Biochemical and Biophysical Research Communications 432 (2013) 643–649

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

SREBP-1a activation by HBx and the effect on hepatitis B virus enhancer II/core promoter

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ARTICLE INFO

Article history:
Received 24 January 2013
Available online 16 February 2013

Keywords:
HBx
SREBP-1a
C/EBP
E4BP4
HBV enhancer II/core promoter
Transcription

ABSTRACT

Hepatitis B virus (HBV) X protein (HBx) plays an important role in HBV pathogenesis by regulating gene expression. Sterol regulatory element binding protein-1a (SREBP-1a) is a key transcriptional factor for modulating fatty acid and cholesterol synthesis. Here we demonstrated that HBx increased mature SREBP-1a protein level in the nucleus and its activity as a transcription factor. We further showed that the up-regulation of SREBP-1a by HBx occurred at the transcriptional level after ectopic expression and in the context of HBV replication. Deletional analysis using SREBP-1a promoter revealed that the sequence from −436 to −398 in the promoter was required for its activation by HBx. This promoter region possesses the binding sequences for two basic leucine zipper (b-ZIP) transcription factors, namely C/EBP and E4BP4. Mutagenesis of the binding sequences on the SREBP-1a promoter and ectopic expression experiments demonstrated that C/EBPα enhanced SREBP-1a activation by HBx, while E4BP4 had an inhibitory effect. C/EBPα was able to significantly reverse the inhibitory activity of E4BP4 on SREBP-1a promoter. These results demonstrated that HBx activates SREBP-1a activity at the transcription level through a complex mechanism involving two bZIP transcription factors C/EBP and E4BP4 with C/EBP being the dominant positive factor. Finally, we showed that knocking down SREBP-1 abolishes HBV enhancer II/core promoter activation by HBx.

1. Introduction

Sterol regulatory element-binding proteins (SREBPs) belong to the family of basic-helix–loop–helix-leucine zipper (bHLH-ZIP) transcription factors [11]. SREBPs directly activate the expression of numerous genes linked to the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids. The mammalian genome encodes three SREBPs, designated SREBP-1a, SREBP-1c, and SREBP-2 [33]. In comparison to SREBP-1c, SREBP-1a has a longer transcription activation domain at its N-terminus which is capable of recruiting co-activators for transcription [33]. As such, SREBP-1a is a more potent activator of all SREBP-responsive genes for fatty acid and cholesterol synthesis, whereas SREBP-1c can only activate fatty acid synthesis [12,29]. SREBPs are synthesized as inactive precursors and the N-terminal portion, released from the SREBP precursor by proteolysis, enters the nucleus and becomes active transcriptional factors [3]. Nuclear SREBPs activate transcription by binding to SRE sequence in the promoter regions of target genes [11].

Hepatitis B virus (HBV) infection is a global health problem with 350–400 million people being chronic carriers [2]. HBV has a partially double-stranded circular DNA genome coding for core, surface, polymerase, and the X (HBx) proteins [6]. Two viral enhancers promote HBV transcription [23]. Enhancer II/core promoter sequence regulates the transcription of 3.5-kb pregenomic RNA, a key step in HBV replication cycle. HBx increases HBV replication and activates HBV enhancer II/core promoter [5,15,21]. HBx also modulates host cellular functions including lipid metabolism [25,32]. In this study, we investigated activation of SREBP-1a by HBx and its involvement in regulating HBV enhancer II/core promoter by HBx.

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http://dx.doi.org/10.1016/j.bbrc.2013.02.030

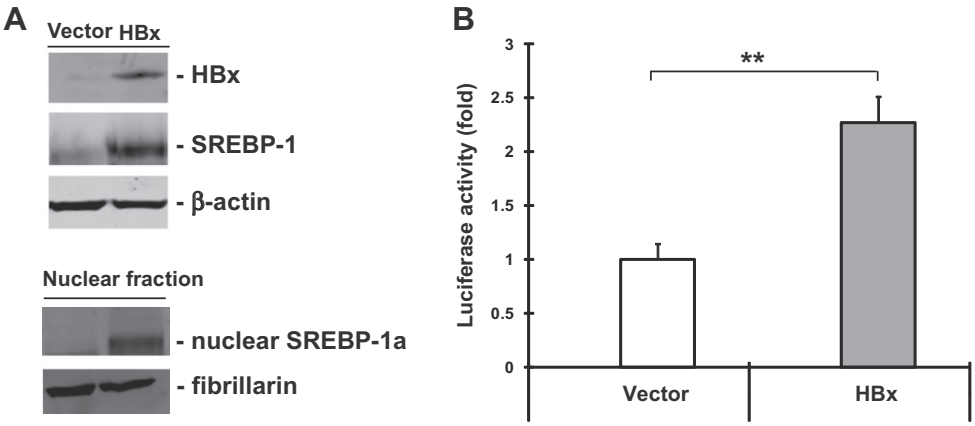

Fig. 1. Expression of HBx increases the level of nuclear SREBP-1a and its transactivation activity. (A) In the top panel, the levels of HBx, SREBP-1, and β-actin in Huh-7 cells after transfection with an HBx-expressing plasmid or the vector were analyzed by immunoblotting. In the bottom panel, levels of SREBP-1a in the nuclear fraction were analyzed by immunoblotting after co-transfection with plasmids expressing Flag-SREBP-1a and HBx or vector. The blots were probed with antibodies against Flag-tag or fibrillarin. (B) A luciferase reporter driven by SRE sequences was co-transfected with a plasmid encoding Flag-SREBP-1a together with HBx-expressing plasmid or vector control into Huh-7 cells. Luciferase assay was performed using the cell lysates. Luciferase activity was expressed as fold change relative to vector control. The statistical difference between samples was demonstrated as ** if $p \leqslant 0.01$.

