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Stronger activation of SREBP-1a by nucleus-localized HBx

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ABSTRACT

We previously showed that hepatitis B virus (HBV) X protein activates the sterol regulatory element-binding protein-1a (SREBP-1a). Here we examined the role of nuclear localization of HBx in this process. In comparison to the wild-type and cytoplasmic HBx, nuclear HBx had stronger effects on SREBP-1a and fatty acid synthase transcription activation, intracellular lipid accumulation and cell proliferation. Furthermore, nuclear HBx could activate HBV enhancer I/X promoter and was more effective on up-regulating HBV mRNA level in the context of HBV replication than the wild-type HBx, while the cytoplasmic HBx had no effect. Our results demonstrate the functional significance of the nucleus-localized HBx in regulating host lipogenic pathway and HBV replication.

1. Introduction

Hepatitis B virus (HBV) is a partially double-stranded circular DNA virus belongs to the Hepadnavirus family [1]. HBV infection causes acute and chronic liver disease and is a high risk factor for the development of hepatocellular carcinoma (HCC) [33]. HBV X protein (HBx) functions as a transcriptional transactivator of host genes and promotes cell growth and possibly HCC [30,31]. HBV gene expression is regulated by two HBV promoters/enhancers [29]. HBx activates both elements and thus increases HBV replication [4,10]. HBx localizes in the nucleus and the cytoplasm, which has functional implications [5,9,21]. Different subcellular localizations of HBx display different transactivation activities. In comparison to the wild-type HBx, nucleus-localized HBx significantly activates HBV enhancer I/X but not NF-κB or AP-1; on the other hand, cytoplasmic HBx is crucial for activating the Ras-Raf-MAP kinase, NF-κB and AP-1 [6,7]. Nuclear, but not cytoplasmic, HBx is required for HBV replication in cell culture [16].

Sterol regulatory element-binding protein-1a (SREBP-1a) is a potent transcription factor for genes involved in fatty acid synthesis and cholesterol synthesis [12,27]. Our previous study showed that HBx activates SREBP-1a at the transcription level involving two transcriptional factors C/EBP and E4BP4 [25]. However, whether the subcellular localization of HBx plays a role in this process has not been investigated. In this study, we demonstrated that, in comparison to the cytoplasmic HBx, the nucleus-localized HBx has stronger effects on up-regulating SREBP-1a, fatty acid synthase (FASN), lipid accumulation, cell proliferation, HBV enhancer I/X promoter, and HBV mRNA in the context of HBV replication.

2. Materials and methods

2.1. Plasmids

A plasmid expressing HBx with a myc-tag at the C-terminus under the control of the elongation factor-1α promoter, HBV enhancer II/core promoter luciferase reporter, SREBP-1a and FASN promoter luciferase reporters, and a greater-than-unit-length HBV genome without expressing HBx (payw 1.2*7) were described previously [13,22,25]. A nuclear localization signal (NLS, PKKKRKVFL) [6] or a nuclear export signal (NES, LALKLAGLDI) [16] was added to the N-terminus of the HBx coding sequence to create NLS-HBx or NES-HBx, respectively. The HBV enhancer I/X promoter sequence [23] was cloned upstream of the luciferase gene into the pGL4.14 vector (Promega), generating the pGL4-HBV enhancer I/X promoter.

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2.2. Cell culture, transfection, and nuclear fractionation

HuH-7 cells [19] were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Transfection and nuclear fractionation were described previously [13,32].

2.3. Western blotting and antibodies

Western blotting was performed as described [14,32]. Fibrillarin, β-actin and myc epitope antibodies were from Sigma–Aldrich and Cell Signaling Technology, respectively.

2.4. Reverse transcription and real-time PCR

RNA extraction, reverse transcription, and real-time PCR was performed as described [11,25]. The primers for the real-time PCR were SREBP-1a-FD (5′ CGCTGCTGACCGACAT 3′) and SREBP-1a-rev (5′ CAAGAGAGGAGCTCAATG 3′), FASN-FD (5′ TCATCCCCCTGATGAAGAAG 3′) and FASN-rev (5′ ACTCCACAGGTGGGAACAAG 3′), HBV-FD (5′ AGAAACAACACATAGCGCCTCAT 3′) and HBV-rev (5′ TGCCCCATGCTGTAGATCTTG 3′), and β-glucuronidase (GUSB)-FD (5′ GGTGCTGAGGATTGGCAGTG 3′) and GUSB-rev (5′ CGCACTTCCAACTTGAACAGG 3′).

