Enhancement of gene transactivation activity of androgen receptor by hepatitis B virus X protein

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Abstract

Hepatitis B virus (HBV) X protein (HBx) is a regulatory protein that is required for efficient replication of HBV in its natural host. In this report, we demonstrate by co-immunoprecipitation experiments that HBx can physically bind to the androgen receptor (AR), which is a nuclear hormone receptor that is expressed in many different tissues including the liver. This observation is further supported by confocal microscopy, which reveals that HBx can alter the subcellular localization of the AR both in the presence and in the absence of dihydrotestosterone (DHT). Further studies indicate that HBx can enhance the gene transactivation activity of AR by enhancing its DNA binding activity in a DHT-dependent manner. However, HBx does not remain associated with AR on the DNA. As AR can regulate the expression of a number of cellular genes, our results raise the possibility that HBV pathogenesis may be mediated in part via the interaction between HBx and AR.

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Introduction

Hepatitis B virus (HBV) is an important human pathogen that can cause severe liver diseases including hepatocellular carcinoma (HCC). This virus has a small DNA genome that contains four genes: the S gene encodes the viral envelope proteins; the C gene encodes the viral core protein and a serum e antigen; the P gene encodes the viral DNA polymerase; and the X gene encodes a 16.5-kDa regulatory protein with multiple functions.

The HBV X protein (HBx) does not bind to DNA directly (Yen, 1996). However, it can bind to transcription factors including CREB, ATF-2, AP-2 and HNF1 to modify their activities (Li et al., 2002; Maguire et al., 1991; Seto et al., 1990; Williams and Andrisani, 1995). It can also bind to RBP5, a subunit of all three mammalian RNA polymerases (Cheong et al., 1995), to the proteasome (Fischer et al., 1995; Hu et al., 2006), p53 (Wang et al., 1994), a component of the DNA repair complex UV-DDB (Becker et al., 1998), and a member of the human voltage-dependent ion channel family HVDAC3 (Rahmani et al., 2000). HBx can also stimulate cellular calcium signaling pathways, resulting in the activation of focal adhesion kinase (FAK), prolase-rich tyrosine kinase 2 (Pyk2) and Src, and the downstream mitogen-activated protein kinase (MAPK) signaling pathway and the cyclin A–cdk2 complex (Bouchard et al., 2001, 2006; Klein and Schneider, 1997). HBx can also activate NFκB (Su et al., 2001). Studies on the related woodchuck hepatitis virus B virus (WHV) indicate that the X gene is required for efficient replication of WHV in woodchucks (Chen et al., 1993; Zhang et al., 2001; Zoulim et al., 1994). Further studies using transgenic mice carrying the entire HBV genome demonstrate that HBx can enhance HBV gene expression and the subsequent viral DNA replication (Xu et al., 2002).
HBV can induce HCC, which is male preponderant, suggesting a possible role of androgen and/or its receptor in HBV pathogenesis. Androgen receptor (AR) is a member of the nuclear receptor superfamily. This protein is approximately 110 kDa in size. It contains several functional domains including the ligand binding domain, the DNA binding domain, the transcription modulation domain and a nuclear localization signal (Lee and Chang, 2003). After binding to its ligand testosterone or 5α-dihydrotestosterone (DHT), AR is activated and transported into the nucleus where it binds to the promoters of its target genes to activate their expression (Lee and Chang, 2003). AR is expressed in many different tissues including the liver and has a wide range of activities. In the liver, it can upregulate the expression of transforming growth factor β1 (TGF-β1), sex-limited protein (Slp) and various drug- and steroid-metabolizing enzymes including specific cytochrome P450 isozymes (Chatterjee et al., 1996; Yoon et al., 2006). AR is believed to play an important role in hepatocellular carcinogenesis, as there is an increased risk for hepatocellular carcinoma (HCC) in patients treated with androgens (Touraine et al., 1993), and a positive correlation between the HCC risk and the serum testosterone level (Tanaka et al., 2000; Yu et al., 2000, 2001). Furthermore, a lower number of the CAG trinucleotide repeats in exon 1 of the AR gene, which increases the gene transactivation activity of AR (Beilin et al., 2000; Chamberlain et al., 1994; Irvine et al., 2000), is also associated with an increased risk for HCC (Yu et al., 2000, 2001).

