Biochemical and Biophysical Research Communications 423 (2012) 6–12

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

The interaction between hepatitis B virus X protein and AIB1 oncogene is required for the activation of NFκB signal transduction

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ARTICLE INFO

Article history:
Received 25 April 2012
Available online 22 May 2012

Keywords:
Hepatitis B
X protein
AIB1
NCoA-3
SRC-3
Interaction
NFkB
Hepatocellular carcinoma

ABSTRACT

We identified the interaction between HBV X (HBx) protein and the oncogene AIB1 (amplified in breast cancer 1). A serine/proline motif (SSPSPS) in HBx was found to be required for the interaction. Two LXD motifs [LLXX(X)L, X means any amino acids], LLRNSL and LLDQLHTLL in AIB1 were also found to be involved in the HBx–AIB1 interaction. The HBx–AIB1 interaction was important for the activation of NFκB signal transduction, the HBx mutant that did not interact with AIB1showed dramatically lower NFκB activation activity than the WT HBx. These findings contribute to the new understanding on signal transduction activation mechanisms of HBx.

1. Introduction

Hepatitis B virus (HBV) is one of the most common types of hepatitis viruses causing chronic liver infections in 350 million people worldwide. Chronic hepatitis B can cause cirrhosis, liver failure and hepatocellular carcinoma or HCC [1].

HBV is part of the *Hepadnaviridae* family, with eight know genotypes of the virus, from genotype A to H. It has a 3.2-kb partially double stranded circular DNA genome enclosed within the virus particle envelope. The four viral mRNAs encode the viral core (Pre core/C), envelope structural proteins (Pre S1/S2/S), polymerase-reverse-transcriptase and X nonstructural viral protein. Among all HBV proteins, the X (HBx) is one of most active proteins regulating host cell signals. The HBx gene from most HBV strains encodes 154 amino acids with a molecular weight about 17 kD [1,2]. The X protein is a transcriptional *trans*-activator able to upregulate the HBV genome transcription activity; it has also been found that the X protein is involved in tumorigenesis through protein–protein interactions and signal transduction activations such as AP1, AP2, cAMP response element-binding protein (CREB) and nuclear factor kappa B (NFκB)-related proteins [2,3].

Most of the above transcription and signal transduction factors have been found to interact with the p160 proteins or steroid receptor coactivators (SRCs), these activators have been found to carry a variety of molecular functions such as transcriptional activation and signal transduction regulation [4,5]. Among the coactivators, AIB1 (amplified in breast cancer 1) is an important member in the p160 family. AIB1 is a protein composed of about 1400 amino acids, and also defined as steroid receptor coactivator (SRC-3), receptor associated activator 3 (RAC-3) and thyroid hormone receptor activator molecule 1 (Tram-1). AIB1 has been reported to be involved in several cancer progressions, such as breast cancer, prostate cancer, colorectal carcinoma and ovarian cancer [6,7]. However whether AIB1 is involved in the molecular pathogenesis of HBV is yet to be characterized.

In this study, we investigated the interaction between hepatitis B X protein and AIB1, and the relationship between their interaction and the regulation of NFκB. We also mapped the interacting motif and further identified that other viral proteins utilize the same motif for protein–protein interactions.

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http://dx.doi.org/10.1016/j.bbrc.2012.05.021

2. Materials and methods

2.1. Tissue Culture

Hepatocellular carcinoma cells (HuH-7) from Riken Cell (Japan) were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen). All cell cultures were maintained in a humidified 5% CO_2 incubator at 37 °C.

2.2. Cloning HBV X genes into a mammalian two-hybrid vector

The cloning of HBx gene was carried out using HBV clone pEco63 as a template (Accession No. AY108092). The X gene was cloned in frame into pVP16 vector (Clontech) and designated as pVP-HBx.

2.3. Cloning of AIB1 into the mammalian two-hybrid vector

The gene template of AIB1 was purchased from Origene. The PCR-amplified AIB1 was cloned in frame into mammalian two hybrid vectors pM (Clontech) and designated as pM-AIB1.

2.4. Transfection

1×10^5 HuH-7 cells were used for transfection using Attractene transfection reagent according to the manufacturer's protocol (Qiagen). The experiments triplicated for each transfection were repeated at least 3–5 times with SD (Standard Deviation) being less than 10%.

