The carboxy-terminus of the hepatitis B virus X protein is necessary and sufficient for the activation of hypoxia-inducible factor-1α

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Abstract Hepatitis B virus X protein (HBx) of the hepatitis B virus is strongly implicated in angiogenesis and metastasis during hepatocarcinogenesis. Previously, we reported that HBx enhances activity of hypoxia-inducible factor-1α (HIF-1α), a potent transactivator that induces angiogenic factors. Here, we delineate the structural region of HBx that potentiates HIF-1α. The carboxy-terminus of HBx increased the stability of HIF-1α protein, probably through inhibiting interaction with von Hippel-Lindau protein. Further, the carboxy-terminus of HBx enhanced the transactivation function of HIF-1α by enhancing its association with CREB binding protein (CBP). Finally, we demonstrated the physical association of HBx with the basic helix–loop–helix/PER–ARNT–SIM domain, the inhibitory domain, and the carboxy-terminal transactivation domain of HIF-1α in vivo.

Keywords: Hepatitis B virus X protein; Hypoxia-inducible factor-1α; Hepatitis B virus

1. Introduction

Hepatitis B virus (HBV) is one of the major causes of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [1]. Among the HBV proteins, HBV X protein (HBx), which encodes the X gene of the HBV genome, is indispensable for viral replication in the natural host and is highly conserved among mammalian Hepadnaviridae [2]. HBx has been shown to be expressed both during viral infection and in HBV-associated HCC [1,3]. HBx is distributed in the cytoplasm but to some extent in the nucleus, suggesting an important function of the protein in modulating wide range of intracellular events of host cells [1,4,5].

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Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBx, hepatitis B virus X protein; HIF-1, hypoxia inducible factor-1; ARNT, aryl hydrocarbon nuclear translocator; VHL, product of von Hippel-Lindau; HPH, HIF-1 prolyl hydroxylases; FIH-1, factor inhibiting HIF-1; CBP, CREB binding protein; HA, hemaglutinin; β-gal, β-galactosidase; GST, glutathione *S*-transferase; HRE, hypoxia response element; MAPK, mitogen-activated protein kinase; bHLH/PAS, basic helix–loop–helix/PER–ARNT–SIM; ODD, oxygen-dependent degradation domain; ID, inhibitory domain; CTAD, C-terminal transactivation domain

Large and advanced HCCs are richly supplied with blood vessels, indicating that angiogenic factors are involved in the HBV-associated hepatocarcinogenesis. HBx has been strongly implicated in angiogenesis and metastasis. Recently, we and others have shown that HBx enhances protein stability as well as the transactivation function of hypoxia inducible factor-1α (HIF-1α), a major transcriptional factor that regulates expression of angiogenic factors such as vascular endothelial growth factor (VEGF) [6,7]. HIF-1α forms a heterodimer with HIF-1β, the previously described aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1β is constitutively expressed, whereas the transcriptional activity of HIF-1α is tightly regulated by the cellular oxygen concentration [8]. Under normoxic condition, the HIF-1 prolyl hydroxylases (HPHs) induce hydroxylation of Pro-402 and Pro-564 residues in the oxygen-dependent degradation domain (ODD) of HIF-1α, resulting in the binding of HIF-1α to the von-Hippel-Lindau protein (VHL), the recognition component of an E3 ubiquitin protein ligase. Similarly, ARD1, which functions as the acetyltransferase, induces acetylation of Lys-532 residue which enhances interaction of HIF-1α with VHL [9]. Once bound to VHL, HIF-1α undergoes ubiquitination and proteasomal degradation [10,11]. Hydroxylation of Asn-803 within the C-terminal transactivation domain (CTAD) is carried out by the factor inhibiting HIF-1 (FIH-1), which binds the inhibitory domain (ID) and then blocks the association of HIF-1α with p300 [12]. Under hypoxic condition, HIF-1α accumulates and associates with the ARNT and the complex interacts with hypoxia response element (HRE) of target genes in the nucleus [13,14].

