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Hepatitis B Virus Regulatory HBx Protein Binds to Adaptor Protein IPS-1 and Inhibits the Activation of Beta Interferon▽

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Hepatitis B virus (HBV) encodes the regulatory HBx protein, which is required for virus replication, although its specific role(s) in the replication cycle remains under investigation. An immunoprecipitation/mass spectrometry approach was used to identify four novel HBx binding proteins from the cytoplasmic fraction of HBx transgenic mouse livers. One of these HBx binding partners is beta interferon promoter stimulator 1 (IPS-1), an adaptor protein that plays a critical role in mediating retinoic acid-inducible gene I (RIG-I) signaling, which leads to the activation of beta interferon (IFN-β). The HBx–IPS-1 protein interaction was confirmed in plasmid-transfected HepG2 cells by reciprocal coimmunoprecipitation and Western blotting. We hypothesized that HBx might alter IPS-1 function since proteins of hepatitis C virus and hepatitis A virus similarly bind IPS-1 and target it for inactivation. The effect of HBx on IPS-1-mediated IFN-β signaling was tested in transfected 293T and HepG2 cells, and we show that HBx inhibits double-stranded DNA (dsDNA)-mediated IFN-β activation in a dose-dependent manner when expressed either alone or within the context of HBV replication. However, HBx does not inhibit poly(I:C)-activated IFN-β signaling. These results demonstrate that HBx interferes with the RIG-I pathway of innate immunity. Hepatitis B virus now joins hepatitis C virus and hepatitis A virus in targeting the same innate immune response pathway, presumably as a shared strategy to benefit replication of these viruses in the liver.

Hepatitis B virus (HBV) is a small (3.2-kb) DNA virus that causes acute and chronic inflammation of the liver, and the latter is a risk factor for the development of hepatocellular carcinoma (HCC) (39). Worldwide, an estimated 350 million people have chronic HBV and are at risk for severe liver disease (39). New insight into the virus-host interactions underlying chronic virus replication was provided with the demonstration that HBV infection fails to activate the innate immune response in chimpanzees (52). This observation was recently confirmed in acutely infected humans (10) and primary human hepatocytes exposed to HBV (17). Several studies have clearly demonstrated that HBV replication is controlled by an activated adaptive immune response (4, 14, 36, 53, 54), suggesting that HBV has evolved a strategy to dampen activation of the innate immune response.

The sole HBV regulatory protein, the 17-kDa HBx protein, plays an essential role in virus replication in HepG2 cells (3) and in hydrodynamically injected mice (22). Given the abundance of properties attributed to HBx (reviewed in reference 2), it is likely that HBx has more than one function during the virus life cycle. These functions may be mediated, in part, through HBx interactions with cellular proteins. Indeed, screening protocols such as the yeast two-hybrid assay have been used to identify over 30 HBx-interacting proteins (reviewed in reference 13). The biologic importance of these virus-host protein interactions is difficult to assess due to a paucity of virus replication assays. In the related woodchuck virus replication model, it was demonstrated that the HBx interaction with cellular DDB1 is critical for virus replication (42), and this was confirmed in plasmid-transfected HepG2 cells (27). However, the role of other HBx binding partners in virus replication remains unknown.

Cells respond to virus infection through the recognition of viral pathogen-associated molecular patterns (PAMPs). Several cytoplasmic host pattern recognition receptors (PRRs) are responsible for this, including the Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I) and other RIG-I-like receptors (RLRs), such as MDA-5 and LGP2 (43; also reviewed in reference 55). Following recognition of the viral DNA or RNA, the PRRs undergo conformational changes that activate downstream pathways, ultimately leading to the induction of type I interferon (IFN) and proinflammatory cytokines. A key adaptor protein in this process is the beta interferon promoter stimulator 1 (IPS-1) protein (21), also known as mitochondrial antiviral signaling protein (MAVS) (40), VISA (58), and Cardiff (35; also reviewed in reference 20). Upon its activation, IPS-1 recruits kinases that phosphorylate latent transcription factors required for the production of IFN-β (11, 21, 25). Interestingly, IPS-1 is targeted for interaction by several viral proteins, effectively inactivating the innate antiviral immune response (43).

The goal of the present study was to identify HBx-interacting proteins in the liver of the HBx transgenic mouse by using an immunoprecipitation (IP)/mass spectrometry (MS) approach. Four novel HBx binding partners, including IPS-1, were iden-

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tified from the cytoplasmic fraction of the livers of HBx transgenic mice. We confirmed the HBx–IPS-1 interaction in human HepG2 cells and demonstrated that HBx is able to inhibit the double-stranded DNA (dsDNA) activation of IFN-β. We further show that HBx localized to the cytoplasm is responsible for this effect and that the effect of HBx is apparent within the context of HBV replication. Our results suggest a role for HBx in dampening the innate immune response, thereby permitting virus spread throughout the liver while avoiding the host immune response.