2.5. Mammalian two hybrid analysis

The Mammalian Two-Hybrid Assay System (Clontech) was used. The pM vector carries a Gal4 DNA binding domain (BD), the pVP16 vector carries a herpesvirus VP16 DNA activation domain (AD). When two proteins interact, the BD and AD domains form a transcriptional activation complex and activate the secreted alkaline phosphatase (SEAP) reporter gene provided on the pG5SEAP reporter vector; SEAP can be secreted into the cell culture supernatant and measured by a chemiluminescence assay using GreatEscape Chemiluminescence Detection Assay (Clontech). The level of chemiluminescence was measured by a microplate reader [8]. Results were analyzed using Microsoft Office Excel. The readout of chemiluminescence activity was converted into ratios by setting the value of vector control as 1.

2.6. Signal transduction analysis on NFκB pathway

HuH-7 cells were plated at 30% confluency in a 96-well plate, and incubated for 24 h in a 37 °C incubator with 5% CO_2. Hundred nano grams of the NFκB reporter plasmid pGL 4.32[*luc2p*/NF-κB-RE/Hygro] (Promega) was transfected into each well using 0.3 μl Attractene transfection reagent (Qiagen) following the manufacturer's protocol. The cells were incubated for 48 h and then treated with TNF-α at a concentration of 20 ng/ml for a period of 5 h. Detection of luciferase was completed using the Luciferase Assay System from Promega, following the manufacturer's protocol. Luminescence was detected within 5 min of adding the reagent, using a GENios Pro plate detector (Tecan) set for 100 ms luminescence reading. Results were analyzed using Microsoft Office Excel. The readout of chemiluminescence activity was converted into ratios by setting the value of vector control as 1.

2.7. Co-immunoprecipitation

AIB1 was cloned into pFlagCMV-10 vector (Sigma) and designated as pFlagAIB1. HBx was cloned into pcMyc vector (Clontech) and designated as pMyc-HBx. The HBx and corresponding mutant were subsequently cotransfected with the AIB1 and its mutation constructs into 1.5×10^6 HuH-7 cells for 48 h. Cells were harvested in the coimmunoprecipitation buffer (1% digitonin, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8.0), and precipitated with the anti-Flag antibody (Abcam) and protein A/G (Invitrogen). The immunoprecipitated samples were subjected to a 4–12% SDS PAGE (Invitrogen) and transferred to the nitrocellulose membrane using iBlot (Invitrogen). The blot was probed with an anti-Myc primary antibody (Abcam) and developed with ECL Plus system (Thermo Scientific).

2.8. Detection of HBx from HCC liver tissues

Samples were collected from liver transplantation patients (in Beijing Chao Yang Hospital) who suffered from HBV-associated hepatocellular carcinoma (HCC). Control samples were from the previous liver biopsy samples of non-HBV infected patients stored in the Pathology Department of Chao Yang Hospital. The consent of the patients was obtained prior to this study. After liver transplantation operation, the HCC liver was used for the study. The cancerous sites and non-cancerous sites from the same liver were sectioned into 4 nm-thick slides and subjected to HBx staining. The slides were treated with mouse anti-HBx antibody (Abcam) for overnight at 4 °C, washed for 3 times with PBX and then incubated wit rabbit anti-mouse antibody conjugated with horseradish peroxidase (HRP) for 30 min at room temperature. Slides were washed 3 times with PBX and then developed with 3,3′ diamino benzidine (DAB), the slides were also treated with haemaxylin for the nuclei staining [9,10].

3. Results

3.1. HBV X protein interacts with AIB1

We performed mammalian two-hybrid assays to investigate whether HBx interacts with AIB1. *AIB1* was cloned in frame into pM vector and designated as pM-AIB1, while HBx gene and its sequential deletion constructs were cloned into pVP16 vector. These constructs were designated as pVP-HBx, and ΔpVP-HBx-1 to ΔpVP-HBx-4 as illustrated in Fig. 1A. The HBx constructs were then co-transfected with pM-AIB1 into HuH-7 cells. Supernatants were harvested 48 h post transfection and subjected to measure the SEAP chemiluminescence activity. As shown in Fig. 1B, pVP-HBx demonstrated over 10 times higher SEAP activity than the mock-transfected sample and the negative control sample cotransfected with pM and pVP16. This result suggested that HBx interacts with AIB1.

