Hepatitis B virus X protein Enhances Transcriptional Activity of Hypoxia-Inducible Factor-1α through Activation of Mitogen-Activated Protein Kinase Pathway

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SUMMARY

Hepatitis B virus X protein (HBx) of the hepatitis B virus (HBV) was strongly implicated in angiogenesis and metastasis during hepatocarcinogenesis. Here, we explored the possibility of cross-talk between HBx and hypoxia inducible factor-1α (HIF-1α), a potent transcriptional inducer of angiogenic factors. First, we showed that stability of HIF-1α protein was increased by HBx in HBx-inducible Chang liver cells as well as in transient HBx expression system of non-hepatic cells. Immunofluorescence studies revealed that the HBx-induced HIF-1α was partially translocated into nucleus in majority of cells, while additional CoCl₂-induced hypoxic condition caused complete nuclear translocation. Second, HBx induced both phosphorylation of HIF-1α and activation of p42/p44 mitogen-activated protein kinases (MAPKs), which were synergistically enhanced in the presence of CoCl₂. Further, HBx enhanced transcriptional activity of HIF-1α in the reporter genes encoding hypoxia response element or VEGF promoter. Either treatment of MEK inhibitor, PD98059, or coexpression of dominant-negative MAPK mutants abolished the HBx-induced transcriptional activity, protein stability, as well as nuclear translocation of HIF-1α, suggesting that HBx activates HIF-1α through MAPK pathway. Third, the association of HIF-1α with VHL was decreased but that with CBP was enhanced in the presence of HBx, suggesting the molecular mechanism by which HBx enhances the protein stability and transactivation function of HIF-1α. Finally, we demonstrated that expression of HIF-1α and VEGF was increased in the liver of HBx-transgenic mice, suggesting that the cross-talk between HIF-1α and HBx may lead transcriptional activation of HIF-1α target genes, which play a critical role in hepatocarcinogenesis.
Hepatitis B virus (HBV) is strongly associated with the development of hepatocellular carcinoma (HCC), and yet, the mechanism by which HBV induces events leading to HCC is not clearly elucidated. One of the open reading frames encoded by the HBV genome is a regulatory X protein (HBx), which is a multifunctional transactivator of 16.5 kDa that is required for the transcription of the viral genome (reviewed in 1). HBx activates signal transduction cascades, including the Ras/Raf/mitogen activated protein kinase (MAPK), c-Jun N-terminal kinase, Src-dependent pathways and Jak1 tyrosine kinase, thereby results in the activation of AP-1, NF-κB, and signal transducers and activators of transcriptions (STATs), the major transcriptional factors associated with the proliferation and differentiation signals of liver cells (reviewed in 2). HBx protein also interacts with the tumor suppressor p53, resulting in loss of the protein function (3-5). Such pleiotropic effects of HBx may contribute to HBx-induced hepatocarcinogenesis.

Hypoxic stress in solid tumors is known to be a strong stimulus for new blood vessel formation which is termed angiogenesis (reviewed in 6, 7). One of the most angiogenic growth factors in tumors could be vascular endothelial growth factor (VEGF) that induces endothelial cell proliferation (8). The expression of VEGF is increased during exposure to hypoxia, and the hypoxia-mediated response depends on hypoxia regulated element (HRE) sequences in the 5’ and 3’ regions of the VEGF gene that specifically bind to and transcriptinally activated by the hypoxia inducible factor-1 (HIF-1) (9, 10). HIF-1 comprises α and β subunit, both of which belong to the basic helix-loop-helix (bHLH)-PAS (PER, ARNT, SIM) protein family. HIF-1α, the previously described aryl hydrocarbon receptor nuclear translocator (ARNT), can dimerize with several different bHLH-PAS proteins, whereas HIF-1α is the specific oxygen-regulated subunit. While HIF-1α is quite stable in normoxic conditions, HIF-1α is extremely unstable and quickly degraded by the ubiquitin-proteasome system (11, 12). Recently, it has been shown that the tumor suppressor von Hippel-Lindau (VHL) protein interacts with hydroxylated HIF-1α on 546 proline residue in the presence of oxygen and leads to proteolysis of HIF-1α (13-15). Further activation of HIF-1 involves nuclear translocation,
dimerization with HIF-1β, DNA binding, and recruitment of transcriptional co-activators such as CREB binding protein (CBP)/p300 (16). These processes are regulated by post-translational modifications in that phosphorylation of HIF-1α via the Ras/Raf/-MEK-p42/p44 signaling pathway leads to enhanced trans-activation function of HIF-1α (17-19). Eventually, HIF-1 activates the transcription of diverse genes encoding proteins that function to increase oxygen delivery, such as erythropoietin, to allow metabolic adaptation such as glucose transporters and glycolytic enzymes and to promote cell survival such as insulin-like growth factor II (IGF-II) in response to hypoxia (reviewed in 20). Upregulation of HIF-1α was observed in a broad range of cancers including breast and prostate cancer, which was correlated well with increased vascularity and metastatic potential, indicating that the expression of HIF-1α is associated with tumor progression (21, 22).

HBx has been strongly implicated in angiogenesis and metastasis during hepatocarcinogenesis. The expression of IGF-II, which has a potential angiogenic activity, was enhanced in the HBx-stable transfectants (23, 24). HBx bearing cells showed decreased adhesion to fibronectin and induced a migratory phenotype in a CD44-dependent manner, indicating a role of HBx in invasion and metastasis (25, 26). Importantly, the expression of VEGF and new blood vessel formation were increased in the hepatocytes that stably express HBx (27). Although HIF-1 is a major transcription factor known to regulate angiogenic factors such as VEGF and IGF-II, the role of HBx in transcriptional function of HIF-1 has not been investigated. In the present investigation, therefore, we explored the possibility of cross-talk between HBx and HIF-1α and showed for the first time that the HBx enhances transcriptional activity of HIF-1α probably through activation of MAPK pathway. Our results support the important role of HBx in angiogenesis and tumor progression in the HBV-associated hepatocarcinogenesis.
EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture--Chang (ATCC CCL 13), and Chang X-34 and Chang X-31 in which HBx gene expression is under the control of a doxycycline-inducible promoter, were described previously (4, 28). Human hepatocellular carcinoma cell line, HepG2 (ATCC HB 8065), human cervical carcinoma cell line, HeLa (ATCC CCL-2), and human embryonal kidney cell line, 293 (ATCC CRL-1573) were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified eagle's medium containing 10% fetal bovine serum (FBS) at 37°C in a humid atmosphere of 5% CO₂.