Mutations and deletions in the HBx open reading frame are frequently found in naturally occurring human HCC [15–22], which may indicate differential functions of structural domains of HBx that contribute to the development of HCC. In the present investigation, therefore, we analyzed the functional domain of the HBx that induces transcriptional activity of HIF-1α to provide further insights on the role of HBx in the HBV-associated hepatocarcinogensis.

2. Materials and methods

2.1. Cell culture

HepG2 (ATCC HB-8065), 293 (ATCC CRL-1573), and NIH3T3 (CRL-1658) cells were obtained from the American Type Culture Collection. Chang X-34, which expresses HBx under the control of doxycycline (Doxy)-inducible promoter, was described previously [6]. Cells were maintained in Dulbecco's modified Eagle's medium

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containing 10% fetal bovine serum at 37 °C in a humid atmosphere of 5% CO_2. Hypoxic condition was induced chemically by treating cells with 100 μM $CoCl_2$.

2.2. Plasmids and reporter gene assays

The pCMV-Myc-HBx, the truncated deletion constructs of HBx, i.e., HBx_{NT} (amino acids 1–57) and HBx_{CT} (amino acids 57–154), the p3XFLAG^{TM}7.1-HIF-1α, the truncated pEBG-HIF-1α constructs, pGal4-HIF-1α, and pCMV-HA-VHL, were described previously [6,23,24]. The HRE-Luc, the VEGF promoter-Luc, and the Gal4-*tk*-Luc were described previously [6]. Transient transfection and luciferase assay were performed as described previously [6].

2.3. Western blot analysis and immunoprecipitation

Cells were lysed in a lysis buffer and whole cell lysates were obtained by subsequent centrifugation as described previously [6]. 50 μg of protein was subjected to 8–15% sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Membranes were probed with specific antibodies against HIF-1α, Myc, hemaglutinin (HA), phosphorylated p42/p44, p42/p44, CREB binding protein (CBP) (Santa Cruz Biotech., Santa Cruz, CA), FLAG (Sigma, St. Louis, MO), or α-tubulin (Oncogene, Boston, MA). For immunoprecipitation, 500 μg of whole cell lysates was incubated with 1 μg of anti-HIF-1α, anti-FLAG, anti-Myc or anti-glutathione *S*-transferase (GST) and immunoreactive proteins were detected as described previously [6].

3. Results

3.1. The carboxy terminus of HBx induces transcriptional activity of HIF-1α

Based on the previous observation that the carboxy-terminus of HBx is crucial for the transactivation function [21,22,25], we constructed two HBx mutants, HBx_{NT} and HBx_{CT}, as shown in Fig. 1A. First, we carried out reporter gene analysis by transient transfection of the truncated HBx expression vectors together with a reporter gene containing HRE sequences located in the promoter of erythropoietin [6]. Consistent with our previous report, the wild-type HBx induced the HRE reporter dose-dependently (Fig. 1B). To compare with the wild-type HBx, the HBx_{NT} induced the reporter slightly only at the highest dose examined. However, the HBx_{CT} enhanced the reporter as strongly as the wild-type HBx (Fig. 1B). Similar results were obtained using the VEGF promoter-Luc reporter (Fig. 1B).