The sequential deletion mutants from ΔpVP-HBx-1 to ΔpVP-HBx-4 showed different SEAP activity. The ΔpVP-HBx-1 had almost the same SEAP activity of the full-length HBx, while from ΔpVP-HBx-2 to ΔpVP-HBx-4, the SEAP activity dropped significantly (Fig. 1B). This result suggested that the sequence between ΔHBx-1 and ΔHBx-2 (aa. 32–64) may contain a motif(s) that is required for the HBx and AIB1 interaction. We performed a sequence analysis and identified a serine-proline motif, SSPSPS from aa 38–43. Sequence alignment with HBx from all 8 serotypes of HBV showed that Ser41 is highly conserved (Fig. 1C). To further confirm that this motif is important for the interaction between HBx and AIB1, we mutated the Ser41 from SSPSPS to SSPAPS in the full-length pVP-HBx construct and designated the mutant as

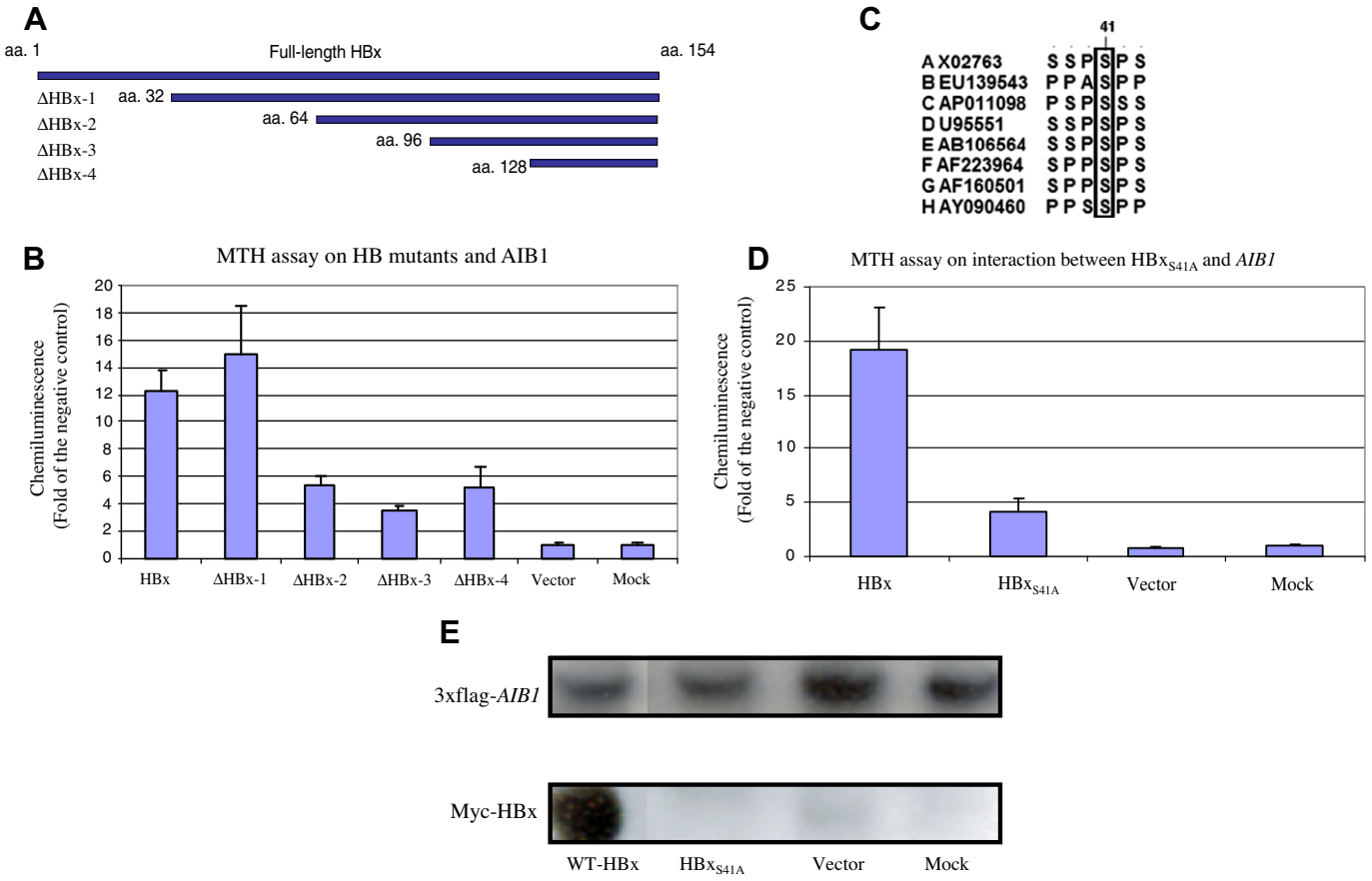


Fig. 1. Interaction motif mapping on HBx. (A) Schematic explanation on sequential HBx truncation from the amino-terminus. (B) Mammalian two-hybrid assay. Full length HBx and HBx truncation mutants ΔpVPHBx1-4 were cotransfected with pM-*AIB1* and the pG5SEAP reporter plasmid for 48 h. Supernatants were harvested for SEAP chemiluminescence analysis. (C) Amino acid sequences of the serine-proline motif from eight genotypes of HBV were aligned, the conserved serine 41 is highlighted in the frame. (D) Mammalian two-hybrid assay on *AIB1* interaction with HBx site directed-mutagenesis mutant pVP-HBx$_{S41A}$. The pVP-HBx and the mutant pVP-HBx$_{S41A}$ were cotransfected into Huh7 cells with pM-*AIB1* and the reporter plasmid pG5SEAP for 48 h. Supernatants were harvested and subjected to the SEAP chemiluminescence analysis. Negative control is pM and pVP vector supplied by the manufacturer. The results were average of three repeats; the standard deviation was calculated with Microsoft Office Excel using STDEV function. (E) The pFlag-*AIB1* was cotransfected with pMyc-HBx and pMyc-HBx$_{S41A}$ into Huh7 cells for 48 h. The harvested cells were used for co-immunoprecipitation with an anti-Flag antibody. The HBx and the mutant were detected by Western blot using an anti-Myc antibody.

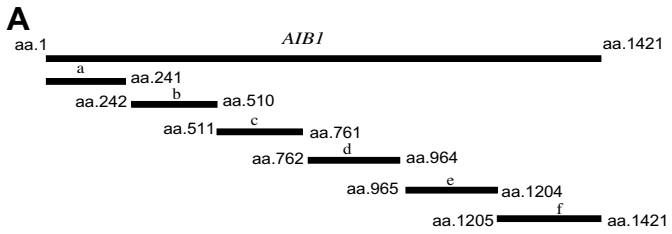
pVP-HBx$_{S41A}$, following which the mutant construct was cotransfected with pM-AIB1 in a mammalian two-hybrid assay. As shown in Fig. 1D, the above mutation significantly reduced the signal of interaction in the mammalian two-hybrid assay, suggesting that Ser41 is required for the interaction between HBx and AIB1.