2.5. Luciferase assay

Cells were lysed in a Passive Lysis Buffer (Promega) and the luciferase activity was determined using the luciferase assay reagents (Promega) in a TD 20/20 Luminometer (Turner Designs). After normalization against the total protein concentration in the same sample, the luciferase results were analyzed for statistical differences using Student *t* test. A *p* value of ≤ 0.05 was considered statistically significant.

2.6. Oil RED O staining and MTT assay

The amounts of the neutral lipids were measured at 500 nm [17] after Oil Red O (ORO) staining as previously described [13]. The MTT assay was performed as described [11].

3. Results and discussion

3.1. HBx subcellular localization analyzed by nuclear fractionation

Previous studies have shown that HBx in different subcellular compartments may have different functions [6,7,16]. However, the impact of the subcellular localization of HBx on lipogenic gene expression has not been studied. To specifically study the function of nucleus- and cytoplasm-localized HBx, a nuclear localization signal (NLS, PKKKRKVFL; NLS-HBx) [6] or a nuclear export signal (NES, LALKLAGLDI; NES-HBx) [16] was added to the N-terminus of the HBx coding sequence [25]. This is a widely used experimental approach [2,6,16,24]. To determine the intracellular distribution of the HBx proteins, nuclear and cytoplasmic fractions as well as total cell lysates were prepared and subjected to Western blotting after transfecting HuH-7 cells with the HBx-expressing plasmids. As shown in Fig. 1, wild-type HBx was present in both nuclear and cytoplasmic fractions as expected; NES-HBx was found predominantly in the cytoplasmic fraction, whereas NLS-HBx was predominantly nuclear. These results demonstrated that addition of a nuclear export signal to HBx efficiently excludes HBx from the nucleus and a nuclear localization signal renders nuclear localization of HBx.

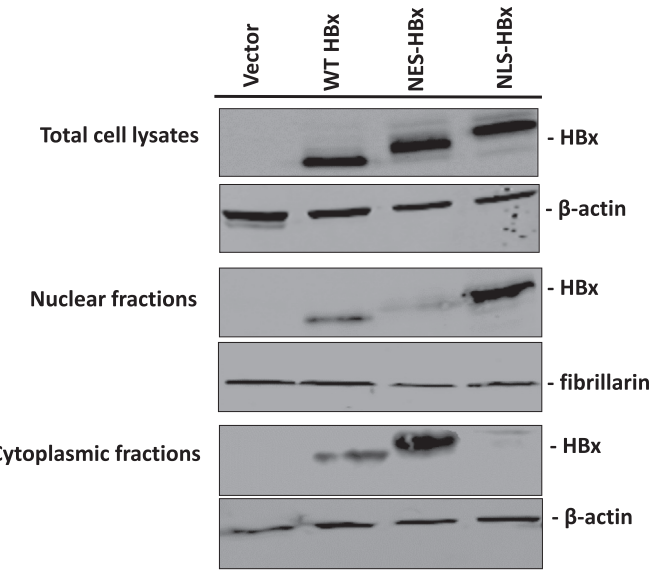

Fig. 1. Expression and subcellular localization of HBx proteins. HuH-7 cells were transfected with plasmids expressing wild-type (WT) HBx, HBx with a nuclear export signal (NES-HBx) or a nuclear localization signal (NLS-HBx) at the N-terminus, or vector. A myc tag is present at the C-terminus of HBx. At 48 h after transfection, total cell lysates, nuclear and cytoplasmic fractions were analyzed by Western blotting using antibodies against the myc tag, β-actin or fibrillarin.