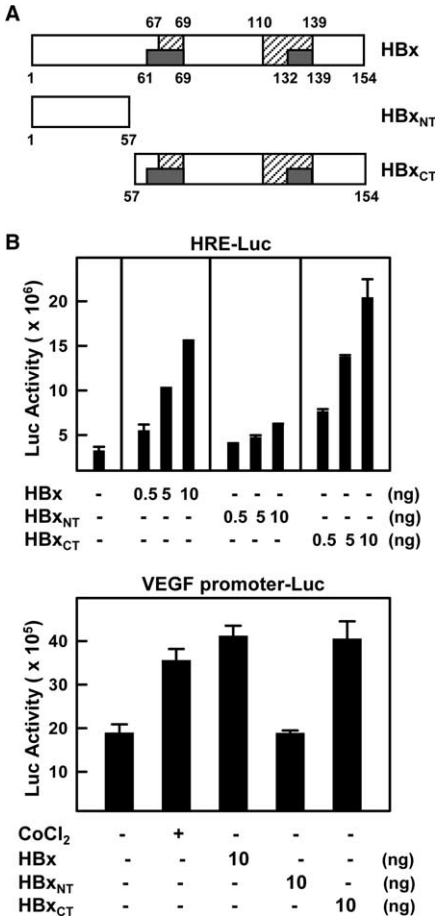

Fig. 1. The carboxy-terminus of HBx is essential for enhancing transcriptional activity of HIF-1α. (A) A schematic representation of the wild-type HBx and the truncated constructs. The shaded and striped regions represent Kunitz domain homologous region and essential transactivation domain (TAD), respectively. (B) The HRE-Luc reporter gene (0.1 μg) (upper panel) or VEGF promoter-Luc (0.3 μg) (lower panel) was co-transfected with the indicated amount of the expression plasmid for HBx, HBx_{NT}, or HBx_{CT} into HepG2 cells. After 24 h of transfection, the cells were incubated in the presence or absence of 100 μM $CoCl_2$ for 24 h, and then cell lysates were obtained and assayed for luciferase activity. Data represent means ± S.D. of three independent experiments.

3.2. The carboxy terminus of HBx increases stability of HIF-1α protein

Next, we examined whether the HBx_{CT} enhanced stability of HIF-1α protein, since transcriptional activity of HIF-1α is primarily regulated by the stability of protein. The HBx_{CT} induced the protein-level of HIF-1α as strongly as the wild-type HBx, while the HBx_{NT} did not (Fig. 2A). When the stability of HIF-1α was measured in the presence of cycloheximide (CHX), which blocks new protein synthesis, the HBx_{CT} blocked degradation of HIF-1α as efficiently as the wild-type HBx, whereas the HBx_{NT} did not at all (Fig. 2B). Because the stability of HIF-1α is regulated by VHL which subsequently drives the ubiquitin–proteasomal degradation of HIF-1α, we examined whether the HBx_{CT} reduced the binding of HIF-1α to VHL. As in our previous report, when cells were treated with MG132, which blocks proteasome function, the binding of HIF-1α and VHL was strong, but the binding was largely diminished in the presence of hypoxia-mimicking agent, $CoCl_2$, the wild-type HBx, and the HBx_{CT} (Fig. 2C) [6]. We previously demonstrated that HBx induces stability of HIF-1α through activation of mitogen-activated protein kinase (MAPK) signaling pathways [6]. When either the wild-type HBx or the HBx_{CT} was overexpressed, the phosphorylation of p44/p42 was strongly enhanced, which was in the same pattern of the expression of HIF-1α (Fig. 2D). The result was consistent with the previous observation that amino acids 58–119 of HBx were sufficient to activate MAPK signaling pathways in mouse liver [26].

3.3. The carboxy terminus of HBx induces transactivation function of HIF-1α

Transcriptional activation of HIF-1α can also be achieved by enhancing transactivation functions of HIF-1α. Therefore, we examined whether the HBx and its mutants directly enhanced transactivation function of HIF-1α using a Gal4-driven reporter system [6]. Gal4-HIF-1α largely enhanced Gal4-*tk*-Luc activity in the presence of either $CoCl_2$ or the