Plasmids and Transient Transfection--The FLAG-tagged full-length HIF-1α was constructed by inserting a PCR amplified full-length HIF-1α fragment into the KpnI/BamHI site of p3XFLAG™-7.1 (Sigma, St. Louis, MO). The Myc-tagged HBx was constructed by inserting an EcoRI/KpnI fragment of pCMV-HA-HBx to the corresponding restriction sites of pCMV-Myc (Clontech, PaloAlto, CA). The Myc-tagged VHL was constructed by inserting a PCR amplified VHL fragment into the EcoRI/Xhol site of pCMV-Myc. All the construction was confirmed by sequencing. For transient transfection, 293 (2 x 10⁶ cells/dish) or HeLa cells (8 x 10⁵ cells/dish) were seeded in 60-cm² dishes and incubated overnight. The cells were transfected with 2-µg expression vectors using Polyfect® (Qiagen Inc., Chatsworth, CA) according to the manufacturer’s instructions.

Western Blot Analysis and Immunoprecipitation--Cells/tissues were lysed in a lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 1% NP-40, protease inhibitors, for 30 min on ice, and whole cell lysate was obtained by subsequent centrifugation. Fifty microgram of protein from whole cell lysates was subjected to 12% Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), unless otherwise indicated, and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Blocking was performed in 5% (w/v) non-fat dried milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20 and then incubated with specific antibodies against HIF-1α, VEGF, hemaglutinin (HA), phosphorylated p42/p44, p42/p44, Myc, CBP (Santa Cruz Biotech., Santa Cruz, CA), FLAG (Sigma), or α-
tubulin (Oncogene, Boston, MA). Secondary antibodies conjugated with horseradish peroxidase (Zymed Lab., South San Fransisco, CA) were used and immunoreactive proteins were detected using the Super Signal (Pierce, Rockford, IL). The protein concentration was quantified by bicinchoninic acid assay (Pierce). For immunoprecipitation, five hundred microgram whole cell lysates were incubated with 2-µg anti-HIF-1α antibody (Santa Cruz Biotech.). The resulting immune complex was precipitated by adding 40-µl protein-A agarose slurry. To precipitate FLAG-tagged HIF-1α, anti-FLAG M2 affinity gel (Sigma) was used. The resulting immune complex was washed five times with lysis buffer, subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with either anti-Myc or anti-CBP antibody (Santa Cruz Biotech.).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)--Total RNA was prepared using RNeasy kit (Qiagen Inc.). PCR reaction was performed as described previously with specific primers for HIF-1α (forward: 5'-CAAGTGCAATAGACTG-3’, reverse: 5'-GGCTGCTCCAGGTACGGCG-3’), VEGF (forward: 5'-TGAGTGGCTAAACGTGCTG-3’, reverse: 5'-GTAGCTTATCTACTTTGTCC-3’), HBx (forward: 5'-GCTCTAGAAATGGCTGCTG-3’, reverse: 5'-CCCAAGCTTTTACGGCAGAGTG-3’) and β-actin (5'-CGTGGCCCGCCTAGGACCA-3’, reverse: 5'-TTGCTTGGGTTCCAGGCG-3’) (28). Genes were analyzed under the same conditions used to exponentially amplify the PCR products.

Flow Cytometric Analysis for HIF-1α Expression--Cells were detached by trypsinization and washed twice with PBS containing 3% FBS. Cells were fixed in 2% paraformaldehyde in PBS for 15 min on ice. Cells were washed twice in cold PBS containing 3% FBS, and resuspended in a permeabilization buffer (0.1% Triton X-100 in PBS) for 10 min on ice. Then cells were stained with the anti-HIF-1α antibodies (1:100 dilution, Santa Cruz Biotech.), followed by secondary antibody consisted of biotinylated anti-rabbit IgG (1:200 dilution, Vector Laboratories, Inc., Burlingam, CA) and Streptavidin-fluorescein (1:200 dilution, Boeringer Mannheim GmbH, Mannheim, Germany). The fluorescent intensity of the stained cells was measured in a FACStar PLUS flow cytometer and data were analyzed using the PC-Lysis software program (Becton-Dickson, Mountain View, CA). This procedure was carried out on normal rabbit IgG in
parallel as a negative control.

**Immunocytochemistry**—Chang and Chang X-34 cells (7.5 x 10⁴ cells/chamber) that had been plated the previous day on 4-chamber slide glass were treated with 100 µM CoCl₂ or vehicle in the presence or absence of 2 µg/ml doxycycline for 24 h. The cells were fixed with 50% Aceton/50% Methanol for 5 min and then permeabilized with 0.1% Triton X-100 in PBS for 10 min at RT. The cells were incubated in blocking solution of 3% FBS in PBS for 1 h and then stained with anti-HIF-1α antibody diluted into 1:100 in PBS containing 3% FBS overnight, followed by a secondary antibody consisting of biotinylated anti-rabbit IgG (Vector Laboratories, Inc.) diluted to 1:200 in PBS containing 3% FBS. Streptavidin-fluorescein (1:200 dilution, Boeringer Mannheim) was used to visualize the stained cells by confocal microscopy (Nikon, Japan). 4',6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei.