3.2. Nuclear HBx is more effective in up-regulating SREBP-1a and FASN

We previously demonstrated that HBx activates SREBP-1a transcription [25]. To examine the role of subcellular localization of HBx in this process, we co-transfected HuH-7 cells with plasmids expressing wild-type HBx, NES-HBx, NLS-HBx, or empty vector, together with an SREBP-1a promoter luciferase reporter [25]. Consistent with our previous study [25], we observed significant activation of SREBP-1a promoter by wild-type HBx (Fig. 2A). Addition of a nuclear localization signal to HBx resulted in significantly higher SREBP-1a promoter activation than the wild-type HBx (Fig. 2A). In contrast, HBx with a nuclear export signal, NES-HBx, did not activate the SREBP-1a promoter (Fig. 2A). Since FASN is one of the target genes of SREBP-1a, we examined the effects of HBx subcellular localization mutants on FASN promoter activity using a FASN promoter luciferase reporter [13]. As shown in Fig. 2C, wild-type HBx resulted in significantly higher FASN promoter activity in comparison to vector. Addition of an NLS further enhanced FASN promoter activation by HBx, whereas the NES-HBx could no longer activate the FASN promoter (Fig. 2C). To substantiate the promoter-reporter results, we determined the transcript levels of endogenous SREBP-1a and FASN. As shown in Fig. 2B and D, the effects of HBx in different subcellular compartments on endogenous SREBP-1a and FASN transcript levels were in agreement with those obtained from promoter-reporter based assays (Fig. 2A and C). These results indicated that nucleus-localized HBx is more effective on the activation of SREBP-1a and FASN transcription. Since we previously showed that HBx activates SREBP-1a through transcriptional factor C/EBP [25] and others showed that HBx can enhance C/EBP activity through direct protein–protein interaction presumably in the nucleus [4], it is conceivable that the nuclear HBx is more readily for interacting with and activating C/EBP, which in turn increases SREBP-1a transcription.

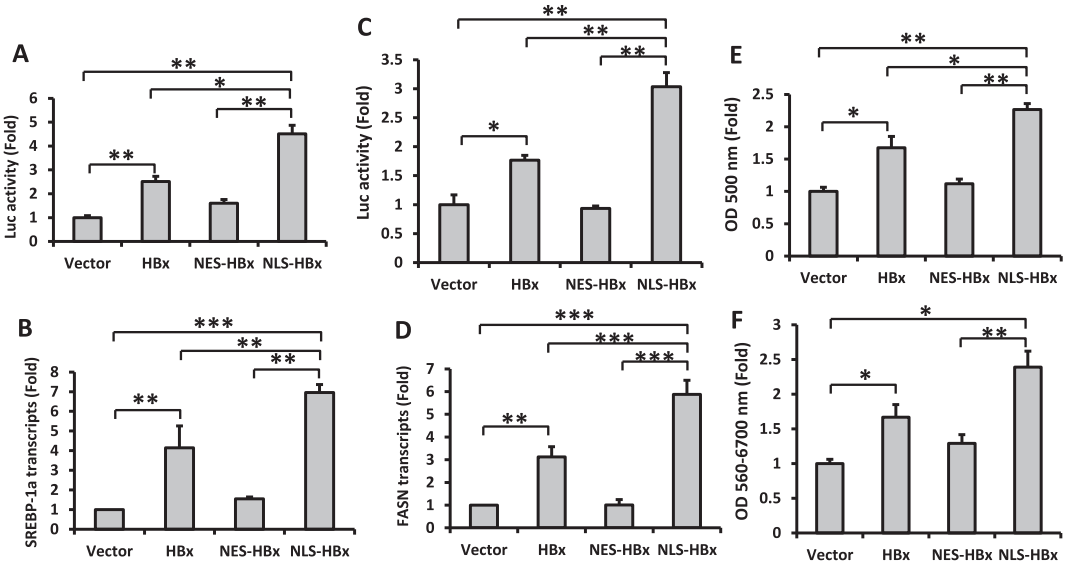

Fig. 2. Nuclear HBx has a stronger effect on SREBP-1a and FASN transcription activation, intracellular lipid accumulation and cell proliferation than wild-type and cytoplasmic HBx. (A and C). HuH-7 cells were co-transfected with plasmids expressing WT, NES-, or NLS-HBx, or vector, together with a SREBP-1a promoter (A) or a FASN promoter (C) luciferase reporters. Luciferase assay was performed at 48 h after transfection and normalized against the protein concentration to determine the promoter activities. (B and D). HuH-7 cells were transfected with plasmids expressing WT, NES-, or NLS-HBx, or vector. At 16 h after transfection, the transcript levels of SREBP-1a (B) and FASN (D) were determined by reverse transcription real-time PCR. The levels of β-glucuronidase (GUSB) were also determined and used for normalization. (E and F). HuH-7 cells were transfected with plasmids expressing WT, NES-, or NLS-HBx, or vector. At 48 h after transfection, cells were subjected to either Oil Red O staining (E) or MTT assay (F). Statistical differences between samples were analyzed by Student's *t* test and demonstrated as * if $p \leq 0.05$, or ** if $p \leq 0.01$.