MATERIALS AND METHODS

Transgenic mice. Transgenic mice expressing the HBV HBx protein under the control of the liver-specific alpha-1-antitrypsin regulatory region (ATX mice) have been described previously (26). ATX mice and their nontransgenic littermates were used in this study. All procedures were performed following federal regulations for the care and treatment of laboratory animals. ATX mice were crossed with outbred ICR mice, and the progeny were genotyped by PCR analysis of high-molecular weight tail DNA using X-specific primers (24). Genotypes were confirmed by IP/Western blotting for HBx protein, as described below.

Cell culture and plasmids. Human embryonic kidney 293T and liver HepG2 cells were maintained in Eagle's minimum essential medium (EMEM) with 10% fetal bovine serum (FBS), as previously described (23). Plasmid DNA encoding IPS-1 containing hemagglutinin (HA) and FLAG epitopes was obtained from Zhijian Chen (28). Plasmid DNA encoding HBx (subtype adw2) was prepared by cloning into plasmid vector pSI (Promega) as previously described (23). pSI-X plasmids with an in-frame nuclear localization signal (pSI-NLS-X) or a nuclear export signal (pSI-NES-X) were previously described (23). A reporter plasmid encoding firefly (F) luciferase under the control of the IFN-β regulatory region (IFN-β-Luc) was obtained from Michael Gale (12). A plasmid encoding *Renilla* (R) luciferase (used for normalization) was created from pRL-CMV (Promega) by PCR cloning into the EcoRI-NotI sites of pSI. A plasmid encoding the avian influenza virus nonstructural 1 (NS-1) protein was obtained from Andrew Rice. Plasmids carrying small hairpin RNA (shRNA) specific for IPS-1 or control noncoding shRNA were obtained from Open Biosystems (Thermo Fisher Scientific).

Plasmid transfections and luciferase assays. Plasmid DNAs were transfected into triplicate wells (24-well format) of HepG2 or 293T cells by using Trans*IT*-LT-1 (for HepG2 cells) or Trans*IT*-293 (for 293T cells) reagent according to the manufacturer's protocol (Mirus Corporation). Activation of IFN-β by dsDNA or dsRNA was measured as described by Cheng et al. (7). In brief, cells were transfected with the firefly and *Renilla* luciferase reporter plasmids and, 36 h later, transfected with poly(dAT:dAT) (Sigma) or poly(I:C) (Sigma). Cells were harvested 24 h later and processed using a dual-luciferase assay according to the manufacturer's protocol (Promega Corporation). Mean values of *Renilla*-normalized firefly luciferase counts were determined from triplicate wells. To test the effect of HBx (or control NS-1) on IFN-β activation, luciferase plasmids were cotransfected with either empty vector pSI or pSI-X (or NS-1- or epitope-tagged versions of HBx). The total amount of DNA transfected per well remained constant. Luciferase results were obtained from cotransfection of 2 μg/ml dsRNA or dsDNA, and results were confirmed in experiments using 1 μg/ml (not shown). Results were confirmed in at least 3 independent experiments for both 293T and HepG2 cells.

Antibodies, coimmunoprecipitation (co-IP), and Western blotting. HepG2 cells were grown overnight in 60-mm plates and transfected with plasmid DNAs using Trans*IT*-LT-1. After 48 h, cells were harvested and lysed using extraction buffer containing 1% NP-40 as described previously (32). IPs were performed using rabbit antibodies specific for HBx (26) and IPS-1 (anti-MAVS ab31334; Abcam, Inc.). The antibody-antigen complex was precipitated by formalin-fixed, heat-killed *Staphylococcus aureus* protein. SDS-polyacrylamide gels of 15% and 10% were used to identify HBx and IPS-1, respectively. Western blot detection of HBx and IPS-1 was carried out as described previously (32), using rabbit anti-HBx and anti-HA antibodies (Covance, Inc.), respectively, and a Pierce West Femto kit. To affinity purify the rabbit anti-HBx, GST-X protein was purified from *Escherichia coli* BL21(DE3) by using glutathione Sepharose 4B beads and immobilized by the dimethylpimelimidate (DMP) coupling method. In brief, GST-X binding glutathione beads were washed with 0.2 M HEPES (pH 8.2) and incubated with 100 mM DMP in 0.2 M HEPES (pH 8.2) for 2 h at 4°C. The beads were then washed three times with 0.2 M HEPES (pH 8.2) and incubated with 40 mM ethanolamine in 0.1 M borate buffer (pH 8.0) for 1 h at 4°C. The conjugated beads were then washed twice with cold phosphate-buffered saline (PBS) and three times with 0.2 M glycine-HCl (pH 2.5) and 1 M K_2HPO_4 (pH 10) and equilibrated with cold PBS. Then, a total of 2 ml of rabbit anti-X antiserum was mixed with 100 μl of GST-X-conjugated glutathione beads for 1 h at 4°C. The reaction mixture was then transferred to a Bio-Spin chromatography column (Bio-Rad) and washed with 500 ml of cold PBS. The washed beads were then eluted with 1 ml of 200 mM glycine (pH 2.0) solution. Each 100-μl fraction was collected, and 20 μl of 1 M Tris-HCl (pH 8.0) and 20 μl of glycerol were added immediately to prevent antibody denaturation. Three fractions containing higher levels of antibody were then combined, and the typical antibody concentration was 0.1 mg/ml.

