Archives of Biochemistry and Biophysics 528 (2012) 156–162

Contents lists available at SciVerse ScienceDirect

Archives of Biochemistry and Biophysics

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

Pro-apoptotic or anti-apoptotic property of X protein of hepatitis B virus is determined by phosphorylation at Ser31 by Akt

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ARTICLE INFO

Article history:
Received 13 June 2012
and in revised form 14 August 2012
Available online 13 September 2012

Keywords:
HBx
Akt
Phosphorylation
Pro-apoptotic
Anti-apoptotic

ABSTRACT

The X protein of hepatitis B virus (HBx) has been specifically implicated in either pro-apoptotic or anti-apoptotic activity in an experimental system, but the underlying mechanism is yet uncertain. Activations of survival and proliferation signaling pathways appear to account partly for its anti-apoptotic property. Change in mitochondrial membrane potential may be responsible for its apoptotic property. In this study, we isolated two HBx isoforms from an HBV carrier, one of which contains Akt phosphorylation site at Ser31 and functions as an anti-apoptotic protein (designated HBx-S31). The other does not contain Akt phosphorylation site and functions as an apoptotic protein (designated HBx-L31). HBx-S31 can activate Akt, whereas HBx-L31 cannot; the former enhances tumor growth, whereas the latter suppresses tumorigenesis. Our study provides evidence that HBx plays dual roles, namely pro-apoptotic and anti-apoptotic, through different isoforms in which HBx with Ser31 transduces survival signal.

Introduction

The human hepatitis B virus (HBV)[1] is associated with a major etiological factor of hepatocellular carcinoma (HCC) in the world [1]. The current knowledge of the HBV replication has been well documented [2], in contrast with poor understanding of the mechanisms of HBV-associated carcinogenesis. The 17-kDa X protein (HBx) has focused much attention in HBV-mediated HCC, and many different biological properties have been ascribed to the protein, affecting transcription [3–7], signal transduction [8–12], DNA repair [13–15], cell cycle control [16–18] and apoptosis [19–22]. However, several reported properties of HBx look contradictory between each other. For instance, HBx was found by one group to counteract p53-induced apoptosis [23], whereas another group reported a p53-dependent pro-apoptotic effect of the viral protein [24].

HBx affects viral and cellular gene expression. A direct effect of HBx on the transcription process has been based on physical and functional interactions of HBx with several components of the transcriptional machinery [3–7], including the CREB transcription factor [6], the common subunit of RNA polymerases RPB5 [3], the p53 tumor suppressor [25], and the UV-damaged DNA binding protein (UVDDB) [26], whereas an indirect effect involving activation of signaling cascades has also been documented, including the Jak-Stat [8], Ras/MAP kinase [9], PI-3 kinase/Akt [10], SAPK/JNK [11], and NF-κB [12] , and Notch [27] signaling pathways. Blockade of Notch signaling has been shown to promote HBx-mediated apoptosis [27].

Difficulty in unraveling HBx functions might come in part from the fact that many investigations were based merely on one isoform of HBx. The HBV polymerase possesses two distinct functions, namely DNA-dependent RNA polymerase and RNA-dependent DNA polymerase. Both DNA and RNA syntheses lack proof-reading activity [30]. Thus, mutation may occur and result in diverse isofoms of HBx and other viral proteins encoded by HBV DNA. A number of reports showed that HBx triggers apoptosis in hepatoma cell lines [19–22], however, other groups later gave opposite results [8–11,27]. Shih et al. showed that HBx activates survival signal by linking SRC to PI-3 kinase [10], and Chung et al. showed that HBx induces matrix metalloproteinase-9 gene expression through activation of ERK and PI-3 K/AKT pathways [8], suggesting HBx is involved in invasive potential of HCC. In addition, HBx shifts TGF-β signaling from the tumor-suppressive pSmad3C pathway to the oncogenic pSmad3L pathway in early hepatocarcinogenic process [28], and contradictorily HBx also upregulates expression

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[1] *Abbreviations used:* HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBx, X protein; UVDDB, UV-damaged DNA binding protein; Dox, doxycycline; STS, staurosporine.

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http://dx.doi.org/10.1016/j.abb.2012.08.008

of Fas and Fas ligand [29]. However, the detailed mechanism of HBx-mediated tumor invasion remains uncertain. In this report, we show that pro- or anti- apoptotic property of HBx is determined by phosphorylation of Ser-31 by Akt, a serine/threonine kinase involved in signal transduction of cell survival.

Experimental

HBV DNA isolation and PCR amplification of HBx DNA

Blood samples were obtained from a patient with chronic infection of hepatitis B virus. Serum was collected by centrifugation at 1000*g*. To 0.5 ml serum was added proteinase K to final 10 μg/ml and SDS to final 1%. The resulting serum was incubated at 55 °C overnight and then processed for phenol/chloroform extraction. DNA was precipitated with 0.1 volume of 3 M sodium acetate, pH 5.2 and one volume of isopropanol, and the pellet was washed with 70% ethanol and then air dried. The HBV DNA pellet was dissolved in 20 μl TE (20 mM Tris, 1 mM EDTA, pH 8.0), 1 μl of which was used for HBx PCR. The primers for PCR were forward 5′-AAGCTTGCTGCTCGGGTGTGCTGCCAA (flanked by Hind III) and reverse 5′-GGTACCGGCAGAGGTGAAAAAGTTGCA (flanked by Kpn I). The conditions for the reaction were 95 °C 1 min, 55 °C 1 min, and 72 °C 1 min; 25 cycles.