3.3. Nuclear HBx is more effective on enhancing intracellular lipid accumulation and cell proliferation

To determine the effects of HBx subcellular localization on intracellular lipid accumulation, we measured the amounts of neutral lipids. As expected, wild-type HBx expression was associated with significantly more neutral lipids than vector (Fig. 2E). Lipid accumulation was further enhanced by the NLS-HBx or returned to baseline by the NES-HBx (Fig. 2E). Since increased lipogenesis and lipid accumulation have been recognized as one of the major factors driving cell proliferation [18], we also determined the effects of HBx and its mutants on cell proliferation by a standard MTT assay. As shown in Fig. 2F, wild-type HBx could significantly increase cell proliferation than vector control. Once again, this effect was enhanced by a nuclear localization signal and dampened by a nuclear export signal (Fig. 2F). These results indicated that nucleus-localized HBx is more effective on stimulating lipid accumulation and cell proliferation, probably as a consequence of enhanced SREBP-1a and FASN activity.

3.4. Nuclear HBx is more effective on up-regulating HBV mRNA

HBx also activates HBV enhancers and thus increases HBV replication [4,10]. To examine how HBx in different subcellular compartments regulates HBV enhancers, we co-transfected HuH-7 cells with plasmids expressing HBx, NES-HBx, NLS-HBx, or empty vector, and luciferase reporters under the control of HBV enhancer I/X [4] or HBV enhancer II/CP [5,25]. As expected, we observed more than 1.5-fold activation of HBV enhancers I and II by the wild-type HBx (Fig. 3A and B). NES-HBx did not activate either enhancers (Fig. 3A and B). NLS-HBx increased HBV enhancer I activity more than 2-fold than baseline (Fig. 3A); although higher than the wild-type HBx, it just fell short of reaching statistical significance ($p = 0.052$). NLS-HBx did not significantly increase HBV enhancer II/CP activity than vector or wild-type HBx (Fig. 3B). These results indicated that while the cytoplasmic HBx does not activate HBV enhancers/promoters, nuclear HBx can increase HBV enhancer I/X activity, but has a marginal effect on HBV enhancer II/CP activity. It has been shown that nuclear HBx can no longer activate NF-κB [6]. Interestingly, HBV enhancer II has an NF-κB binding motif whereas HBV enhance I does not [26]. Therefore, it is possible that the differential effects of nuclear HBx on two HBV enhancers/promoters are due to its ability to activate NF-κB activity. However, the exact mechanisms warrant further investigation. It is worth mentioning that only nuclear, but not cytoplasmic, HBx can rescue HBx-deficient virus replication [16]. The demonstration of HBV enhancer I/X activation by NLS-HBx by our results may have provided a possible mechanistic explanation.

To further understand the biological significance of the results we have so far, we determined the effect of HBx in different subcellular compartments on HBV mRNA levels in the context of HBV replication in a plasmid-based HBV replication system using a greater-than-unit-length HBV genome plasmid. We used an HBV genome without expressing HBx, payw 1.2*7 [22], which is a common approach to study the role of HBx in HBV replication [15,16]. HuH-7 cells were co-transfected with plasmids expressing WT, NES-, or NLS-HBx, or vector, together with HBV genome plasmid payw 1.2*7. At 48 h after transfection, the level of HBV mRNA was determined. As shown in Fig. 3C, wild-type HBx significantly increased HBV mRNA level by more than two folds in comparison to vector. This is consistent with the enhancing role of HBx in HBV replication [28]. While cytoplasmic HBx had no effect on HBV mRNA level, nuclear HBx increased HBV mRNA level by six folds, which was significantly higher than wild-type HBx (Fig. 3C). These results demonstrated that the nuclear HBx is more effective on enhancing HBV mRNA levels in the context of HBV replication than the wild-type HBx. While our results are in agreement with those from the Slagle's group [16], they are not consistent with the results from the Ryu's group [2]. The reason for this discrepancy is not clear, but it may be due to different cell lines and/or the assays used. This has been thoroughly discussed recently [28].