Liver tissue preparation and cell harvesting for IP/MS. ATX and wild-type (WT) mice were anesthetized by intraperitoneal injection using 0.75 to 1.5 ml/kg body weight of anesthetic (37.5 mg ketamine, 1.9 mg xylazine, and 0.37 mg acepromazine in 5 ml of sterile water). Mice were then subjected to intracardiac perfusion with aprotinin-PBS prior to collection of liver tissue. The livers were exposed, removed, and minced into small pieces on ice to release cells. Cells were further dissociated by aspiration through a large-bore pipette and a 100-μm-pore-size cell strainer. Isolated cells were collected by centrifugation (1,000 × *g*, 5 min) and washed three times in cold PBS. Cytoplasmic fractions (S100) and nuclear extracts (NE) from livers were prepared by mechanical lysis. A packed cell volume (PCV) was resuspended in 2.5 PCVs of lysis buffer (10 mM HEPES, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5% NP-40, 0.34 M sucrose, 10 mM phenylmethylsulfonyl fluoride [PMSF], 10 mM 2-mercaptoethanol) and homogenized with a Dounce homogenizer. The lysate was centrifuged at 500 × *g* for 10 min at 4°C to generate the supernatant (for S100) and the packed nuclear pellet (for NE). The supernatant was removed and centrifuged at 100,000 × *g* for 20 min to eliminate plasma membrane contamination. The ATX and WT supernatants were removed and then precleared by the addition of 15 μl of protein A-Sepharose bead slurry (50%), and samples were then rotated for 1 h at 4°C, followed by centrifugation at 100,000 × *g* for 20 min. For IP, 10 mg of each cleared supernatant was mixed with 8 μg of affinity-purified antibody and rotated for 2 h at 4°C. The reaction mixture was then centrifuged at 100,000 × *g* for 20 min at 4°C. The antibody/protein/beads were then washed three times with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40), boiled with Laemmli buffer, and subjected to SDS-PAGE with 4%- to 20%-gradient SDS-acrylamide gels made with Tris-glycine buffer (Novex gel; Invitrogen). Gels were stained with Coomassie brilliant blue to identify lanes, and lanes were cut into 10 fragments. The excised fragments were destained and subjected to in-gel digestion using 100 ng trypsin in 50 mM NH_4HCO_3, pH 8.5, for 4 h. Peptides were then extracted with acetonitrile and dried in a Speed-Vac dryer (Thermo Savant).

Protein identification by nano-high-performance liquid chromatography/MS analysis. Dried samples were resuspended in 20 μl of 5% methanol-95% water-0.1% formic acid (FA), loaded onto an 0.3-mm by 5-mm C_{18} trap column (Zorbax 300SB; Agilent Technologies), equilibrated in 0.1% FA in water at 10 μl/min, and washed for 5 min at the same flow rate, and then the trap column was switched in-line with an 100-mm by 75-μm column packed with BioBasic C_{18} matrix (PicoFrit; New Objective) equilibrated in 0.1% FA-water. The peptides were separated with a 50-min discontinuous gradient of acetonitrile-0.1% FA (5 to 60% acetonitrile for 20 min) at a flow rate of 200 nl/min. Separated peptides were electrosprayed directly into a mass spectrometer using a nanospray source with a voltage of 2.5 kV applied to the liquid junction. The mass spectrometer was operated in the data-dependent mode, acquiring fragmentation spectra of the top 20 strongest ions. The obtained tandem MS (MS/MS) spectra were analyzed against the NCBI reference protein sequence database using the BioWorks database search engine (BioWorksBrowser version 3.2; Thermo Electron). All peptide hits with stringent BioWorksBrowser filtering criteria with a peptide probability of $>5 \times 10^{-5}$ and an Xcorr score of >2.5 for 2+ ions and >4.5 for 3+ ions were further examined manually, and all peptides had to be assigned by consecutive b or y ions to eliminate false positives.

HBV plasmid replication assay. HBV replication was measured using a plasmid replication system, as described previously (22, 23). Briefly, HepG2 cells grown in six-well plates were transfected in triplicate with a plasmid carrying greater-than-genome-length HBV DNA that drives the expression of all HBV proteins (payw1.2), including HBx (33), or with an HBx-deficient plasmid that does not express HBx due to a stop codon (payw1.2*7) (34). In the present study, these plasmids are referred to as pHBV and pHBVΔX, respectively. Comparison of virus replication between pHBV and pHBVΔX revealed replication that was due to HBx, and the HBx-deficient replication was restored by cotransfection of a plasmid encoding HBx (pSI-X), as described previously (22, 23). On day 3 posttransfection, cells were lysed with NP-40-containing buffer, and viral capsids