In this report, we have investigated the possible interaction between the HBV regulatory protein HBx and AR. Our results indicated that HBx could bind to and enhance the gene transactivation activities of AR in the presence of DHT. This enhancement of the AR activity by HBV in a DHT-dependent manner may explain the gender difference of HCC incidence among HBV carriers.

Results

Co-immunoprecipitation of HBx and AR

To investigate whether HBx and AR can bind to each other, we performed the co-immunoprecipitation experiments. Huh7 cells, which are human hepatoma cells that do not express androgen receptor (Xie et al., 2001), were co-transfected with the AR expression plasmid and the expression plasmid for HA-tagged HBx. Cells were lysed 48 h after transfection and immunoprecipitated with the anti-AR antibody, followed by the Western blot analysis using the anti-HA antibody. As shown in Fig. 1A, AR could be immunoprecipitated by the anti-AR antibody in the presence of HA-tagged HBx (lane 6) but not in the absence of it (lane 5). This result indicated that AR and HBx could physically bind to each other. This result was confirmed by a reciprocal experiment, in which the cell lysates were first immunoprecipitated with the anti-HA antibody followed by the Western blot analysis using the anti-AR antibody. As shown in Fig. 1B, the HA-tagged protein could be immunoprecipitated by the anti-AR antibody only in the presence of AR (lane 4) and not in the absence of it (lane 2), confirming that HBx and AR could bind to each other.

Co-localization of HBx and AR in cells

To further investigate how HBx and AR may interact with each other in the cell, we also performed the confocal microscopy. Huh7 cells were first transfected with either the HA-tagged HBx expression plasmid or the AR expression plasmid. As shown in Fig. 2A, HBx displayed a predominantly cytoplasmic localization whether or not the cells were treated with dihydrotestosterone (DHT) (panels a and b). The staining pattern of HBx in the cytoplasm was granular, which is consistent with the previous report (Rahmani et al., 2000). In contrast, AR displayed a more diffuse cytoplasmic localization in the absence of DHT (panel c). In agreement with the previous report (Tomura et al., 2001), AR was localized predominantly to the nucleus after cells were treated with DHT (panel d).

If HBx and AR can indeed bind to each other in the cell, they will likely have similar subcellular localizations when they are co-expressed in the same cell. For this reason, we also co-transfected Huh7 cells with the HA-tagged HBx expression plasmid and the AR expression plasmid. As shown in Fig. 2B, in the absence of DHT, HBx and AR displayed the same granular cytoplasmic staining pattern (panels a–c), indicating the co-localization of HBx and AR in the cytoplasm. In the presence of DHT, most AR was localized to the nucleus. However, a fraction of AR was also found co-localized with
HBx in the cytoplasm (panels d–f, denoted by an arrow), indicating a partial retention of AR in the cytoplasm by HBx. On rare occasions, HBx was found to co-localize with AR in the nucleus (panels g–i). These results further indicate the physical interaction of these two proteins in Huh7 cells.

**Enhancement of gene transactivation activity of AR by HBx**

To further investigate whether the interaction between HBx and AR affects the biological activities of AR, we conducted a gene transactivation assay using the firefly luciferase reporter, which has been linked to a promoter that contains the androgen responding element. Huh7 cells were co-transfected with the HA-tagged HBx expression plasmid and the AR expression plasmid. Red color, HBx staining; green color, AR staining; yellow or orange color, co-localization of HBx and AR. Panels a–c, without DHT treatment; and panels d–i, with DHT treatment. The arrow in panels d–f denotes a cell that co-expressed AR and HBx. The partial colocalization of AR and HBx in the cytoplasm of this cell was clearly visible. Most of HBx and AR double-positive cells displayed this staining pattern (unpublished observation). The unmarked cell in panels d–f expressed only AR, which was detectable only in the nucleus. On rare occasions, HBx and AR co-localized in the nucleus (g–i).