**Reporter Gene Assays**—The HRE-Luc, containing four copies of the erythropoietin hypoxia-responsive element, i.e., 5'-GATCGCCCTACGTGCTGTCTCA-3’, Gal4-tk-Luc containing Gal4 binding site, and the VEGF promoter (-2068 to +50)-Luc reporter constructs have been described elsewhere (17, 29, 30). The eukaryotic expression vector for wild type HBx, HpSVX, the vector encoding frameshift mutant of HBx, pSVXkB, and the dominant negative mutants of p42 and p44, pERK1KR and pERK2KR, respectively, were as described previously (17, 31). The eukaryotic expression vector encoding full-length HIF-1α, and pGal4-HIF-1α, containing the DNA binding domain (1-147 amino acids) of Yeast Gal4 linked to the full-length coding region of mouse HIF-1α as described previously (17). HepG2 cells (1.5 x 10⁵ cells/well) were seeded in a 12-well culture plate and transfected with reporter plasmid (0.1 or 0.3 µg), β-galactosidase (β-gal) expression vector (0.2 µg) in the presence or absence of HBx expression vector using LipofectaminePlus® (GIBCO BRL, Grand Island, NY). After 24 h of transfection, the cells were treated with 100 µM CoCl₂ for 24 h and then lysed in the cell culture lysis buffer (Promega, Madison, WI). Luciferase activity was determined using an Analytical luminescence luminometer according to the manufacturer’s instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-gal activity.

**Animals and Immunohistochemistry**—Transgenic mice carrying the HBx gene were maintained in a
laboratory animal facility in the Department of Laboratory Animal Medicine, Medical Research Center, Yonsei University College of Medicine, Seoul, Korea, under specific pathogen free conditions (28, 32). We used the HBx homozygote transgenic mice at different ages and age-matched wild-type controls, which were maintained in a barrier room under constant temperature (22±2°C) and humidity (55±2%). Food (Purina diet) and water were supplied ad libitum. The experimental protocol was approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University, according to the Guide for Animal Experiments edited by Korean Academy for Medical Sciences. Mice were sacrificed under ether anesthesia and the liver was removed and fixed in 10% neutral buffered formalin. Three portions of each liver tissue were embedded in paraplast. To visualize the expression of VEGF in liver tissue of the HBx transgenic mice, a standard immunohistochemistry protocol was used, as previously described (33). The primary antibody binding was localized by a mouse-anti-human VEGF antibody (1:100 Dilution) (Santa Cruz Biotech.) at 4°C overnight, followed by treatment with anti-mouse IgG antibodies (Vector Laboratories, Inc.). Sections were incubated with an avidin-biotin complex conjugated to horseradish peroxidase. To better visualize the positive cells, color development was enhanced by diaminobenzidine and counter-stained by hematoxylin.

RESULTS

HBx increases stability of HIF-1α protein --Since the role of HBx is implicated in angiogenesis and metastasis during hepatocarcinogenesis, we examined whether HBx directly modulate the transcriptional activity of HIF-1α which is a critical inducer of tumor angiogenesis. Transcriptional activity of HIF-1α is primarily regulated by accumulation of HIF-1α protein under hypoxic stress, which otherwise rapidly degraded by ubiquitin-proteasome pathway in normoxic cell (11, 12). Therefore, we tested whether HBx induced accumulation of HIF-1α. For this purpose, we employed the Chang X-34 cell line, in which the expression of HBx gene is under control of an inducible doxycycline promoter (4, 28, 31). After Chang X-
34 cells were treated with doxycycline, the expression of HIF-1α was measured by Western blot analysis (Fig. 1A). When the HA-tagged HBx was stained with specific antibodies against HA, the protein was slightly detectable in the absence of doxycycline treatment, which probably represented leaky expression of HBx, being consistent with the previous reports (4, 34). With doxycycline, the expression of HA-HBx was significantly induced. When HBx was expressed at the lower level without doxycycline treatment, the expression of HIF-1α was detected. When HBx was expressed at higher level after doxycycline treatment, the amount of HIF-1α protein was increased. Consistent with the expression level of HIF-1α, VEGF expression was increased after doxycycline treatment. However, treatment with doxycycline alone did not induce expression of HIF-1α or VEGF in Chang cells (data not shown). Consistent with the result of Western blot analysis, the increase of HIF-1α protein in the presence of HBx was demonstrated by flow cytometry (Fig. 1B). To further confirm the results, we introduced HBx into a non-hepatic origin HeLa cell line and measured protein-level of HIF-1α. As shown in Fig. 1C, expression of HIF-1α as well as VEGF was increased upon HBx expression. Although the mRNA level of VEGF was largely increased, that of HIF-1α was remaining unchanged in the presence of HBx. Since these results indicate that HBx increases the protein-level of HIF-1α perhaps through stabilization of HIF-1α, we measured HIF-1α protein in the presence of cycloheximide which blocks new protein synthesis. As shown in Fig. 1D, HBx induced strong expression of HIF-1α and it blocked degradation of the protein as efficiently as CoCl₂ treatment which mimicked hypoxic condition.

**HBx increases nuclear translocation and phosphorylation of HIF-1α**—For transcriptional activation of HIF-1α, the protein is required to translocate into nucleus (35). Therefore, we analyzed whether HBx influenced the subcellular localization of HIF-1α by immunofluorescence studies. As shown in Fig. 2, Chang cells showed very little HIF-1α immunoreactivity which was diffused into cytoplasm. Chang X-34 cells that expressed HBx by doxycycline showed increased expression of HIF-1α, which was variably distributed in nucleus and cytoplasm. HIF-1α was located in nucleus in about 25% of cells and in both
cytoplasm and nucleus in approximately 60% of cells (Fig. 2). These results further confirmed that HBx increases the expression of HIF-1α protein and demonstrated that HBx enhances the nuclear translocation of HIF-1α. After treatment with CoCl2, the expression of HIF-1α in the doxycycline-treated Chang X-34 cells was further enhanced and the protein was almost completely localized in nucleus. The result was largely in contrast to that observed in Chang cells in which no significant induction or nuclear translocation of HIF-1α was seen after CoCl2 treatment (data not shown).