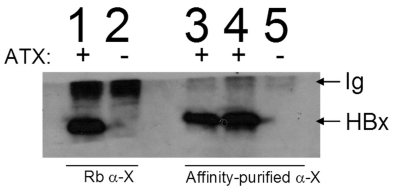

FIG. 1. Detection of HBx in ATX mouse liver extracts using affinity-purified antibody. Rabbit (Rb) antibody prepared against full-length HBx protein (α-X) detects the 17-kDa HBx protein in extracts of ATX but not nontransgenic livers (lanes 1 and 2, respectively) when used for IP/Western blotting, as described previously (16). Following affinity purification of the antibody (see Materials and Methods), α-X retained reactivity with HBx (lanes 3 and 4) and did not react with extracts of nontransgenic livers (lane 5). IP was performed with 2 mg liver lysate, using 1 μl (lanes 1, 2, 4, and 5) or 0.5 μl (lane 3) of α-X. The migration of HBx is indicated at the right.

were purified. Capsid-associated viral DNA was purified using a QIAamp MinElute virus spin kit (Qiagen Corporation) and quantitated by real-time PCR as described previously (22, 23). Results were confirmed in at least three independent experiments.

Statistics. Error bars in the figures represent standard errors of the means within a representative experiment. Statistical significance, measured from a minimum of 3 independent experiments, was determined by Student's *t* test using Microsoft Excel software. Significance (asterisk) was assigned for *P* values of less than 0.05.

RESULTS

The hydrodynamic tail vein injection model (29) has been used to establish that mouse liver can support high levels of HBV replication (59) that is dependent upon the expression of HBx (22, 23). We reasoned that the liver of HBx transgenic mice is an appropriate tissue from which to identify HBx-interacting proteins that are relevant to virus replication.

ATX transgenic mice. Livers from alpha-1-antitrypsin–HBx (ATX) mice (26) and control nontransgenic (wild-type [WT]) littermates were used for the identification of HBx-interacting proteins *in vivo*. Expression of HBx was confirmed by a combination IP/Western blot analysis of liver lysates, carried out using either whole rabbit anti-HBx serum (Fig. 1, lane 1) or the same antibody that had been affinity purified by binding to and eluting from GST-HBx (see Materials and Methods) (Fig. 1, lanes 3 and 4). No HBx was detected in the livers of nontransgenic mice (Fig. 1, lanes 2 and 5). The amount of liver lysate needed for IP (2 mg per lane) is similar to that needed for IP of the woodchuck X protein from livers of woodchucks chronically infected with the related woodchuck hepatitis virus, indicating that HBx is expressed at near-physiologic levels in the livers of ATX mice. These results establish that affinity-purified antibody retains its reactivity to HBx (Fig. 1, lanes 3 and 4).

HBx-interacting proteins *in vivo*. The affinity-purified antibody was next used to IP lysates from ATX and control WT livers. Liver cytoplasmic extracts were prepared to preserve protein complexes (see Materials and Methods). MS analysis of recovered proteins was performed for both the ATX and the WT livers. Four proteins that copurified with HBx from ATX livers, but not from IPs of WT livers lacking HBx, were identified (Table 1). The IPS-1 protein (also known as MAVS [40], Cardiff [35], and VISA [21]) was selected for follow-up because of its established role in the innate immune response (reviewed in references 21 and 44) and the knowledge that it is targeted for inactivation by other viruses that replicate in the liver, including hepatitis A virus (HAV) (37, 60), hepatitis C virus (HCV) (6, 28, 30), and GB virus B (5).

The interaction of HBx with IPS-1 was confirmed by reciprocal co-IP experiments in transfected human liver HepG2 cells. Cells were transfected with plasmids encoding IPS-1 or HBx and harvested at 48 h. In cells receiving both plasmids, HBx was identified by IP/Western blotting when the IP was performed with either rabbit anti-IPS-1 or rabbit anti-HBx (Fig. 2A, lanes 4 and 5, respectively). Specificity controls included a negative-control antibody (Fig. 2A, lane 1) and the demonstration that rabbit anti-IPS-1 did not cross-react with HBx (Fig. 2A, lane 6). In the reciprocal experiments, IP with either rabbit anti-IPS-1 or rabbit anti-HBx led to Western blot detection of IPS-1 (Fig. 2B, lanes 4 and 5, respectively), confirming that HBx and IPS-1 were in a complex. Neither the negative-control antibody nor the rabbit anti-HBx reacted with IPS-1 (Fig. 2B, lanes 1 and 3, respectively). Densitometer scanning of anti-HBx IPs from pSI-X-transfected HepG2 cells revealed that less than 5% of endogenous IPS-1 was present in a complex with HBx (data not shown). These results support the IP/MS results and confirm that HBx and IPS-1 interact directly or indirectly in human liver HepG2 cells.