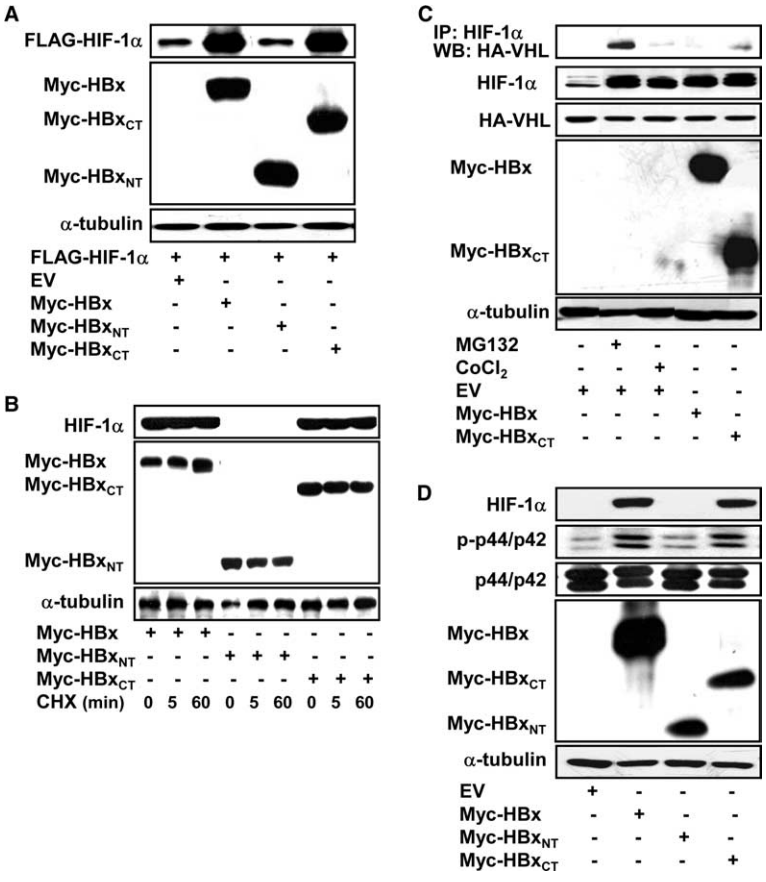

Fig. 2. HBx_{CT} increases the stability of HIF-1α. (A) 293 cells (1×10^6 cells/well) were seeded in 6-well plates and incubated overnight. The cells were transfected with 3 μg each of p3XFLAG^{TM}7.1-HIF-1α, pCMV-Myc-HBx, -HBx_{NT}, -HBx_{CT}, and empty vector (EV) with the indicated combination. After 24 h of transfection, 50 μg of whole cell lysates was analyzed for the expression of the indicated proteins. (B) 293 cells (1×10^6 cells/well) were seeded in 6-well plates and incubated overnight. The cells were transfected with 3 μg each of pCMV-Myc-HBx, -HBx_{NT} or -HBx_{CT}. After 24 h of transfection, the cells were treated with 10 μM CHX for the indicated period. 50 μg of whole cell lysates was analyzed for the expression of the indicated proteins. (C) NIH3T3 cells (8×10^5 cells/dish) were seeded in 60-cm^2 dishes and incubated overnight. The cells were transfected with 3 μg pCMV-HA-VHL with 3 μg each of pCMV-Myc-HBx, -HBx_{CT}, or EV. After 1 h of transfection, the cells were treated with 10 μM MG132 for 1 h or 100 μM $CoCl_2$ for 24 h as indicated. 500 μg of whole cell lysates was immunoprecipitated with anti-HIF-1α antibody and then analyzed using anti-HA antibody. 50 μg of whole cell lysates was analyzed for the expression of the indicated proteins. (D) 293 cells (1×10^6 cells/well) were seeded in 6-well plates and incubated overnight. The cells were transfected with 3 μg of each pCMV-Myc-HBx, -HBx_{NT}, -HBx_{CT}, or EV. After 24 h transfection, 50 μg of whole cell lysates was analyzed for the expression of the indicated proteins. The expression of α-tubulin was monitored as a control. One representative of at least three independent experiments with similar results is shown.

wild-type HBx. The HBx_{NT} did not induce the reporter, but the HBx_{CT} induced it as strongly as the wild-type HBx (Fig. 3A). The association of HIF-1α with coactivator CBP increased in the presence of either the wild-type HBx or the HBx_{CT}. These results indicate that the carboxy-terminus of HBx increases the transactivation of HIF-1α by enhancing the association of HIF-1α with CBP.