fig. 2. Confocal microscopy of the subcellular localization of HBx and AR in Huh7 cells. (A) Subcellular localization of HBx and AR. Panels a and b, cells expressing HA-tagged HBx; panels c and d, cells expressing AR. Panels a and c, without DHT treatment; panels b and d, with DHT treatment. (B) Co-localization analysis of HBx and AR. Huh7 cells were co-transfected with the HA-tagged HBx expression plasmid and the AR expression plasmid. Red color, HBx staining; green color, AR staining; yellow or orange color, co-localization of HBx and AR. Panels a–c, without DHT treatment; and panels d–i, with DHT treatment. The arrow in panels d–f denotes a cell that co-expressed AR and HBx. The partial colocalization of AR and HBx in the cytoplasm of this cell was clearly visible. Most of HBx and AR double-positive cells displayed this staining pattern (unpublished observation). The unmarked cell in panels d–f expressed only AR, which was detectable only in the nucleus. On rare occasions, HBx and AR co-localized in the nucleus (g–i).

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with DHT (Fig. 3, also data not shown). These results indicated that the effect of HBx on the reporter required DHT and was mediated by AR.

Enhancement of the DNA binding activity of AR by HBx

To further investigate how HBx enhanced the gene transactivation activity of AR, we also conducted the chromatin immunoprecipitation (ChIP) assay. In this study, Huh7 cells were transfected with the pMMTV-luciferase reporter plasmid with or without the AR expression plasmid and the HBx expression plasmid, and with or without the DHT treatment. The pMMTV-luciferase contains only one copy of the androgen responding element and was used in this particular study. After cell lysis, the AR–DNA complex was immunoprecipitated with the anti-AR antibody and the DNA was quantified by the polymerase chain (PCR) reaction. The PCR results are shown in Fig. 4A and the quantification results are shown in Fig. 4B. As shown in Fig. 4A, no reporter DNA was detected in this ChIP assay if this reporter was co-transfected with the AR expression plasmid and immunoprecipitated with a control antibody (lane 2) or if this reporter was co-transfected with the control pRc/CMV expression vector and immunoprecipitated with the anti-AR antibody (lane 3). These two experiments served as the negative controls. In contrast, a basal level of the reporter DNA was detected if this reporter was co-transfected with the AR expression plasmid into Huh7 cells and immunoprecipitated with the anti-AR antibody (lane 4). The amount of the reporter DNA precipitated by the anti-AR antibody was doubled if cells were treated with DHT for 16–18 h (Fig. 4A, lane 5; also Fig. 4B). This increase was further enhanced by HBx (Fig. 4A, lane 8; also Fig. 4B). HBx had little effect on the DNA binding activity of AR in the absence of DHT (Fig. 4A, lane 6; also Fig. 4B). Thus, these results indicated that HBx could enhance the DNA binding activity of AR only in the presence of DHT.

As HBx could bind to AR, we also investigated whether HBx remained associated with AR on the reporter DNA. As HBx was fused to the HA-tag, the anti-HA antibody was used to immunoprecipitate HBx for the ChIP assay. As shown in Fig. 5, except for the positive control, almost no reporter DNA could be detected in this ChIP assay, indicating that HBx did not remain associated with AR on the reporter DNA.

Discussion

HBx is a regulatory protein with multiple functions. By conducting the co-immunoprecipitation experiments, we now demonstrate that HBx can also bind to the steroid hormone receptor AR (Fig. 1). Our observation was further confirmed by confocal microscopy. AR had a diffused cytoplasmic localization in the absence of DHT (Fig. 2A). However, when it was co-expressed with HBx, it became co-localized with HBx, which had a more granular staining pattern in the cytoplasm (Fig. 2B). AR is translocated into the nucleus after its activation by DHT (Fig. 2A) (Tomura et al., 2001). In the presence of HBx, it was partially retained by HBx in the cytoplasm (Fig. 2B). Interestingly, HBx was also found on rare occasions to co-localize with AR in the nucleus (Fig. 2B). It is possible that a small amount of HBx, which is not detectable by confocal microscopy, is also co-localized with AR in the nucleus in the presence of DHT.