To further confirm the protein–protein interaction, we also performed co-immunoprecipitation with WT HBx. HBx-S41A mutant and AIB1, HBx and HBx$_{S41A}$ mutant were subcloned into pMyc vector (Clontech) and designated as pMyc-HBx and pMyc-HBx$_{S41A}$. AIB1 was cloned into p3xFlag-CMV10 vector (Sigma) and designated as pFlag-AIB1. The two HBx constructs were co-transfected with pFlag-AIB1 into HuH-7 cells for 48 h. Cells were harvested for co-immunoprecipitation with anti-Flag antibody (Sigma), the precipitated samples were subjected to a Western blot for the detection of Myc-HBx with an anti-Myc antibody (Abcam). As shown in Fig. 1E, the WT co-immunoprecipitated with AIB1, while HBx$_{S41A}$ mutant showed significant reduction of interaction. This result confirmed that HBx interacted with AIB1, mutation of Ser41 affected the interaction.

3.2. Mapping of interaction domain in AIB1

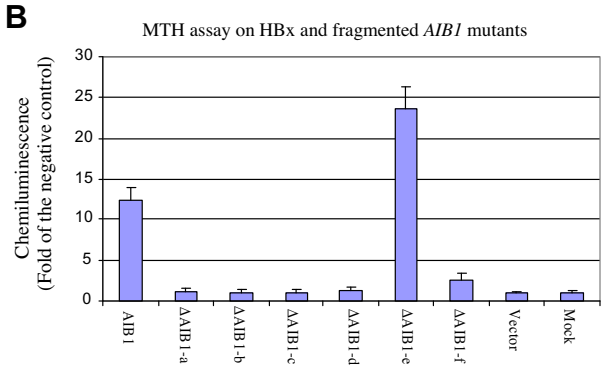
In addition to the mapping of the interaction domain within HBx, we also characterized the interaction motif in AIB1. As depicted in Fig. 2A, the full-length AIB1 was segmented into six fragments and cloned into pM vector and designated as ΔpM-AIB1-1 to ΔpM-AIB1-6. These constructs were subsequently co-transfected with pVP-HBx into HuH-7 cells. As shown in Fig. 2B, the fragment 5 of AIB1 (aa 965–1204) showed significantly higher SEAP activity than the other fragments, suggesting that this region may contain the interaction motif(s). Further analysis on the fragment 5 (Fig. 2C) sequence indicated that the region contains two LLXX(X)L/LXX(X)LL domains (X means any amino acids) or LXD (^{1029}LLRNSL^{1034} and ^{1053}LLDQLHTLL^{1061}). LXDs in p160 proteins are known to be involved in ligand-binding and protein–protein interactions [11]. To further characterize whether these motifs are responsible for the interaction with HBx, we individually mutated the leucine 1034 and 1057 into alanine (LLRNS<u>A</u>^{1034}, LLDQ<u>A</u>^{1057}HTLL) in the full-length pM-AIB1 construct and designated the mutant as pM-AIB1$_{L1034A}$ and pM-AIB1$_{L1057A}$. The mutant constructs were co-transfected into HuH-7 cells with the full-length pVP-HBx to perform a mammalian two-hybrid assay. As demonstrated in Fig. 2D, both single amino acid mutations in AIB1 reduced its chemiluminescence activity to HBx, the pM-AIB1$_{L1057A}$ showed more significant reduction than pM-AIB1$_{L1034A}$. This result indicated that both LXDs in AIB1 may be involved in the interaction between HBx and AIB1.