3.4. The carboxy-terminus of HBx interacts with HIF-1α protein

Finally, we examined whether HIF-1α and HBx were physically associated for the cross-talk. We employed the Chang X-34, in which the expression of HBx gene is under control of an inducible Doxy promoter [6]. As shown in Fig. 4A, HBx was immunoprecipitated with HIF-1α in the Chang X-34 cells in the presence of Doxy. The specificity of the association was demonstrated by the fact that normal rabbit IgG did not precipitate HBx (Fig. 4A). The result was confirmed by cotransfection of expression vectors for HBx and HIF-1α in 293 cells. The HBx_{NT} was not associated with HIF-1α, whereas the HBx_{CT} strongly interacted with HIF-1α in vivo (Fig. 4B). To examine which domain of HIF-1α bound to the HBx protein, six GST-fused HIF-1α deletion chimeras were tested (Fig. 5A) [23]. Coimmunoprecipitation assay showed that HBx bound to all the GST-fused HIF-1α deletion chimeras except one that contained aa 401–603, which is known to bind VHL and ARD1 (Fig. 5B).

4. Discussion

Previously, we and others reported that HBx enhances transcriptional activity as well as expression of target genes of HIF-1α, which could be one of the important mechanisms by which HBx induces hepatocarcinogenesis [6,7]. Several in vitro studies indicate that the first 50 amino acids at the amino-terminus of HBx are sufficient for cell transformation, while the carboxy-terminus of HBx is essential for maintaining

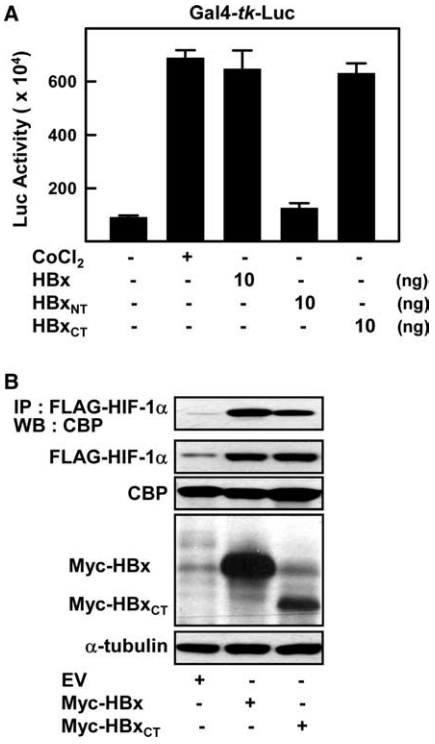

Fig. 3. HBx_{CT} increases transactivation function of HIF-1α. (A) The Gal4-*tk*-Luc reporter gene (0.2 μg) was co-transfected with the indicated amount of pCMV-Myc-HBx, -HBx_{NT}, or -HBx_{CT} into HepG2 cells. After 24 h of transfection, the cells were incubated with 100 μM $CoCl_2$ for 24 h, and then cell lysates were obtained and assayed for luciferase activity. Data represent means ± S.D. of three independent experiments. (B) 293 cells (3×10^6 cells/dish) were seeded in 100-cm^2 dishes and incubated overnight. The cells were transfected with 3 μg p3XFLAG^{TM}7.1-HIF-1α with 3 μg each of pCMV-Myc-HBx, -HBx_{CT}, or EV. After 24 h of transfection, 500 μg of whole cell lysates was immunoprecipitated with anti-FLAG antibody and then analyzed using anti-CBP antibody. 50 μg of whole cell lysates was analyzed for the expression of indicated proteins. The expression of α-tubulin was monitored as a control. One representative of at least three independent experiments with similar results is shown.