It has been reported that the Raf/MEK/p42/p44 MAPK signaling pathway stimulates transcriptional activity of HIF-1α (17-19). HIF-1α was phosphorylated by p42/p44 MAPK in vitro (18). The phosphorylation and the resulting mobility shift of HIF-1α were inhibited by PD98059, a specific inhibitor of MAPK pathway, indicating a direct effect of this signaling pathway on the trans-activation function of HIF-1α (18). Since HBx activates extracellular signal-regulated kinases (ERKs) by stimulating ras-GTP complex formation (36), we asked whether the HBx protein induced phosphorylation of HIF-1α through the p42/p44 MAPK signaling pathway. First, we re-examined the migration pattern of HIF-1α in Chang X-34 cells by SDS-PAGE with an 8% gel (Fig. 3). Interestingly enough, the HIF-1α band was clearly separated into a fast and a slow migrating band. The slow migrating band which represents phosphorylated and active status of HIF-1α appeared in Chang X-34 cells, and it became the most striking in the presence of both doxycycline and CoCl2. In the same cell lysates, the p42/p44 MAPK was strongly activated when evaluated by Western blotting analysis using anti-phospho p42/p44 antibodies. When we employed a different doxycycline-inducible Chang cell clone, Chang X-31, basically the same results were obtained. However, the degree of both HIF-1α and p42/p44 MAPK activation was lower without doxycycline treatment in Chang X-31, which could be due to the lower leaky expression of HBx in the clone (34). These results may suggest that HIF-1α is phosphorylated by p42/p44 MAPK in the presence of HBx.

HBx induces transcriptional activity of HIF-1α--To further investigate the effect of HBx in transcriptional activity of HIF-1α, we carried out reporter gene analysis by transient co-transfection of the
HBx expression vector together with a reporter gene containing HRE sequences that locates in the erythropoietin gene promoter (29). As shown in Fig. 4, co-transfection of HBx expression vector into HepG2 cells activated the HRE reporter gene activity in a dose-dependent manner, while the frameshift mutant HBx expression vector did not induce significant changes. To test whether the induction was directly due to transcriptional activation of HIF-1α, we employed a Gal4-driven reporter system (17). We co-transfected a pGal4/HIF-1α plasmid that encodes full-length mouse HIF-1α linked to the DNA binding domain of yeast Gal4 together with the reporter plasmid containing Gal4 binding sequences and thymidine kinase (tk) promoter. Since the Gal4 fusion protein is able to bind Gal4 binding site, the reporter gene is transcribed only when HIF-1α has transcriptional activity (17). Co-transfection of wild-type HBx enhanced Gal4-tk-Luc activity in a dose-dependent manner. Similar results were obtained using a luciferase reporter containing a VEGF promoter fragment (-2068 to +50), which includes a HRE (30). Together, these results indicate that HBx induced transcriptional activity of HIF-1α through direct regulation of HIF-1α. Interestingly, the expression of HBx in the presence of CoCl2 remarkably activated all three reporter activities (Fig. 4). The synergistic increases of the reporter activity by HBx and hypoxia may suggest a strong cooperation of hypoxia and HBx for maximal activation of HIF-1α.

HBx induces transcriptional activity of HIF-1α through activation of MAPK signaling pathway—
To further test the involvement of p42/p44 MAPK signaling pathway in HBx-induced transcriptional activity of HIF-1α, we examined whether the HBx-induced HIF-1α activity was repressed by blocking p42/p44 MAPK pathway. As shown in Fig. 5A, PD98059 efficiently blocked the Gal4-HIF-1α driven Gal4-tk-Luc activity that was induced by HBx. Further, the dominant negative mutants of p42/p44 MAPK, ERK1KR and ERK2KR repressed the HBx-induced HIF-1α activity dose-dependently, suggesting that HBx induces transcriptional activity of HIF-1α through activation of MAPK signaling pathway (Fig. 5B). To check whether MAPK pathway affects the protein-level of HIF-1α, HIF-1α protein was measured after PD98059 treatment. Surprisingly, PD98059 decreased the protein-level of HIF-1α in Chang X-34 cells when
measured by both flow cytometry and Western blot analysis (Figs. 6A and 6B). The decrease was more striking in the HBx-induced HIF-1α protein level to compare with that induced by both HBx and CoCl2, suggesting that the HBx-induced MAPK signaling pathway contributed to stabilization of HIF-1α, which may be distinct from the Hypoxia-mediated MAPK signaling pathway. Similarly, the level of HIF-1α protein that induced by HBx in HeLa was decreased in the presence of PD98059. In contrast, Wortmannin, an inhibitor of phosphatidylinositol 3-kinase, did not reduce the level of HIF-1α protein (Fig. 6C). Consistent with the results, PD98059 largely decreased the HBx-induced stability of HIF-1α that measured in the presence of cycloheximide (Fig. 6D). When we examined the localization of HIF-1α after PD98059 treatment, most of the protein was present in cytosol, although the intensity of fluorescence was significantly diminished (Fig. 6E). Taken together, these results demonstrated that p42/p44 MAPK signaling pathway was strongly involved in the HBx-induced HIF-1α activation.