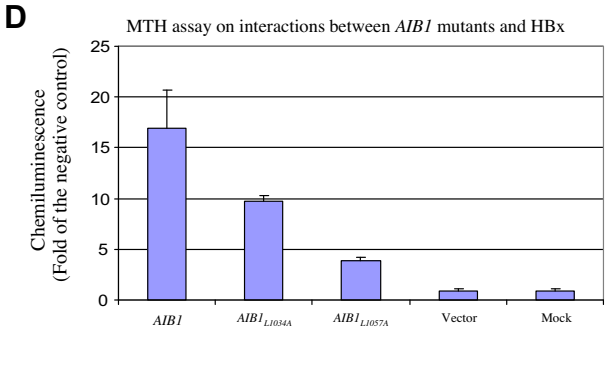
To confirm the above finding, we performed a co-immunoprecipitation with WT AIB1, AIB1$_{L1034A}$ and AIB1$_{L1057A}$ mutants. Full-length AIB1, AIB1$_{L1034A}$ and AIB1 $_{L1057A}$ mutants were subcloned



C

(N)aa965SNSIPGARPVLQQQQQMLQMRPGEIPMGMGANPYGQAAASNQLGSWP
DGMLSMEQVSHGTQNRPLLRNSL[1034]L[1057]DDLVGPPSNLEGQSDERALLDQL[1057]HTLL
SNTDATGLEEIDRALGIPELVNQGQALEPKQDAFQGQEAAVMMDQKAGLYGQT
YPAQGPPMQGGFHLQGQSPSFNSMMNQMNQQGNFPLQGMHPRANIMRPRTNTP
KQLRMQLQQRLQGQQFLNQSRQALELKMENPTAGGAA aa1204 (C)





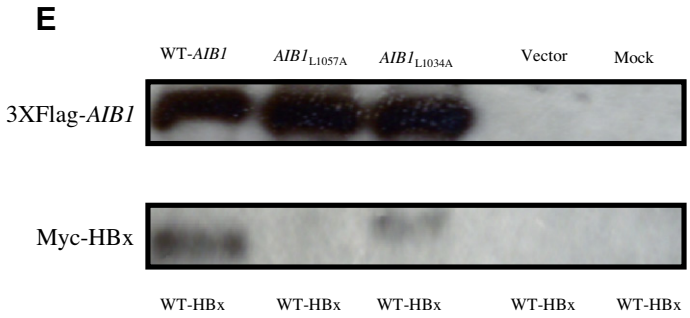


Fig. 2. Interaction motif mapping on *AIB1*. (A) Schematic diagram on truncation of *AIB1* into six fragments. (B) mammalian two-hybrid assay on HBx and the fragmented AIB1. AIB1 was truncated into six fragments and designated as pM-*AIB1*-1-6. The truncation constructs were cotransfected into Huh7 cells with full-length pVP HBx the and the reporter plasmid pG5SEAP, Supernatants were harvested 48 h post-transfection and subjected to SEAP chemiluminescence analysis. (C) Sequence that contains motifs that are important for the HBx–*AIB1* interaction. Two LXX(X)LL/LLXX(X)L motifs are underlined, the mutated amino acids are in red color. (D) MTH assay, pM-*AIB1*, pM-$AIB1_{L1034A}$, and pM-$AIB1_{L1057A}$ were cotransfected with pVP-HBx and the reporter plasmid pG5SEAP respectively. Negative control was made by pM, pVP16 and mock-transfected cells. The reading value of the SEAP chemiluminescence were calculated in relevance to reading from the mock-transfected cells (relative value of 1). (E) The pFlag-*AIB1*, pFlag-$AIB1_{L1034A}$ and pFlag-$AIB1_{L1057A}$ were co-transfected with pVP-HBx into Huh7 cells for 48 h. The immunoprecipitated samples were subjected to a Western blot detected by an anti-Myc antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