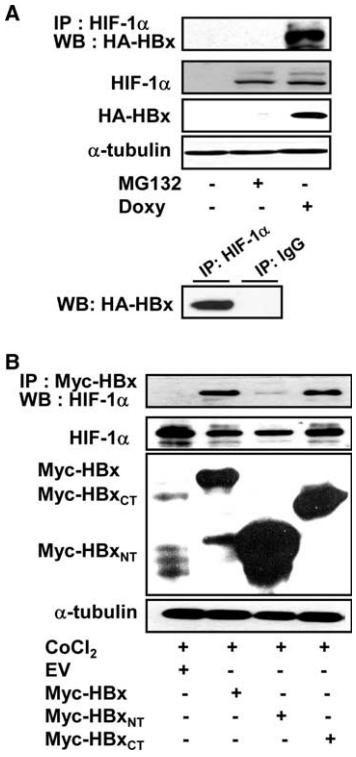

Fig. 4. HBx interacts with HIF-1α protein. (A) Chang X-34 cells (2×10^6 cells/dish) were seeded in 100-cm^2 dishes and incubated overnight. The cells were treated with 2 μg/ml Doxy for 24 h or 10 μM MG132 for 1 h as indicated. 500 μg of whole cell lysates was immunoprecipitated with anti-HIF-1α antibody and then analyzed using anti-HA antibody. 50 μg of whole cell lysates was analyzed for the expression of indicated proteins (upper panel). Normal rabbit IgG was used for immunoprecipitation of the whole cell lysates obtained from the Doxy-treated Chang X-34 cells (lower panel). (B) 293 cells (3×10^6 cells/dish) were seeded in 100-cm^2 dishes and incubated overnight. The cells were transfected with 3 μg each of pCMV-Myc-HBx, -HBx_{NT}, -HBx_{CT} or EV. After 1 h of transfection, cells were incubated in the presence of 100 μM $CoCl_2$ for 24 h as indicated. 500 μg of whole cell lysates was immunoprecipitated with anti-Myc antibody and then analyzed using anti-HIF-1α antibody. 50 μg of whole cell lysates was analyzed for the expression of indicated proteins. The expression of α-tubulin was monitored as a control. One representative of at least three independent experiments with similar results is shown.

transactivation function of the HBx [25]. These data may suggest that distinct functions of structural domains of HBx may play different roles in the HBV-related hepatocarcinogenesis. Thus, we investigated the functional domain of HBx that induces transcriptional activity of HIF-1α and found that the carboxy-terminus of HBx is important in the transactivation as well as stability of HIF-1α protein.

Since it was reported that the number of HBV genomes was low in HBsAg negative tumorous livers of HCC patients [20,27], the biological relevance of our findings might be questioned. However, the expression of HBx in chronic HBV infection and in progression of HCC remains controversial. Several studies have shown that HBx protein was detected in a high portion of samples, for instance, 10 of 18 (58.8%) of livers from HCC patients and 69% of samples from patients with chronic HBV infection [28–32]. The X gene is the most frequently integrated portion of HBV DNA found in hepatocyte chromosomes during the development of HCC [33]. It has been shown that most, if not all, HBx isolated from integrated HBV DNA in HCC patients was in a truncated form [15–22]. Interestingly enough, Lee et al. demonstrated that hypoxia stimulated the expression of HBx gene in HBV-integrated liver cancer cells obtained from HCC patients [34]. They also showed that hypoxia increased the activity of HBV Enhancer1 and suggested that hypoxia may be a stimulus for the induction of HBx expression. Since inflammatory cytokines such as IFN-γ, TNF, and IL-1β induce stabilization of HIF-1α even in the absence of hypoxic stress, these cytokines may enhance the expression of HBx [35,36]. Taken together, a significant portion of HCC liver expresses HBx, and the cross-talk between HBx and HIF-1α may play an important role in the progression of HCCs.