2. Materials and methods

2.1. Plasmids and antibodies

The coding sequence of HBx was amplified by PCR from plasmids pRBK HBx or pawy1.2 [8,28] and cloned in-frame with the myc tag into the pEF/cyto/myc vector (Invitrogen). HBV enhancer II/core promoter sequence [27] was cloned into pGL4.14 vector (Promega), generating pGL4-HBV EN2/CP where the expression of luciferase gene was controlled by HBV enhancer II/core promoter. Flag-tagged SREBP-1a (aa. 1–517) was amplified from an SREBP-1a plasmid [30] and inserted into the pCMV2 Flag vector (Sigma–Aldrich) [14]. Plasmid pSRE-Luc containing three copies of SRE sequences was provided by Shimano [1]. Human SREBP-1a promoter – luciferase reporters containing different lengths of the SREBP-1a promoter were described previously [10]. Mutant SREBP-1a promoters with mutations for the binding sequences for C/EBP (CCAAT/enhancer binding protein) and E4BP4 (Adenovirus E4 promoter binding protein 4) were generated by site-directed mutagenesis and confirmed by DNA sequencing (Fig. 3). Plasmids expressing C/EBPα [4] and E4BP4 (Open Biosystems) were used. SREBP-1-targeting microRNA (miRNA) with target sequence of 5′ CCTGGTCTACCATAAGCTGCA 3′ was constructed in pcDNA6.2-GW/EmGFP miR vector (Invitrogen).

SREBP-1, Flag (M2), fibrillarin, β-actin and Myc epitope antibodies were from Santa Cruz Biotechnology, Sigma–Aldrich, and Cell Signaling Technology, respectively. Anti-HBx antibody was provided by Richardson [8].

2.2. Cell culture, transfection and nuclear fractionation

Huh-7 cells [20] were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Huh-7 cells were transfected using the calcium phosphate precipitation method as previously described [13]. Nuclear fractions were isolated as described [36].

2.3. Immunoblotting analysis

Huh-7 cells were collected with a Cell Lysis Buffer (Cell Signaling Technology) containing 1 mM phenylmethylsulphonyl fluoride (PMSF). Immunoblotting was performed as described [14,36]. For SREBP experiments, cells were treated with a protease inhibitor ALLN (25 μg/ml, Calbiochem) for 1 h prior to lysis.

2.4. Reverse transcription and real-time PCR

RNA was isolated from Huh-7 cells with Trizol (Invitrogen) followed by DNase I (Invitrogen) digestion. Reverse transcription was carried out by Superscript II (Invitrogen) and random priming. Real-time PCR was performed with primers SREBP-1a-FD (5′ CGCTGCTGACCGACAT 3′) and SREBP-1a-rev (5′ CAAGAGAGGAGCTCAATG 3′) using SYBR Green based detection system. Housekeeping gene GUSB was amplified in parallel by primers GUSB-FD (5′ GGTGCTGAGGATTGGCAGTG 3′) and GUSB-rev (5′ CGCACTTCCAACTTGAACAGG 3′). Data was analyzed by Bio-Rad iQ5 program.

2.5. Luciferase assay

Huh-7 cells were lysed in a Passive Lysis Buffer (Promega) and luciferase activity was determined using luciferase assay reagents (Promega) in a TD 20/20 Luminometer (Turner Designs). Results were analyzed for statistical differences using Student t test. A p value of $\leqslant 0.05$ was considered statistically significant.

3. Results

3.1. HBx increases the level of SREBP-1a in the nucleus

HBx coding sequence was cloned into the pEF-cyto-myc vector in-frame with the myc tag. The resulting plasmid was transfected into Huh-7 cells and the cell lysates were analyzed in immunoblotting. As shown in Fig. 1A, a specific protein band was recognized by an HBx-specific antibody and a myc-tag antibody (not shown) in cells transfected with HBx-expressing plasmid, but not in vector-transfected cells. The level of β-actin was used as loading control. These results demonstrated the expression of HBx protein after transfection.

Given the importance of SREBP-1a in modulating lipid metabolism, we explored the role of HBx in SREBP-1a activation. Huh-7 cells were transfected with HBx-expressing plasmid and vector control. The level of SREBP-1 was analyzed by immunoblotting using an anti-SREBP-1 antibody. As shown in the upper panel of Fig. 1A, expression of HBx was associated with increased level of SREBP-1 compared to control. Because we were interested in SREBP-1a levels especially in the nucleus as the active form, however the SREBP-1 antibody cannot distinguish SREBP-1a from another isoform SREBP-1c. Therefore we used a plasmid expressing