into p3xFlag-CMV10 vector (Sigma) and designated as pFlag-AIB1, pFlag-$AIB1_{L1034A}$ and pFlag-$AIB1_{L1057A}$. The AIB1 and its mutant were co-transfected with WT pMyc-HBx into HuH-7 cells for 48 h. Cells were harvested for co-immunoprecipitation with the anti-Flag antibody (Sigma). The immunoprecipitated samples were subjected to a Western blot probed with anti-Myc antibody (Abcam). As shown in Fig. 2E, compared with WT AIB1, the $AIB1_{L1034A}$ and $AIB1_{L1057A}$ both mutant showed reduced interaction, $AIB1_{L1057A}$ showed significantly reduced interaction with HBx. This result is consistent with the findings from the MTH assay and confirmed that aa 1034 and 1057 in AIB1 are important for the protein–protein interaction with HBx.

3.3. NFκB signal transduction analysis

HBx has been shown to activate of NFκB pathway which plays important roles in tumorigenesis [12]. To analyze whether the interaction between HBx and AIB1 is important for the activation of NFκB, we employed a SEAP-based NFκB signal transduction reporter system (Clontech), which measures the NFκB activations through chemiluminescence analysis. The pVP-HBx and pVP-HBx_{S41A} were co-transfected respectively into HuH-7 cells with

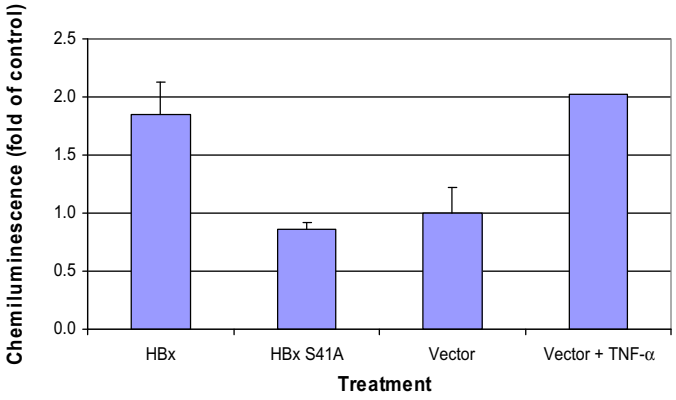


Fig. 3. NFκB activation. pVP-HBx and pVP-HBx_{S41A} mutant were cotransfected with NFκB reporter plasmid pGL 4.32 and incubated 48 h. The vector control was treated with TNF-α at a concentration of 20 ng/ml for a period of 5 h. Luciferase activities were measured, results were analyzed using Microsoft Office Excel. The readout of chemiluminescence activity was converted into ratios by setting the value of vector control as 1.

the NFκB-SEAP reporter plasmid. As shown in Fig. 3, the WT HBx protein significantly up-regulated the NFκB activity in comparison to the HBx_{S41A} mutant. This result suggested that the interaction between HBx and AIB1 may be important for the activation of NFκB.

3.4. HBx expression is much higher is HCC tissue than the non-HCC tissue from the same patients

HBx transcript, anti-HBx antibody and HBx protein expression have all been found to be higher in HCC patients, suggesting that HBx is involved in the progression of HBV-induced HCC [13–16]. However the comparison of HBx expression in the HCC tissue to the surrounding non-HCC tissue in the same patients has yet to be revealed. Demonstration on the magnitude of the difference will provide direct evidence on the role of HBx in HCC development. We collected five HCC liver samples from liver transplantation operations for patients who suffered from HBV-related HCC in 2011 in Beijing Chao Yang Hospital. The cancerous tissue and non-cancerous tissue samples were sectioned into 4 nm slides and probed with anti-HBx antibody. Five non-HBV infected biopsy samples were also used as negative controls. As shown in Fig. 4, the non-HBV infected samples did not show the detection of HBx, (with traces of background), the tissue from non-cancerous sites of the liver showed low level to back ground level of HBx, while the tissue from the cancerous site demonstrated significantly higher HBx than the surrounding non-HCC tissue. The scale of difference on HBx expression between HCC and non-HCC varies, in Fig. 4, P-3 showed the least amount of difference. Even though the increased HBx expression has been reported previously, the direct comparison between the cancerous and noncancerous tissue from the same HCC liver has yet to be characterized, our study demonstrated for the first time that HBx expression is higher in the cancerous site of the liver. This preliminary observation showed that HBx expression is important in the development of HCC.