HBx does not inhibit dsRNA-induced IFN-β. IPS-1 is a key adaptor protein that interacts with cytoplasmic receptors, such as RIG-I, to mediate the activation of IFN-β (Fig. 3). We next tested whether HBx would alter IPS-1 function. Cells grown in culture cannot be infected by HBV, and so the innate signaling pathway was instead induced using poly(I:C). The use of this synthetic dsRNA is relevant since HBV replication proceeds through a pregenomic RNA intermediate (reviewed in reference 39) that might trigger the RIG-I signaling pathway. Cells were cotransfected with plasmid DNAs encoding firefly (F)

TABLE 1. HBx-interacting proteins identified by coimmunoprecipitation from ATX, but not WT, liver cytoplasmic extracts[a]

Gene[b]	Protein[c]	Putative function	Accession no.[d]
VISA	IPS-1	Beta interferon promoter stimulator 1[e]; involved in IFN-β signaling	MGI 2444773
Ank3	ANK3	E-cadherin partner; role in polarization of membrane proteins	MGI 88026
PRIC285	PR285	ATP-dependent helicase; peroxisomal proliferator-activated receptor A-interacting complex 285	MGI 63178
PM20D1	PM20D1	Contains peptidase M20 domain; probable carboxypeptidase	MGI 2442939

[a] Determined by co-IP and mass spectrometry sequencing.
[b] Full-length cDNA and shRNA clones for all four genes are commercially available from Open Biosystems.
[c] Commercial antibodies are available for IPS-1 and ANK3 only.
[d] MGI, Mouse Genome Informatics database, available at http://www.informatics.jax.org.
[e] Also known as mitochondrial antiviral signaling protein (MAVS), VISA, and Cardiff; referred to as IPS-1 in the current study.

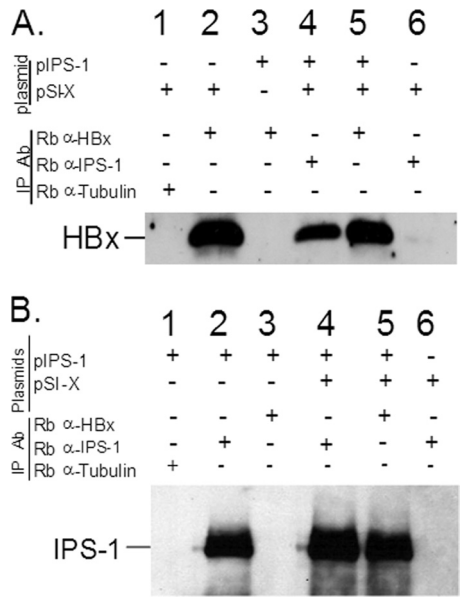

FIG. 2. Reciprocal co-IP of HBx and IPS-1 in transfected HepG2 cells. (A) Western blot detection of HBx. Cells were transfected with the plasmids indicated, and IP was performed using the antibodies (Ab) listed. Complexes were separated by 15% SDS-PAGE and transferred to nitrocellulose, and HBx was detected by Western blotting (see Materials and Methods). (B) Western blot detection of IPS-1. Cells were transfected with the plasmids indicated, and IP was performed using the antibodies listed. Complexes were analyzed by 10% SDS-PAGE and transferred to nitrocellulose, and IPS-1 was detected by Western blotting using anti-HA, which detected HA-tagged IPS-1 (see Materials and Methods).

luciferase under the control of the IFN-β regulatory elements. A plasmid encoding *Renilla* (R) luciferase was included as a control for normalization. After 36 h, cells were transfected with increasing amounts of poly(I:C). At 24 h posttransfection, cells were harvested and analyzed for luciferase (F/R) activity by use of a dual-luciferase assay (see Materials and Methods). Poly(I:C) induced IFN-β luciferase in a dose-dependent manner (Fig. 4A), as expected. This activation was significantly reduced by cotransfection of a plasmid DNA carrying an IPS-1-specific shRNA (Fig. 4B), indicating that the activation of IFN-β occurred through an IPS-1-dependent pathway, as previously reported (21). We first tested the hypothesis that HBx might inhibit poly(I:C)-activated IFN-β. Cells were cotransfected with the luciferase reporter plasmids and plasmids encoding full-length HBx (pSI-X) or empty vector (pSI). A plasmid encoding influenza virus NS-1, which is reported to inhibit poly(I:C)-induced IFN-β (46), was included as a positive control and led to a significant decrease in poly(I:C)-induced IFN-β (Fig. 4D). However, HBx did not inhibit poly(I:C)-induced IFN-β but instead led to a dose-dependent increase in IFN-β (Fig. 4C). This 2.5-fold increase in IFN-β luciferase is consistent with numerous reports that HBx is a weak but promiscuous transcriptional transactivator (reviewed in reference 2). Indeed, we found that in the absence of poly(I:C), HBx exerts a modest dose-dependent activation of the IFN-β promoter in 293T (Fig. 4E) and HepG2 (data not shown) cells. We conclude that HBx expression does not interfere with poly(I:C)-induced IFN-β.

