNA were analyzed for NFkB luciferase activity. All luciferase activities were normalized to the activities of β-galactosidase. The results are the means of three separate experiments.

Yeast two-hybrid assay

LexA yeast two-hybrid system was obtained from Clontech and applied to identify the cellular proteins capable of binding to HBx from a human HeLa cDNA library. A full length of HBx gene was cloned into pLexA. Yeast two-hybrid assay was performed using yeast strain EGY048 according to the manufacturer's instructions. Screened positive clones were further characterized by sequencing analysis and possible homologous genes were searched through the NCBI GeneBank BLAST programs.

In vitro binding assay

GST-VBP1 and GST proteins expressed in E.coli BL21 cells were purified with glutathione sepharose 4B beads (Peptron). 293T cells were transfected with pFLAG-HBx and cell extracts were prepared by lysing cells in lysis buffer (50mM Hepes pH7.5, 50mM NaCl, 0.1% NP-40) containing a protease inhibitor cocktail (Roche Molecular Biotech). GST-VBP1 or GST protein was incubated with the cell lysates containing HBx. Glutathione sepharose 4B beads were added to the reaction mixture and proteins bound to glutathione sepharose 4B beads were analyzed by Western blotting with anti-FLAG antibody.

Immunoprecipitaton and Western blot analysis

At 36 h after transfection, cells were harvested and resuspended in lysis buffer (50mM Tris-HCl pH8.0, 150mM NaCl, 5mM EDTA, 0.5% NP-40, 0.1mM PMSF). Cell extracts were prepared by centrifugation at 12,000rpm at 4°C for 10 min and subjected to immunoprecipitation with anti-FLAG antibody. The immunocomplexes were resuspended in gel-loading buffer, resolved by SDS-PAGE, and transferred to PVDF membranes. The membranes were immunoblotted with anti-GST monoclonal antibody (Sigma).

MTT assay

The extent of cell proliferation was determined by MTT assay. Briefly, cells were plated in 96-well microplates (1×10^4 cells per well). Cells were transfected with pFLAG-HBx and pGST-VBP1 using Lipofectamine transfection reagent (Gibco). At 36 h after transfection, MTT [3-(4,5-dimethylthiazol-2yl)-2,3-diphenyltetrazolium bromide] was added to each well and incubated at 37°C for 1 h. To suspend MTT-formazan crystals that were generated, DMSO was added to wells along with glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). Viable cells were determined using an ELISA reader at 495 nm. The proliferation rates were calculated from the OD readings with various concentrations of CPT-L2-BA3 using the untreated cells as 100%. Three independent experiments were performed.

Luciferase reporter assay

Briefly, NFκB-driven luciferase reporter plasmid and pSV2-β-gal plasmid were transfected into cells along with various plasmids. At 36 h after transfection, cells were harvested and cell ex-

tracts were prepared. Luciferase assays were performed according to the manufacturer's directions (Promega). NFκB transcriptional activities were determined by normalizing the luciferase activities to the corresponding β-galactosidase activities. Each experiment was repeated three times.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts containing 10 μg of proteins were added to binding buffer containing 10 mM HEPES (pH 7.5), 50 mM KCl, 0.5 mM EDTA, 0.3 mM BSA, 1 mM DTT, 10% (vol/vol) glycerol, and 3 μg of poly (dI-dC). The sequence of oligonucleotides used for NFκB DNA binding was 5'-AGTTGA<u>GGGGACTTTCC</u>CAGGC-3' (NFκB binding site is underlined). The oligonucleotide was annealed with its complementary strand and end-labeled by T4 polynucleotide kinase. Binding reactions were started by addition of 32P-labeled DNA probe to binding buffer containing nuclear extracts at room temperature for 30 min. Reaction mixtures were electrophoresed on nondenaturing 5% polyacrylamide gel in 0.5 × TBE buffer at 120 V. Gels were dried and visualized by autoradiography.

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