Upon its activation by DHT, AR is translocated into the nucleus where it will bind to the androgen responding element of its target genes to activate their expression (Kang et al., 2002;
AR is reminiscent of its activities on other transcription factors to remain associated with AR on the DNA. This effect of HBx on AR activity was DHT dependent, as HBx could not enhance the activity of AR in the absence of DHT (data not shown).

HBx is not a DNA-binding protein. However, it has been shown to enhance the DNA binding activity of a number of transcription factors (Yen, 1996). Our ChIP results shown in Fig. 4 demonstrated that HBx could also enhance the DNA binding activity of AR. As shown in Fig. 4, HBx could further enhance the gene transactivation activity of AR 2- to 4-fold in a dose-dependent manner. This effect of HBx on AR was DHT dependent, as HBx could not enhance the activity of AR in the absence of DHT (data not shown).

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this area will likely generate interesting results for understanding HBV pathogenesis in different genders.

Materials and methods

Cell lines and DNA plasmids

Huh7 cells were maintained in Dulbecco’s modified essential medium (DMEM) containing 10% fetal bovine serum (FBS). Depending on the experiments, the FBS might be treated with dextran-coated charcoal (Sigma-Aldrich) for the removal of small molecules. pCMV-HAX (Li et al., 2002), which expresses the HA-tagged HBx under the control of the SV40 early promoter, have been previously described. The expression vector pSG5 was from Stratagen. Probasin-Luc is a plasmid that contains the firefly luciferase reporter linked to the herpesvirus thymidine kinase (tk) promoter and three copies of the rat probasin promoter fragment (nucleotides −244 to −96) that contains the androgen responding elements (Jia et al., 2003). The plasmid pMMTV-Luciferase, which contains 1 copy of the androgen responding element, has been described before (Nordeen, 1988).

Co-immunoprecipitation experiments

Huh7 cells in a 10-cm Petri dish were transfected with pCMV-HAX, pSG5-AR and their control expression vectors in different combinations using the calcium phosphate precipitation method. Forty-eight hours after transfection, cells were rinsed twice with TBS (10 mM Tris–HCl, pH 7.0, 150 mM NaCl) and lysed in 1 ml TBS containing 0.5% Nonidet P-40 (NP40). The cell lysates were briefly centrifuged to remove cell debris. The supernatant was mixed 1:1 with RIPA (10 mM Tris–HCl, pH 7.0, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) and split into two aliquots. The first aliquot was incubated with 1 μl mouse anti-HA monoclonal antibody (USC Norris Cancer Center Cell Culture Core) and the second aliquot was incubated with 1 μl rabbit anti-AR antibody (Affinity Bioreagents). After incubation at 4 °C overnight, the immune complex was precipitated by 4 °C incubation with the respective alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit antibody. The protein bands were visualized using the BioRad AP color kit (BioRad).

Confocal microscopy

Huh7 cells grown on coverslips in a six-well dish were transfected with pCMV-HAX, pSG5-AR and their respective control expression vectors pRc/CMV (Invitrogen) and pSG5 in different combinations by calcium phosphate precipitation. Cells were rinsed twice with DMEM without serum on the second day and further incubated in DMEM with or without 50 nM DHT for 24 h. Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) and then stained with both the mouse anti-HA and the rabbit anti-AR primary antibodies and the rhodamine-conjugated goat anti-mouse and fluorescein-conjugated goat anti-rabbit secondary antibodies for confocal microscopy.

The luciferase reporter assay

Huh7 cells in six-well dishes were co-transfected with 1.5 μg Probasin-luc reporter and either 150 ng pSG5-AR or its control expression vector pSG5. 1.5 μg of pCMV-HAX and its control vector pRc/CMV in different combinations were also included in the co-transfection experiment. Cells were incubated in DMEM containing 0.5% FBS after transfection, treated with 50 nM DHT or its control solvent ethanol in DMEM on the second day for 24 h and then lysed for the luciferase assay. In all the transfection experiments, 150 ng pRL-SV40 (Promega), a plasmid that expresses renila luciferase, was used for co-transfection to monitor the transfection efficiency. The firefly luciferase and the renila luciferase activities were measured using the dual luciferase assay (Promega). The firefly luciferase activities were then normalized against the renila luciferase activities. All the experiments were repeated at least three times.