Next, we investigated the molecular mechanism by which HBx enhances stabilization HIF-1α protein. We checked whether HBx influenced on the association of HIF-1α with VHL which further drives ubiquitin-proteasomal degradation of HIF-1α. As shown in Fig. 7, when cells were treated with MG132 that blocks proteasome function, the binding of HIF-1α and VHL was strong, but the binding was largely diminished in the presence of either HBx or CoCl2. The result may indicate that HBx decreased the prolyl hydroxylation of HIF-1α since VHL interacts with hydroxylated HIF-1α on 546 proline residue (13-15). Intriguingly, however, PD98059 did not recover the association between HIF-1α and VHL, indicating that MAPK pathway may block next steps of the VHL binding such as ubiquitin elongation and/or functional activation of proteasome. We also asked whether HBx increases transcriptional function of HIF-1α by recruiting co-activators. As previously reported, the association of HIF-1α with CBP was increased in the presence of CoCl2 to compare with normoxic condition (Fig. 8). When HBx was present, the HIF-1α binding to CBP was stronger than that under the hypoxic mimicking condition. However, blocking of MAPK pathway using PD98059 decreased the binding, indicating that the MAPK signaling pathway has a
role in association of HIF-1α with CBP, thereby enhancing transactivation function of HIF-1α.

The expression of HIF-1α as well as VEGF was increased in liver tissues of HBx-transgenic mice— Finally, we examined the expression and function of HIF-1α in liver samples obtained from the HBx transgenic mice (32). As expected, the expression of HIF-1α was increased along with the age of the HBx transgenic liver, whereas the expression of HIF-1α was barely detectable in liver samples obtained from the C57BL/6 wild-type control mice (Fig. 9A). To further study the transcriptional function of HIF-1α in the HBx transgenic mice, we examined the expression of VEGF, a downstream target of HIF-1α. As expected, an age-dependent increase of VEGF expression was observed in the HBx-transgenic liver (Fig. 9A). Upon microscopic examination of the liver tissue immunohistochemistry, we found that VEGF proteins diffusely localized in the cytosol of hepatocytes that were located proximal to the central vein in the liver tissues of 7-month old HBx transgenic mice. This observation was increasingly distinct along with the age of the HBx transgenic mice, in that VEGF staining was widely spread in the liver of 13-month old transgenic mice, while the protein was not detected in the liver tissues that were obtained from the age-matched B6 wild-type control mice. Fig. 9B shows the representative figures of the pattern of VEGF expression in the liver of HBx transgenic mice. Taken together, our results demonstrated that the expression of HIF-1α was increased in HBx-transgenic liver, which may induce expression of target genes that are associated with angiogenesis.

**DISCUSSION**

Tumor-associated new vessel formation has been viewed as a central pathogenic step in tumor development, growth, invasion and metastasis (reviewed in 7). While the crucial roles of both HBx and HIF-1α in angiogenesis during hepatocarcinogenesis have been implicated independently, the possible cross-talk between these two molecules has not been previously explored. In this report, we provide evidences, for the first time, that HBx enhances transcriptional activity of HIF-1α, employing HBx-inducible cell lines and transgenic mice carrying HBx gene (4, 28, 32). Also we found that p42/p44 MAPK signaling pathway plays
a critical role in the HBx-induced transcriptional activation of HIF-1α. In addition, we show that the expression of VEGF was induced in the HBx-bearing liver cells and liver tissues, which supports the important role of HBx in new vessel formation in hepatocellular carcinoma.

Recent studies have revealed that transcriptional activity of HIF-1α is primarily regulated by accumulation of HIF-1α protein under hypoxic stress, which is otherwise degraded by the ubiquitin-proteasome pathway in normoxic cells (11, 12). Tumor suppressors, such as VHL and p53, are known to promote ubiquitination and proteasomal degradation of HIF-1α (13-15, 37). In this report, we clearly demonstrated that HBx increases stability of HIF-1α protein (Figs. 2-4), probably through decreasing the association of HIF-1α with VHL (Fig. 7). Our data suggest that HBx may decrease the prolyl hydroxylation of HIF-1α since VHL interacts with hydroxylated HIF-1α on 546 proline residue (13-15). As a result, HBx may block degradation of HIF-1α in HBV-associated hepatocellular carcinoma, which is similar in the case of renal carcinoma cells wherein the loss of VHL function resulted in constitutive expression of HIF-1α (38, 39). Also it is possible that HBx indirectly blocks degradation of HIF-1α by causing loss of p53 function since HBx directly interacts with wild-type p53 in vivo and in vitro, and thereby inhibit p53 function (3-5).

On the other hand, it has been shown that HBx physically interacted with a α proteasome subunit, PSMA7, and an ATPase-like member of the 20S proteasome component. The HBx expression, in turn, inhibited the cellular turn over of c-Jun and ubiquitin-Arg-β-galactosidase, two well known substrates of the ubiquitin-proteasome pathway (40, 41). In an independent study, the α proteasome subunit PSMA7 interacted with HIF-1α and induced proteasome-dependent degradation of HIF-1α (42). These results also raised another possibility that HBx blocks the ubiquitin-proteasome activity by direct interaction with the proteasomal subunit. The MAPK pathway may contribute to this process since PD98059 did not interfere the decreased binding of HIF-1α with VHL that caused by HBx as shown in Fig. 7.

So far, MAPK signal transduction pathway is considered as one of the most important factors in transactivation of HIF-1 under hypoxic condition, which may be independent from stabilization of the protein
HIF-1α was shown to be phosphorylated by the MEK-1/p42/p44 MAPK pathway in vitro and the phosphorylation enhanced transactivation function of HIF-1α without changes in protein-level of HIF-1α (18). Such MEK-1/p42/p44 MAPK activity was necessary for the full activation of HIF-1α as a transactivator under hypoxia and PD98059 did not block the stabilization or DNA binding ability of HIF-1α (17). We also demonstrated that HBx activates MEK-1/p42/p44 MAPK pathway that induces transcriptional activation as well as nuclear translocation of HIF-1α (Figs. 5 and 6). However, the stability of HIF-1α protein in the presence of HBx was very sensitive to decrease by PD98059 treatment (Fig. 6), indicating that the HBx-induced MAPK signaling network plays an important role in the stabilization of HIF-1α, which was contrast to the previous reports. Meanwhile the protein level of HIF-1α that induced by both HBx and CoCl₂ was not decreased so sensitively (Fig. 6B). Therefore, our results suggest that the HBx-induced MAPK signaling network plays an important role in the stabilization of HIF-1α, which may be distinct from that induced by hypoxia. HBx may activate a different set of MAPK signaling molecules that may include yet unknown target molecules which are critical for HIF-1α stabilization.