HBx inhibition of dsDNA-induced IFN-β. It is now appreciated that cytoplasmic sensors that recognize double-stranded DNA (dsDNA) may also lead to activation of the RIG-I pathway (1, 8). Since HBV replication involves the production of core particles that contain partially double-stranded DNA, we next tested the effect of HBx on dsDNA-induced RIG-I signaling. The above-described experiments were repeated using the synthetic dsDNA polymer poly(dAT:dAT) to activate IFN-β, as described previously (7). A dose-dependent IFN-β

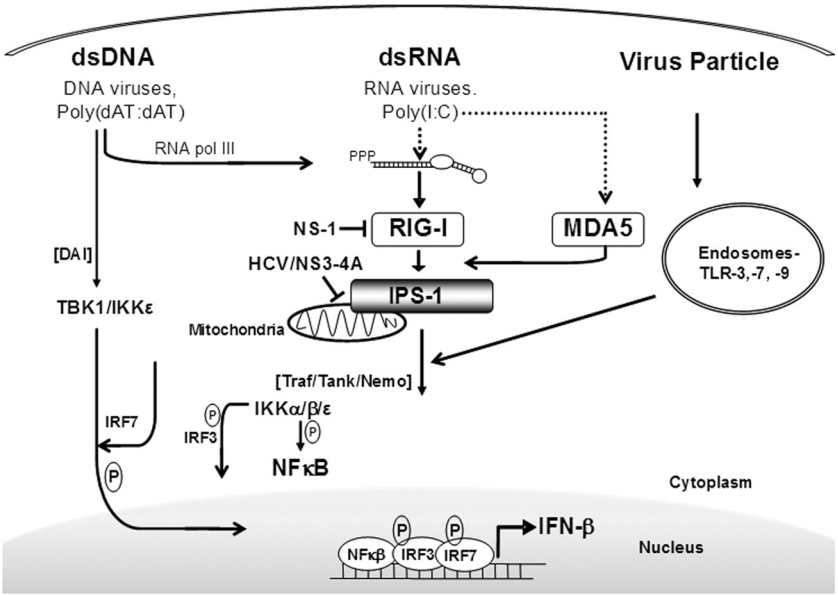

FIG. 3. IFN-β activation pathways. Viral molecules (protein, RNA, and DNA) are detected by cytoplasmic pattern recognition receptors, including RIG-I, MDA-5, TLRs, and DAI. IPS-1 is a key adaptor molecule that binds to activated RIG-I and mediates downstream signaling events, leading to the activation of IFN-β. Other studies have identified the influenza virus NS-1 and the hepatitis C virus NS3-4A proteins that inactivate this signaling (6, 28, 30, 46).

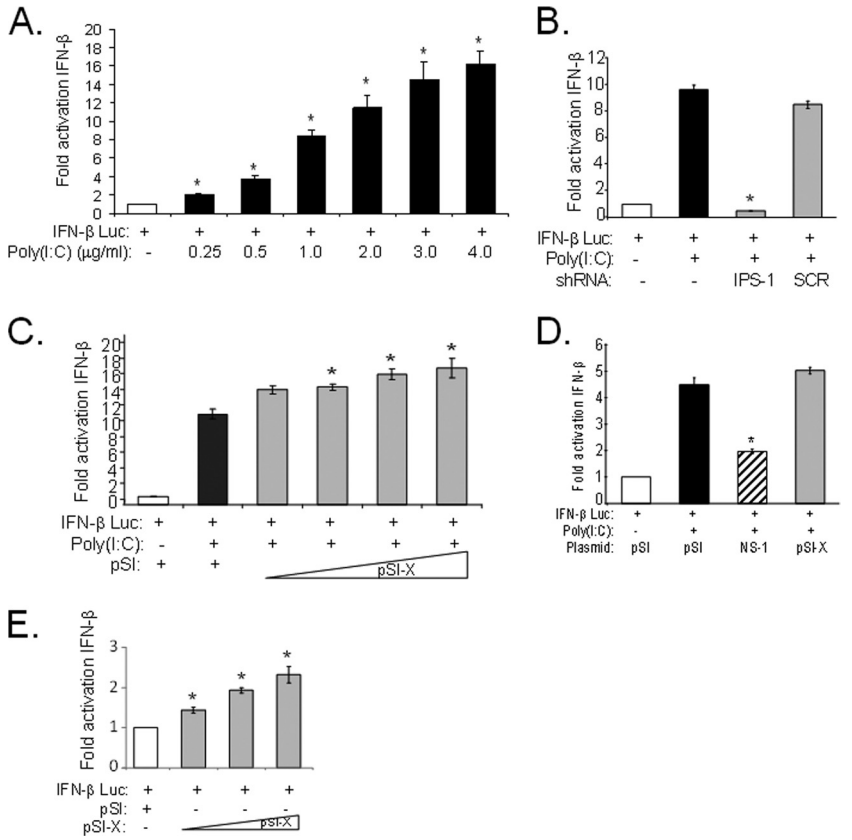

FIG. 4. Effect of HBx on poly(I:C)-induced IFN-β activation. (A) Dose-dependent induction of IFN-β in 293T cells transfected with increasing amounts of poly(I:C). (B) Inhibition of poly(I:C)-induced IFN-β by a cotransfected plasmid carrying IPS-1 shRNA but not scrambled (SCR) shRNA. (C) Effect of increasing amounts of cotransfected pSI-X (0.15, 0.30, 0.45, and 0.60 μg per well) on poly(I:C)-induced IFN-β. No inhibition was observed. (D) A plasmid encoding influenza virus NS-1 inhibits poly(I:C)-induced IFN-β, while a plasmid encoding HBx does not. (E) Dose-dependent activation of the IFN-β promoter by pSI-X. The representative results shown were reproduced in 3 independent experiments and confirmed in HepG2 cells.