Cell lines and cell culture

HEK 293T and the human hepatoma cell line HepG2 were cultured in Dulbecco's modified Eagle medium (GIBCO Invitrogen Corp.) supplemented with 10% fetal bovine serum (GIBCO-BRL) and penicillin/streptomycin (100 U/100 μg/mL, Sigma–Aldrich) at 37 °C and 5% CO_2.

HBx DNA cloning

To generate FLAG-tagged HBx, the HBx DNA synthesized by PCR was cloned in the expression vector p3XFLAG-myc-CMV-27 (abbreviated p3XFLAG, Sigma–Aldrich) via Hind III and Kpn I sites. To produce GST-HBx fusion protein, the HBx DNA was cloned in the pGSTag plasmid. To generate doxycycline (Dox)-induced HBx expression system, FLAG-tagged HBx was cloned in pRetro-X-Tight-Pur plasmid (BD-Clontech).

Site-directed mutations of HBx on Akt phosphorylation sites

Ser31 (Leu31) of HBx was mutated to leucine (serine) with the QuickChange site-directed mutagenesis kit (Stratagene Corp.). Briefly, PCR was done with two complementary primers covering the sites for mutation, and p3XFLAG-HBx was used as a template. The PCR product was treated with the restriction enzyme Dpn I which recognized GATC with methylated adenine. Adenine methylation occurs in plasmid replication of *Escherichia coli* but not in DNA replication of PCR. Thus, the plasmid template was digested by Dpn I. Then, the intact plasmid *i.e.*, the PCR product was used to transform XL-1 blue competent cells.

Immunoprecipitation-Western blotting assay

The p3XFLAG-HBx and control plasmids (4 μg each) were individually transfected into 1×10^6 HEK 293T cells. 36 h after transfection, cells were lysed in a lysis buffer containing 50 mM Tris HCl , pH 7.4, 150 mM NaCl, 1% Triton-X 100, 5 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM sodium vanadate, 50 mM NaF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin . The cell lysate was cleared by centrifugation at 10,000*g*. The supernatant was added 2 μg anti-Akt antibody (Santa Cruz Inc.) and left at 4 °C overnight. The immune complexes were collected on protein A Sepharose CL-4B (Amersham Biosciences) and then resolved with SDS–polyacrylamide gel electrophoresis. The proteins in the gel were transferred to a PVDF membrane in which HBx was detected with anti-FLAG antibody.

In Vitro kinase assay

The HBx DNAs were cloned into the pGSTag plasmid. GST-HBx was induced and purified by the GSH-Sepharose. HBx was separated from GST by thrombin treatment. *In vitro* kinase reaction was performed in 20 μl of kinase buffer containing 3 μg of purified HBx with or without 100 ng of activated Akt1 (Upstate Biotechnology), 200 μM ATP, and 10 μCi [γ-^{32}P]ATP (Perkin–Elmer Life Sciences) at 30 °C for 40 min. The reaction mixtures were subjected to 10% SDS–PAGE. Phosphorylation of the HBx proteins assayed was detected by autoradiography.

Establishment of Dox-inducible HBx expression system

The FLAG-tagged HBx DNA was cloned in the pRetro X-Tight-Pur plasmid (designated pRetro, purchased from BD-Clontech). The pRetro-HBx plasmid was co-transfected with the pRetro-tet-on plasmid into HepG2 cells and then selected with 500 μg/ml G418 and 1 μg/ml puromycin. The resistant clones were picked out and expanded to mass culture. FLAG-HBx was induced by doxycycline (0.1 μg/ml) and detected by Western blot with anti-FLAG antibody.

Western blot analysis

Cells with proper drug treatments were lysed in a buffer containing 50 mM Tris HCl , pH 7.4, 150 mM NaCl, 1% Triton-X 100, 5 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM sodium vanadate, 50 mM NaF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin . Cell lysates were resolved by SDS–PAGE and then transferred to a nitrocellulous membrane. The membrane was treated with a primary antibody, followed by incubation with a peroxidase-conjugated secondary antibody and detection with the enhanced chemiluminescence method.

Apoptosis assay

5×10^4 HepG2 cells (Dox-inducible HBx expression) were plated onto 3-cm tissue culture dishes and then treated with 0.1 μg/ml doxycycline for 48 h. Cells were treated with 200 nM staurosporine for 12 h and subjected to SDS–PAGE and immunoblotting with anti-PARP1 antibody (Santa Cruz Biotech.) for detection of the 89-kDa cleaved PARP1. For inhibition of PI-3 K/Akt signaling, cells were treated with 40 μg/ml of Ly294002 (LY) for 12 h and then with LY and staurosporine for additional 12 h. On the other hand, the cells were grown on coverslides, treated with doxycycline and staurosporine as described above, and then processed for apoptosis assay with Annexin V-cyt3 apoptosis kit (BioVision Inc.).

Immunofluorescence

FLAG-HBx was induced by doxycycline for 48 h. Then cells were grown on coverslides and fixed for 20 min in pre-cold 100% methanol at 0 °C. After rehydration in PBS for 5 min, cells were incubated for 20 min in PBS containing 0.5% Tween-20 (PBST). Cells were blocked in 5% skim milk in PBST and then incubated overnight in a primary antibody (anti-FLAG) prepared in blocking solution. The secondary antibody was conjugated with FITC (fluorescein isothiocyanate).