Distinct structural features are required for protein stability, heterodimerization with ARNT, DNA binding, and transactivation function of HIF-1α. Factors such as HPH, VHL, FIH-1, and coactivator CBP/p300 regulate the function of HIF-1α by direct binding to the specific structural domain of HIF-1α. Therefore, the interaction region of each HIF-1α and HBx may provide a potential molecular mechanism of the cross-talk between these two proteins. Indeed,

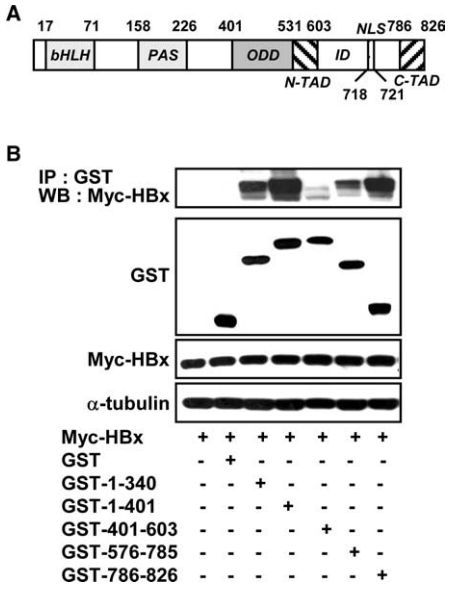

Fig. 5. HBx interacts with both the amino- and carboxy-terminus of HIF-1α. (A) Schematic representation of full-length HIF-1α containing bHLH/PAS, TAD, ODD, and ID [13]. (B) NIH3T3 cells (8×10^5 cells/dish) were seeded in 60-cm^2 dishes and incubated overnight. The cells were transfected with 3 μg of each the pEBG-HIF-1α constructs containing the indicated numbered fragment of HIF-1α. 500 μg of whole cell lysates was immunoprecipitated with anti-GST antibody and then analyzed using anti-Myc antibody. 50 μg of whole cell lysates was analyzed for the expression of indicated proteins. The expression of α-tubulin was monitored as a control. One representative of at least three independent experiments with similar results is shown.

we found that the C-terminus of HBx binds to the basic helix–loop–helix PER–ARNT–SIM (bHLH/PAS), ID, and CTAD (Figs. 4 and 5). The bHLH/PAS domain is required for the heterodimerization with ARNT, which is required for the optimal DNA binding [37]. HBx, therefore, may enhance DNA binding of HIF-1 by direct interaction with this region. Consistent with this result, recently Moon et al. [7] reported that HBx directly interacted with the bHLH/PAS domain of HIF-1α in vitro. Also, the binding of HBx with ID and CTAD in our experiment may suggest that HBx blocks the association of FIH-1 with HIF-1α, which inhibits the hydroxylation of Asn-803, and subsequent recruitment of p300/CBP. In addition, we showed that HBx reduces the binding of HIF-1α with VHL. This may represent a decrease in the activity of HPH, which does not require direct association of HBx with ODD domain of HIF-1α (Figs. 2 and 5). HBx was detected predominantly in the cytoplasm; however, it was also found in the nuclei of positively stained hepatocytes, either exclusively nuclear or localized both in the nucleus and cytoplasm within the same cell [38–40]. Further, HBx interacts with a variety of proteins: cytoplasmic, nuclear, and those that traffic between the cytoplasm and the nucleus [5]. Therefore, our results may suggest that HBx has a dual role in that it may enhance stability of HIF-1α through blocking proteasomal degradation in the cytoplasm and it induces transactivation function of HIF-1 by recruiting coactivator in the nucleus.

In HBV-associated HCC tumor tissues, most of the distal carboxy-terminus of HBx sequences contains deletion, insertion, or point mutation, which contrasts with the full-length HBx isolated from sera and non-tumor tissues [19–22]. Analysis of these naturally occurring HBx mutants for enhancing HIF-1α activity and for functions in new vessel formation may provide new insights on the HBV-associated hepatocarcinogenesis.

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