4. Discussion

In summary, we have found that Hepatitis B virus X protein interacts with AIB1. A highly conserved serine of HBx at Ser41 in

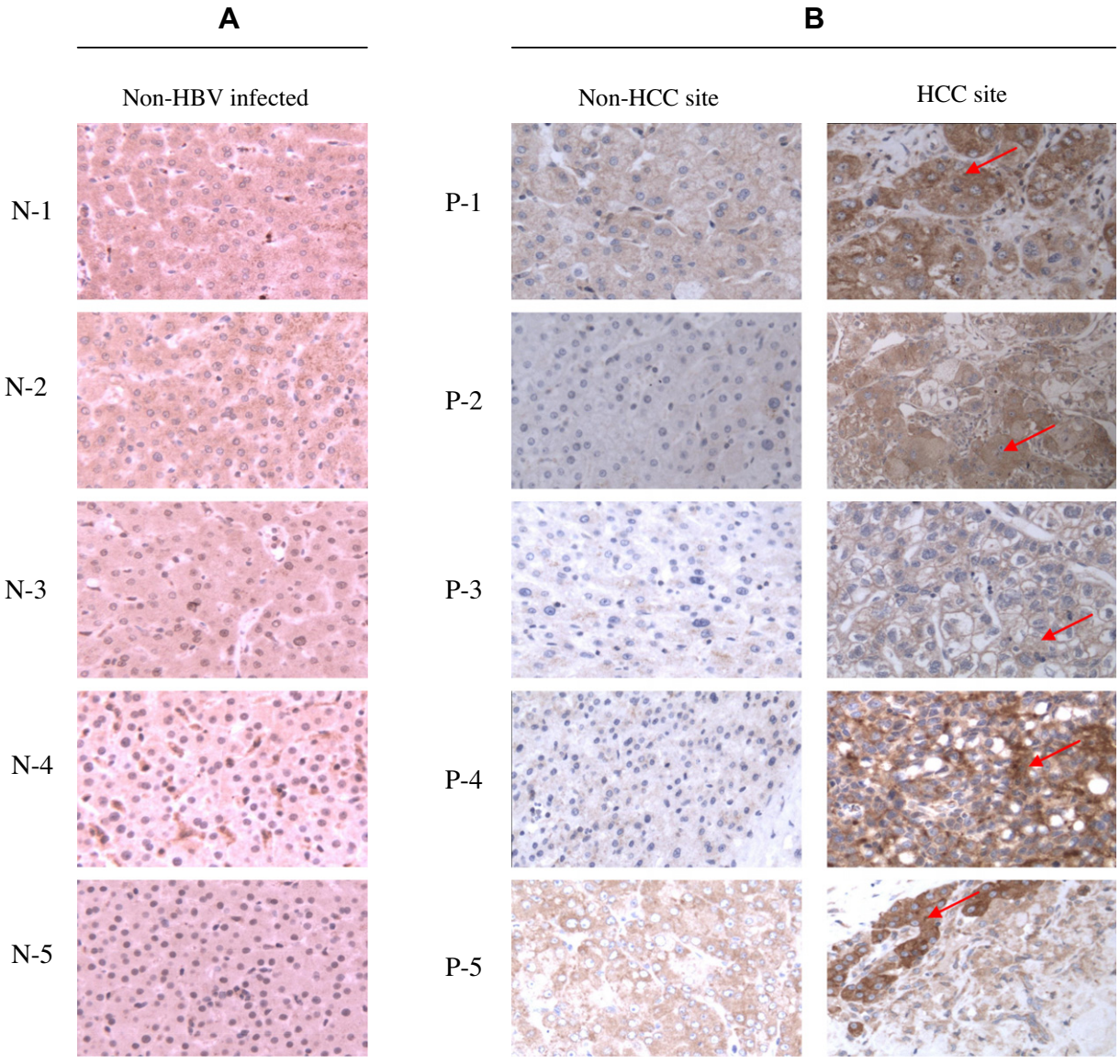


Fig. 4. Expression of HBx in HCC liver tissue. After liver transplantation operation, the HCC liver was used for the study. The cancerous sites and non-cancerous sites from the same liver were sectioned into 4 nm-thick slides and subjected to HBx staining with an anti-HBx antibody, and then developed with 3,3′ diamino benzidine (DAB), the slides were also treated with haemaxylin for the nuclei staining.

a serine-rich motif was required for this interaction. We have also shown that the interaction between HBx and AIB1 is required for the activation of NFκB. The increased HBx expression in the cancerous tissue of HCC liver was also demonstrated to be higher than the non-cancerous tissue from the same liver. To our knowledge, this is the first time that the two types of the tissues from the same liver were compared for HBx expression.