response to increasing amounts of transfected poly(dAT:dAT) was demonstrated (Fig. 5A), confirming a previous study (7). Cotransfection of a plasmid carrying IPS-1 shRNA, but not a scrambled shRNA, inhibited the activation of IFN-β (Fig. 5B), indicating that the activation of IFN-β by poly(dAT:dAT) occurs through an IPS-1 pathway, as previously reported (7). To determine a possible effect of HBx on poly(dAT:dAT)-induced IFN-β, we added increasing amounts of plasmid encoding full-length HBx (pSI-X) or vector pSI (negative control). HBx significantly inhibited dsDNA-induced IFN-β in a dose-dependent manner in 293T (Fig. 5C) and HepG2 (data not shown) cells. Our results demonstrate that HBx can inhibit poly(dAT:dAT)-activated IFN-β. The inability of HBx to completely inhibit IFN-β signaling may be due to limitations of the experimental design. Most cotransfected cells take up both the IFN-β luciferase and pSI-X plasmids, but those containing

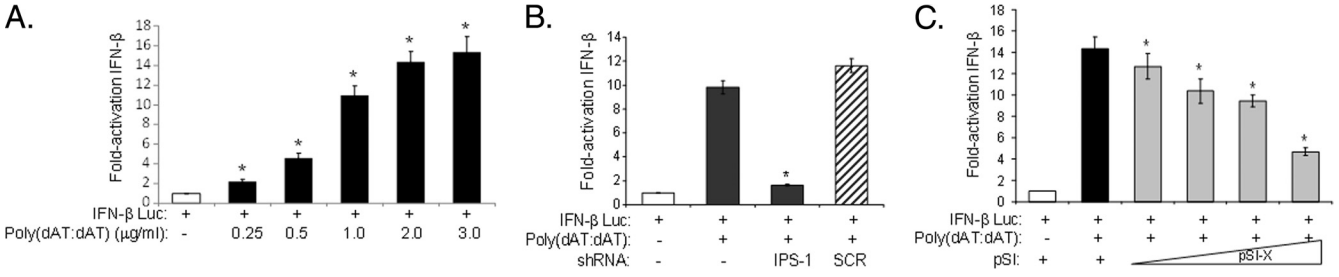

FIG. 5. HBx inhibits poly(dAT:dAT) activation of IFN-β. (A) Dose-response effect of transfected poly(dAT:dAT) on the activation of IFN-β (see Materials and Methods). (B) Poly(dAT:dAT) (2 μg/ml)-activated IFN-β is inhibited by a cotransfected plasmid carrying IPS-1-specific shRNA but not scrambled shRNA. (C) Dose-dependent inhibition of poly(dAT:dAT)-induced IFN-β by cotransfected pSI-X (0.15, 0.30, 0.45, or 0.60 μg per well). All samples were tested in triplicate wells from 24-well plates. Results were reproduced in at least 3 independent experiments and confirmed in HepG2 cells.

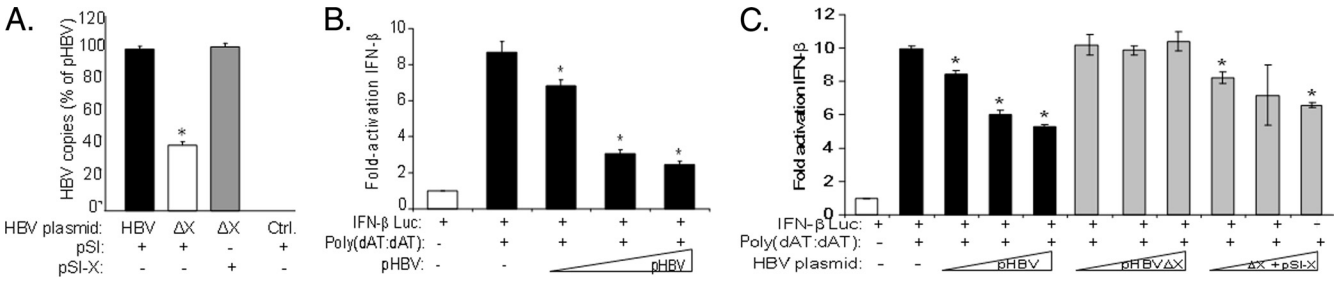

FIG. 6. Inhibition of poly(dAT:dAT)-activated IFN-β in the context of HBV replication. (A) HBV and HBx-deficient HBV (ΔX) replication was measured in HepG2 cells, as described in Materials and Methods. The latter was restored to wild-type levels by cotransfection of pSI-X. (B) Effect of HBV replication on poly(dAT:dAT)-activated IFN-β in transfected HepG2 cells. HBV replication inhibited IFN-β activation in a dose-dependent manner. (C) The experiment described for panel B was repeated using 293T cells. HBV replication with HBx (pHBV) inhibited poly(dAT:dAT)-activated IFN-β, while replication from HBx-deficient pHBVΔX did not alter IFN-β levels. Cotransfection of pHBVΔX with pSI-X restored the inhibition of poly(dAT:dAT)-activated IFN-β luciferase. Representative results shown were reproduced in both 293T and HepG2 cells.

only IFN-β luciferase remain fully responsive to activation by poly(dAT:dAT). In addition, the ability of HBx to transactivate the IFN-β promoter (Fig. 4E) is anticipated to contribute to IFN-β levels above background. We next examined whether the inhibition occurs in the context of HBV replication.