Generally, a local hypoxia is generated during hepatocarcinogenesis due to the shortage of blood supply caused by portal hypertension in cirrhotic lesions and rapid proliferation of tumor cells in liver tissues (22, 23). In relevance, obstruction of hepatic artery during transcatheter embolization of HCC caused extensive ischemic tumor necrosis that accompanied with increases in VEGF level, which may contribute to survival and proliferation of residual tumor cells (43, 44). These examples may suggest a potential cooperation of HBx and hypoxic signal transduction pathways during HBV-associated hepatocarcinogenesis and in the course of therapeutic treatment for liver cancer. Interestingly, we demonstrated that HBx synergistically enhanced transcriptional activity of HIF-1α under hypoxic condition (Fig. 6A), which may provide a molecular mechanism of the cooperation of two signaling pathways. Although the mechanism of the synergism is not yet clarified, HBx may directly or indirectly modulate the factors that regulate the oxygen-dependent transcriptional function of HIF-1α. In this regard, potential target molecules of HBx
could be the HIF-1α regulating factors such as VHL, factor inhibiting HIF-1 (FIH-1), and co-activator CBP/p300. A cooperative binding of VHL and the FIH-1, which recruits histone deacetylases to HIF-1α under normoxic conditions, represses transactivation function of HIF-1α (45). Indeed, FIH-1 was recently identified as an asparaginyl hydroxylase enzyme that modifies Asn803 of HIF-1α using oxygen molecule, which resulted in the dissociation of HIF-1α and co-activator p300 (46, 47). The observation that the association of HIF-1α with VHL was decreased but that with CBP was enhanced in the presence of HBx (Fig. 8), may suggest a potential molecular mechanism of cooperation between HBx and HIF-1α under hypoxic condition.

Although hypoxia is a strong and universal stimulus that activates HIF-1, a significant number of other hormonal, environmental and intracellular stimuli have been reported to induce HIF-1 activation probably in a cell type-specific manner (48, 49). In this report, we show an important role of HBx as a viral inducer of HIF-1 activity in viral pathogenesis as well as in angiogenesis during hepatocarcinogenesis. In addition to HBx, several other viral proteins that induce function of HIF-1α have been reported in literature. Cells transformed by v-Ha ras increased transcriptional activity HIF-1α through phosphatidylinositol 3-kinase/PTEN/AKT/FRAP signaling pathways (50). A constitutively active G protein-coupled receptor encoded by the Kaposi’s sarcoma-associated herpes virus/human herpes virus 8 stimulated HIF-1α through activation of p38 and MAPK signaling pathways, and resulted in expression of VEGF (51). Although the linking with the HIF-1α is unveiled, the human papillomavirus 16 E6 oncoprotein and the simian virus 40 large T antigen enhanced expression of VEGF, which may play a critical role in the virus-associated tumor progression (52, 53). These similarities between viral oncoproteins that are evolutionary different indicate an important role of HIF-1α-mediated cellular signaling in viral pathogenesis.

The expression of HIF-1α is of primary importance in angiogenesis, tumor growth, invasion and metastasis. Since the expression of HIF-1α is high in many carcinoma cells compare to normal tissues, and it is an essential transcriptional factor for VEGF and many glycolytic enzymes which are required for the
continued growth and survival of tumors, HIF-1α has been recognized as a novel and tumor-specific target for anti-cancer therapy (54, 55). Therefore, understanding of the complex cross-talk between the HIF-1α and HBx could not only provide the important implications for angiogenesis and tumor progression but also a valuable strategy to treat HBV-associated hepatocarcinogenesis.

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**References**


Footnotes

The abbreviations used are: HBV, hepatitis B Virus; HCC, hepatocellular carcinoma; HBx, hepatitis B virus X protein; MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor; HRE, hypoxia regulated element; HIF-1, hypoxia inducible factor-1; VHL, von Hippel-Lindau; CBP, CREB binding protein; FBS, fetal bovine serum; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; HA, hemaglutinin; PBS, phosphate-buffered saline; β-gal, β-galactosidase; FIH-1, factor inhibiting HIF-1.

Figure Legends

FIG. 1.  HBx increases stability of HIF-1α protein. Chang and Chang X-34 cells (2 x 10⁶ cells/dish) were seeded in 100-cm² dishes and incubated overnight. The cells were treated with or without 2 µg/ml doxycycline (Doxy) for 24 h. A. Fifty microgram whole cell lysates were analyzed for the expression of HA-HBx, HIF-1α, VEGF or α-tubulin using specific antibodies by Western blot analysis as described in the Materials and Methods. B. The expression of HIF-1α protein was determined by flow cytometry. Dotted histogram, stained with normal rabbit IgG; solid histogram, stained with anti-HIF-1α antibody. C. 293 cells (1 x 10⁶ cells/well) were seeded in 6-well culture plates and incubated overnight. The cells were transfected with 1-µg pCMV-Myc-HBx. After 24 h of transfection, the cells were lysed and fifty microgram cell lysates were analyzed for expression of the indicated proteins by Western blot analysis (left panel). To analyze transcripts, 293 cells (2 x 10⁶ cells/dish) were seeded in 60-cm² dishes and incubated overnight. The cells were transfected with 2-µg pCMV-Myc-HBx. After 24 h of transfection, total RNA was prepared and analyzed for expression of the indicated transcripts by RT-PCR as described in the Materials and Methods (right panel). D. 293 cells (2 x 10⁶ cells/dish) were seeded in 60-cm² dishes and incubated overnight. The
cells were transfected with 2-µg p3XFLAG™7.1-HIF-1α and/or 2-µg pCMV-Myc-HBx. After one hour of transfection, the cells were incubated with or without 100 µM CoCl₂ for 24 h as indicated. At the end of incubation, the cells were treated with 10 µM cycloheximide (CHX) for the indicated period. Fifty microgram of whole cell lysates were analyzed for the expression of FLAG-HIF-1α, Myc-HBx, and α-tubulin by Western blot analysis. One representative of at least three independent experiments with similar results is shown.