The mutation from serine to alanine significantly reduced the interaction activity between HBx and AIB1. The serine domain in HBx was first identified by Pangetal, who found that the motif was responsible for the interaction between HBx and Pin1, where Ser41 was found to be important for the interaction [17]. We also identified that the integrity of a LXD motif (LLRNSL and LLDQLHTLL) was required for the HBx–AIB1 interaction. Mutation of Leu1034 and especially Leu1057 to alanine significantly affected the HBx–AIB1 interaction. More importantly, the HBx–AIB1 interaction was found to be required for the activation of NFκB; while the HBx S41A mutation caused aberrant NFκB activation.

There are several major functional domains in AIB1; (a) bHLH (basic helix-loop-helix region); (b) RID (receptor interacting domain); (c) AD1 (activation domain); (d) Q domain (polyglutamate repeat tract); and (e) the AD2 domain. The L1034 and L1057 are located in two LXDs in the AD1 domain, this domain has been reported to be involved with p300/CBP interaction [18]. Our previous studies found that the LLRNSL and LLDQLHTLL motifs were involved in AIB1 (referred as NCoA-3) interaction with thymine DNA glycosylase (TDG), mutations from LLRNSL to AARNS, and from LLDQLHTLL to AADQLHTLL affected the interaction between TDG and AIB1/NCoA-3 [19]. Our current study found that these LXDs were also involved in the HBx interaction. Single amino acid mutations (from LLRNSL to SSRNSA; and from LLDQLHTLL to LLDQAHTL) in the above two motifs reduced the interaction activity between HBx and AIB1. These results suggested that the LXDs in AD1 domain are involved in the interaction between HBx and AIB1. A GenBank search showed that both LXD sequences are highly conserved only to AIB1 (data not shown). Since the full crystallography structure for AIB1 is not available, it is yet to determine whether these LXDs are the direct interaction domains or allosteric domains that indirectly influence the HBx–AIB1 interaction.

NFκB has been found to be a critical factor in many human malignancies. The upregulated activation of NFκB has been found in a many human cancers and cancerous tissues in experimental animals, such as kidney cancer, hepatocellular carcinoma, breast cancer and lymphoma [20]. NFκB controls cell proliferation, inhibits apoptosis and promote angiogenesis, these activities facilitate the progression of malignancy [21]. NFκB pathway is directly regulated by a group of highly conserved Rel protein family (p50/NFκB1, p52/NFκB2, c-Rel, p65/Rel-A and Rel-B), which bind to a variety of homologous decanucleotide sequences (5′-GGG(A/G)NN(T/C)-(T/C)CC-3′) that regulate downstream gene expressions [21]. The Rel proteins form homo- or heterodimers to control the above regulations. Among all the dimmers, the p50/p65 heterodimer is the most abundant and ubiquitous [20,21]. Before induction, the NFκB–Rel complexes are generally retained in the cytoplasm with their inhibitors such as IκB-α and IκB-β. When cells are induced by kinase-activating agents such as TNFα or PMA, the IκBs are phosphorylated and subsequently degraded by cellular proteases. The degradation of IκBs releases Rel proteins such as p50 and p65 and allows them to relocate into the nucleus and form the p50/p65 heterodimer, which is the functional unit to activate NFκB [22]. In addition to the Rel proteins, other host proteins have also been found to influence the activation of NFκB, for example steroid receptor coactivator 1 or SRC-1 was found to interact with p50 and influenced the activation of NFκB [23]. These data suggested that p160 proteins are involved in the activation of NFκB.

HBx has been reported to be able to activate NFκB [13]; however the path of the activation of NFκB by HBx may be different from the conventional mechanism. Purcell et al. demonstrated that the degradation of IκB induced by the expression of HBx was not related to the phosphorylation of Ser^{32} and Ser^{36} of IκB [24]; Lucito and Schneider also found that protein kinase C was not involved in the HBx-associated NFκB activation [25]. These studies suggested that HBx may activate NFκB through an alternative approach that is yet to be characterized. Our result suggested that the interaction between HBx and AIB1 is required for the activation of NFκB, This result may lead to further understanding of HBx-linked NFκB activation.

Further investigations on signal transduction found that HBx–AIB1 interaction is important for the activation of NFκB. To our knowledge, this is the first time that HBx was found to regulate NFκB activation via AIB1. Our finding in this study also suggests that the regulation of NFκB activity may be influenced by the interaction to a non-Rel protein. This finding is significant to the further understanding of hepatitis B virus pathogenesis.

Acknowledgments

The research was funded by the Research Funding from Public Health Agency of Canada.

We thank Dr. Jingxin Cao in National Microbiology Lab for his support to our project.

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