Chromatin immunoprecipitation (ChIP) assay

2.5–5 × 10^5 Huh7 cells in a 6-well dish were transfected with 1.5 μg/well of DNA plasmids with 0.5 μg each of pMMTV-Luciferase, pCMV-AR and pCMV-HAX. The plasmids pCMV-AR and pCMV-HAX might be replaced by their control vector pRc/CMV, depending on the experiments. Eight to twelve hours after transfection, cells were treated with 10 nM DHT or its control solvent ethanol for 16–18 h in DMEM that contained 10% charcoal-stripped FBS. Cells were then crosslinked with 1% formaldehyde in PBS at room temperature for 10 min on a platform shaker. The cross-linking reaction was stopped by adding glycine to a final concentration of 125 mM with further shaking at room temperature for 5 min. Cells were then rinsed three times with ice cold PBS, scraped in 1 ml PBS and pelleted by centrifugation at 700 × g for 4 min. The cell pellet was then resuspended in the cell lysis buffer (5 mM Pipes, pH 8.0, 85 mM KCl and 0.5% Nonidet-P 40) containing 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM PMSF and incubated on ice for 10 min. Nuclei were then pelleted by centrifugation at 5,000 rpm for 5 min in a microcentrifuge and lysed in the nuclear lysis buffer (50 mM Tris–HCl, pH 8.1, 10 mM EDTA, 1% SDS) containing the same protease inhibitors for 10 min on ice. The chromosomal DNA was pulse-sonicated on ice to an average length of 600 bp. The nuclear debris was removed by centrifugation at full-speed in a microfuge for 10 min at 4 °C. The supernatant was then diluted 5-fold in the ChIP dilution buffer (16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl, 1.2 mM
EDTA, 1.1% Triton X-100 and 0.01% SDS) containing the protease inhibitors. The sample was then pre-treated for 30 min at 4 °C with 80 μl 50% slurry of protein A-agarose that had been pretreated with salmon sperm DNA. After the removal of the protein A-agarose by a brief centrifugation, an aliquot of the supernatant was saved to serve as the input DNA control. The experiments were repeated at 4 °C for 1 h with 60 μl protein A-agarose slurry that had been pretreated with salmon sperm DNA. The agarose beads were then precipitated by a brief centrifugation in a microfuge and washed consecutively for 3–5 min with 1 ml each of the following solutions: low salt wash buffer (20 mM Tris–HCl, pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS); high salt wash buffer (20 mM Tris–HCl, pH 8.1, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS); LiCl wash buffer (10 mM Tris–HCl, pH 8.0, 0.25 M LiCl, 1 mM EDTA, 1% NP40, 1% Na deoxycholate); and TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) twice. The PCR products were then vortexed in 250 μl elution buffer (0.1 M NaHCO3, 1% SDS) for 15 min, pelleted again at 15,000 x g for 3 min. The supernatant was transferred to a new tube. The wash of the pellet was repeated one more time and the two supernatants were combined. The formaldehyde cross-linking was reversed by adding 1 μl 10 mg/ml RNase and 5 M NaCl to a final concentration of 0.3 M followed by incubation at 65 °C water bath for 4–5 h. The DNA was precipitated with 2.5 volumes of 100% ethanol at −20 °C overnight and resuspended in 100 μl water. The input DNA aliquot was treated with the same procedures followed by the addition of 2 μl 0.5 M EDTA, 4 μl 1 M Tris–HCl, pH 6.5 and 1 μl 20 mg/ml proteinase K and further incubated at 45 °C for 1–2 h. The DNA was then purified using the QiaQuick spin column (Qiagen) and eluted in 50 μl 10 mM Tris–HCl, pH 8.0. Two microliters of the DNA was then used for PCR using the following primers: GGTTCCCAGGGCT-

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