FIG. 2. HBx increases the protein-level as well as the nuclear translocation of HIF-1α. Chang and Chang X-34 cells (7.5 x 10⁴ cells/chamber) were seeded in 4-chamber slide glass and incubated for overnight. Chang X-34 cells were treated with or without 100 µM CoCl₂ in the absence of 2 µg/ml doxycycline (Doxy) for 24 h. The cells were fixed, incubated with anti-HIF-1α antibody and analyzed by confocal microscopy as described in Materials and Methods. The nuclear translocation of HIF-1α was quantified by counting at least 400 cells for each experiment. The percentage of cells with the HIF-1α in cytoplasm (C), the nucleus (N), and both (C + N) is shown. One representative of at least three independent experiments with similar results is shown.

FIG. 3. HBx activates p42/p44 MAPK and enhances phosphorylation of HIF-1α. Chang, Chang X-34 and Chang X-31 cells (2 x 10⁶ cells/dish) were seeded in 100-cm² dishes and incubated for overnight. The cells were treated with 100 µM CoCl₂ or vehicle in the presence or absence of 2 µg/ml doxycycline (Doxy) for 24 h. Fifty microgram cell lysates were analyzed for the expression of HIF-1α, phospho-p42/p44 MAPK, p42/p44 MAPK, and HA-HBx using specific antibodies by Western blot analysis as described in Materials and Methods. Representative figures of at least three independent experiments with similar results were shown.
FIG. 4. HBx increases transcriptional activity of HIF-1α. HRE-Luc (0.1 µg), Gal4-tk-Luc (0.2 µg) or VEGF promoter (-2003 ~ 23)-Luc (0.3 µg) reporter was co-transfected with the expression plasmid for HBx or mutant HBx into HepG2 cells as indicated. When Gal4-tk-Luc was used 50 ng pGal4-HIF-1α was co-transfected. After 24 h of transfection, the cells were incubated in the presence or absence of 100 µM CoCl₂ for 24 h, and then cell lysates were obtained and analyzed. Luciferase activity was normalized for transfection efficiency by corresponding β-gal activity. Data shown are the mean ± SD of three independent determinations.

FIG. 5. Blocking of p42/p44 MAPK signaling pathway represses HBx-induced transcriptional activity of HIF-1α. A. The MEK-1 inhibitor PD98059 blocks HBx-induced HIF-1α activity. Gal4-tk-Luc (0.2 µg) reporter and pGal4-HIF-1α (50 ng) were co-transfected with or without the expression plasmid for HBx (5 ng). After 24 h of transfection, the cells were incubated with 100 µM PD98059 for 1h before exposure to 100 µM CoCl₂ for 24 h, and then cell lysates were obtained and analyzed. B. The dominant-negative mutants of p42/p44 blocks HBx-induced HIF-1α activity. Gal4-tk-Luc (0.2 µg) reporter was co-transfected with the expression plasmid for HBx (5 ng), and dominant-negative mutants of p44 (pERK1KR) or p42 (pERK2KR) (100 ng and 250 ng for each) into HepG2 cells as indicated. After 24 h of transfection, the cells were incubated with 100 µM CoCl₂ for 24 h, and then cell lysates were obtained and analyzed. Luciferase activity was normalized for transfection efficiency by corresponding β-gal activity. Data shown are the mean ± SD of three independent determinations.

FIG. 6. PD98059 decreases the HBx-induced stability and nuclear translocation of HIF-1α protein. A. Chang X-34 cells (2 x 10⁶ cells/dish) were seeded in 100-cm² dishes and incubated for overnight and then treated with 100 µM PD98059 or vehicle for 1 h before exposure to 100 µM CoCl₂ in the presence or absence of 2 µg/ml doxycycline (Doxy) for 24 h. The expression of HIF-1α protein was determined by flow
cytometry. Dotted histogram, stained with normal rabbit IgG; solid histogram, stained with anti-HIF-1α antibody.  

B. Chang X-34 cells were treated with the indicated concentrations of PD98059 for 1 h before exposure to 100 μM CoCl₂ in the presence or absence of 2 μg/ml doxycycline (Doxy) for 24 h. Fifty microgram cell lysates were analyzed for the expression of HIF-1α, phospho-p42/p44 MAPK, p42/p44 MAPK, and HA-HBx using specific antibodies by Western blot analysis.  

C. HeLa cells (8 x 10⁵ cells/dish) were seeded in 60-cm² dishes and incubated for overnight. The cells were transfected with 2-μg pCMV-Myc-HBx. After one hour of transfection, the cells were treated with either 100 μM CoCl₂, PD98059, or Wortmannin for 24 h as indicated. Fifty microgram cell lysates were analyzed for expression of HIF-1α, Myc-HBx, and α-tubulin using specific antibodies by Western blot analysis.  

D. 293 cells (2 x 10⁶ cells/dish) were seeded in 60-cm² dishes and incubated overnight. The cells were transfected with 2-μg p3XFLAG™7.1-HIF-1α and/or 2-μg pCMV-Myc-HBx. After one hour of transfection, the cells were incubated with or without 100 μM PD98059 for 24 h. At the end of incubation, the cells were treated with 10 μM cycloheximide (CHX) for the indicated period. Fifty microgram cell lysates were analyzed for the expression of FLAG-HIF-1α, Myc-HBx, and α-tubulin by Western blot analysis.  