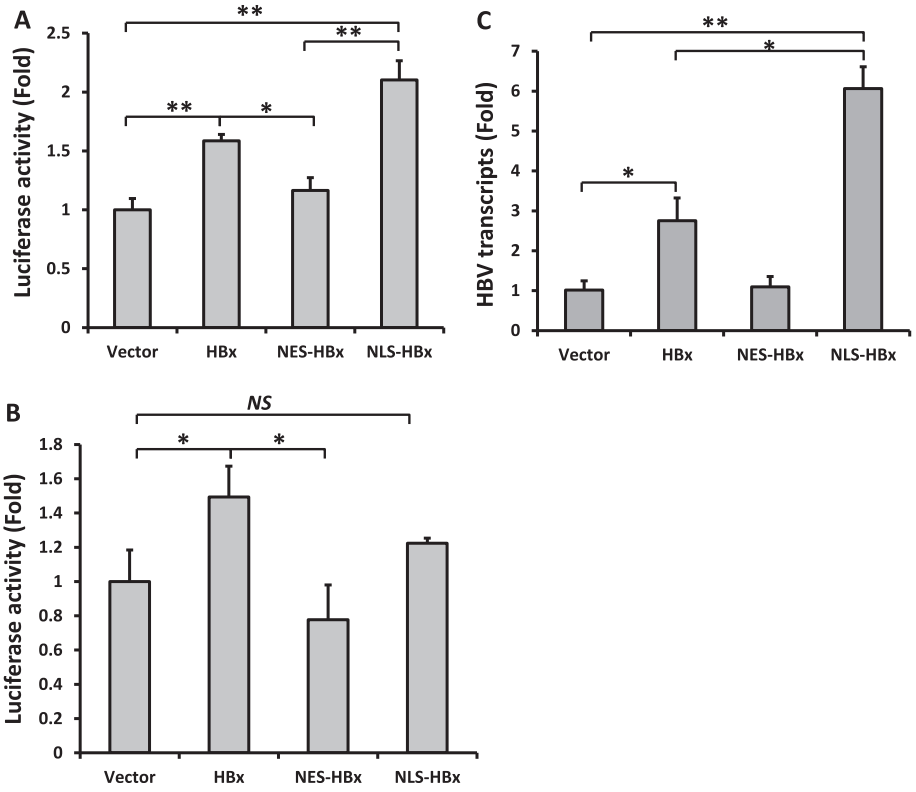

Fig. 3. The effect of HBx on HBV enhancer/promoter and HBV mRNA levels. HuH-7 cells were co-transfected with plasmids expressing WT, NES-, or NLS-HBx, or vector, together with luciferase reporters under the control of HBV enhancer I/X promoter (A) or HBV enhancer II/core promoter (B). Luciferase assay was performed at 48 h after transfection and normalized against the protein concentration to determine the HBV enhancer/promoter activities. (C). HuH-7 cells were co-transfected with plasmids expressing WT, NES-, or NLS-HBx, or vector, together with a greater-than-unit-length HBV genome plasmid payw 1.2*7 (no HBx). At 48 h after transfection, the level of HBV mRNA was determined by reverse transcription real-time PCR. The level of β-glucuronidase (GUSB) was used for normalization. Statistical differences between samples, analyzed by Student's *t* test, were demonstrated as * if $p \leq 0.05$, or ** if $p \leq 0.01$.

Although HBV infection and HBx expression are risk factors for developing hepatocellular carcinoma, the molecular mechanisms are not well understood. A hallmark of carcinogenesis is the abnormally high demand for lipid synthesis by cancer cells. Therefore, activation of SREBP-mediated lipogenic pathways have been recognized as a critical oncogenic mechanism [8]. Clinical studies have found HBx mutations conferring nuclear localization in cancerous tissues at a high frequency [3,20]. Our study demonstrated a stronger activation of the SREBP-1a mediated lipogenic pathway by the nuclear HBx. As such, our results may help understand the biological significance of these clinical findings.

In summary, we have investigated the role of nuclear and cytoplasmic localization of HBx in modulating host lipogenic expression, lipid accumulation, cell proliferation, HBV regulatory elements, and HBV mRNA levels. Our results indicated that nucleus-localized HBx is more effective on activating SREBP-1a and FASN transcription, increasing intracellular lipid accumulation, and cell proliferation. While cytoplasm-localized HBx has no effect on HBV enhancer/promoter activities, nuclear localization plays an important role in enhancing HBV enhancer I/X promoter activity and HBV mRNA level by HBx. Our work sheds more light on the contribution of HBx to the pathogenesis and oncogenesis associated with HBV infection.

Conflict of interest

None.

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