E. Chang X-34 cells (7.5 x 10⁴ cells/chamber) were seeded in 4-chamber slide glass and incubated overnight. Chang X-34 cells were treated with or without 100 μM PD98059 in the presence of 2 μg/ml doxycycline (Doxy) for 24 h. The cells were fixed, incubated with anti-HIF-1α antibody and analyzed by confocal microscopy. The nuclear translocation of HIF-1α was quantitated by counting at least 400 cells for each experiment. The percentage of cells with the HIF-1α in cytoplasm (C), the nucleus (N), and both (C + N) is shown. One representative of at least three independent experiments with similar results is shown.

FIG. 7. HBx interferes the association of HIF-1α and VHL. 293 cells (2 x 10⁶ cells/dish) were seeded in 60-cm² dishes and incubated overnight. The cells were transfected with 2-μg pCMV-Myc-VHL and/or 2-μg pCMV-HA-HBx. After one hour of transfection, the cells were incubated with 100 μM CoCl₂ or 100 μM
PD98059 for 24 h. At the end of incubation, the cells were treated with 10 μM MG132 for 1 h. Five hundred microgram whole cell lysates were immunoprecipitated with anti-HIF-1α antibody, and then analyzed using either anti-HIF-1α antibody or anti-Myc antibody by Western blot analysis. Fifty microgram whole cell lysates were analyzed for the expression of the indicated proteins by Western blot analysis. Representative figures of at least three independent experiments with similar results were shown.

FIG. 8. HBx enhances the interaction between HIF-1α and CBP. 293 cells (2 x 10^6 cells/dish) were seeded in 60-cm² dishes and incubated overnight. The cells were transfected with 2-μg p3XFLAG™7.1-HIF-1α, 2-μg pCMV-Myc-HBx, and/or corresponding empty vectors (EV) as indicated. After one hour of transfection, the cells were incubated with 100 μM CoCl₂ or 100 μM PD98059 for 24 h. At the end of incubation, the cells were treated with 10 μM MG132 for 1 h. Five hundred microgram whole cell lysates were immunoprecipitated with anti-FLAG antibody coated agarose beads for overnight, and the resulting immunocomplex was analyzed using anti-CBP antibody by Western blot analysis. Fifty microgram whole cell lysates were analyzed for expression of the indicated proteins by Western blot analysis. Representative figures of at least three independent experiments with similar results were shown.

FIG. 9. Expression of HIF-1α and VEGF in the liver of HBx transgenic mice. A. One hundred microgram whole cell lysates obtained from liver tissues of the indicated aged transgenic mice and of a 4-month old C57BL/6 (B6) wild-type mouse were fractionated by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and analyzed using specific antibodies for HIF-1α, VEGF, or α-tubulin as described in Materials and Methods. B. Immunohistochemistry of VEGF in the liver of HBx transgenic mice. Expression of VEGF was detected using specific anti-VEGF antibodies in the liver of HBx transgenic mice of 13-month old (a). The expression of VEGF was not detectable either in the liver tissue obtained from a C57BL/6 (B6) wild-type mouse (b) or in the liver of HBx transgenic mice stained without primary antibody (c). Representative figures of at least three independent experiments with similar results were shown.
Figure 2. Yoo et al.
Figure 3. Yoo et al.

Chang  
Chang X-34  
Chang X-31

HIF-1α
phospho-p44/p42
p44/p42
HA-HBx

Doxy  - - - - + + - - + +  
CoCl₂  - + - + - + - + - +

Figure 4. Yoo et al.

HRE-Luc  
Gal4-tk-Luc  
VEGF promoter-Luc

Luc activity (x 10⁴)

HBx (ng)  - 2 5 10 - -  
mHBx (ng)  - - - 2 5 - -

Luc activity (x 10⁵)

HBx (ng)  - - 5 5 - -  
mHBx (ng)  - - - 5 5 - -  
CoCl₂  - + - + - -  

Luc activity (x 10⁶)

HBx (ng)  - - 5 5 - -  
mHBx (ng)  - - - 5 5 - -  
CoCl₂  - + - + - -  

Figure 6. Yoo et al. (cont’d)

C

\[
\begin{array}{ccccccc}
\text{CoCl}_{2} & - & + & - & - & - & - & - \\
\text{Myc-HBx} & - & - & + & + & + & + & + \\
\text{PD98059 (μM)} & - & - & - & 50 & 100 & 200 & - \\
\text{Wortmannin (nM)} & - & - & - & - & - & - & 1 \\
\end{array}
\]

D

\[
\begin{array}{ccccccc}
\text{Myc-HBx} & + & + & + & + & + & + \\
\text{PD98059} & - & - & - & + & + & + \\
\text{CHX} & 0 & 5 & 60 & 0 & 5 & 60 \text{ (min)}
\end{array}
\]
E

HIF-1α

DAPI

Overlay

Chang X-34
(Doxy)

Chang X-34
(PD98059 + Doxy)

Stained cells (%)

Figure 6. Yoo et al. (cont’d)
Figure 7. Yoo et al.

Figure 8. Yoo et al.
Figure 9. Yoo et al.

A

B6 | HBx
---|---
- | -
HIF-1α
VEGF
α-tubulin

4 1 3 5 7 9 11 13 (month)

B

a

b

c

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Hepatitis B virus X protein enhances transcriptional activity of hypoxia-inducible factor-1 alpha through activation of mitogen-activated protein kinase pathway

Young-Gun Yoo, Seung Hyun Oh, Eun Sook Park, Hyeseong Cho, Naery Lee, Hyunsung Park, Dae Kyong Kim, Dae-Yeul Yu, Je Kyung Seong and Mi-Ock Lee

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