A plasmid-based HBV replication assay that permits the study of HBx function in the context of virus replication is now available (3). HepG2 cells were transfected with a plasmid encoding wild-type HBV (pHBV), an HBx-deficient plasmid (pHBVΔX), pHBVΔX plus pSI-X, or a control (non-HBV) plasmid. At day 3 posttransfection, cells were lysed, and capsid-associated DNA was purified and quantitated by real-time PCR (see Materials and Methods). Virus replication from the HBx-deficient pHBVΔX plasmid was significantly reduced relative to that measured from wild-type pHBV, and the lower HBV replication was restored to wild-type levels by cotransfection of a plasmid encoding HBx (Fig. 6A). These results establish the HBx-dependent virus replication previously reported by us (22, 23) and others (3, 27, 47). This plasmid-based HBV replication had negligible effects on the activation of cotransfected IFN-β luciferase (data not shown). We next repeated the above-described experiments in the presence of poly(dAT:dAT)- and poly(I:C)-induced IFN-β activation. HBV replication in HepG2 cells inhibited poly(dAT:dAT)-induced IFN-β in a dose-dependent manner (Fig. 6B). A similar result was obtained when the experiment was performed in 293T cells (Fig. 6C). This inhibition was not apparent when replication was driven from the HBx-deficient plasmid (pHBVΔX), but the inhibition was restored when pHBVΔX was cotransfected with a plasmid encoding full-length HBx (pSI-X) (Fig. 6C). In contrast, there was no effect of HBV replication on poly(I:C)-induced IFN-β (data not shown), a result consistent with the failure of pSI-X to inhibit poly(I:C)-induced IFN-β (Fig. 4C). Together, these results demonstrate that the HBx inhibition of poly(dAT:dAT)-activated IFN-β is apparent within the context of HBV replication.

HBx localized to the cytoplasm inhibits dsDNA-induced IFN-β. The 17-kDa HBx protein localizes to both the nucleus and the cytoplasm, where it presumably has different functions. Since IPS-1 is localized to the cytoplasm (Fig. 3), we hypothesized that HBx localized to that compartment, but not HBx localized to the nucleus, would inhibit dsDNA-induced IFN-β luciferase. We found that HBx targeted to the cytoplasm by virtue of an in-frame nuclear export signal (NES-HBx [23]) suppressed poly(dAT:dAT)-induced IFN-β in a manner similar to that observed for wild-type HBx encoded by pSI-X (Fig. 7A). In contrast, HBx targeted to the nucleus by an in-frame nuclear localization signal (NLS-HBx [23]) was significantly less efficient at inhibiting the dsDNA-induced activation of IFN-β (Fig. 7A). NLS-HBx causes predominant, but not exclusive, localization to the nucleus (23), and the small amount of NLS-HBx in the cytoplasm may explain the partial inhibition of IFN-β by this HBx expression construct. No significant differences in the steady-state levels of NES-HBx and NLS-HBx were noted by IP/Western blotting (Fig. 7B). These results

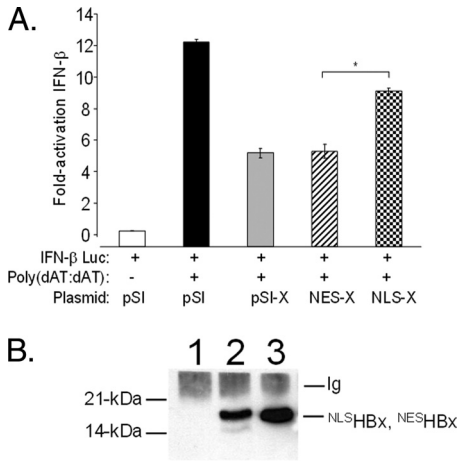

FIG. 7. Effect of HBx localization on poly(dAT:dAT)-induced IFN-β. (A) 293T cells were transfected with the plasmids indicated, and the ability of HBx, nucleus-excluded HBx (NES-X), or nucleus-localized HBx (NLS-X) to inhibit poly(dAT:dAT)-induced IFN-β was measured as described in Materials and Methods. The representative result shown was confirmed in 4 independent experiments. (B) Cells grown in 60-mm plates were transfected with pSI (lane 1), pSI-NLS-HBx (lane 2), or pSI-NES-X (lane 3). Lysates from 3 transfected plates were pooled and analyzed by IP/Western blotting for HBx (see Materials and Methods). No reproducible differences in steady-state levels of NLS-X and NES-X were detected in multiple experiments. The migration of molecular size markers is shown at the left.

support the idea that HBx inhibition of dsDNA activation of IFN-β is mediated by cytoplasm-localized HBx.

DISCUSSION

IPS-1 is an adaptor protein that plays an essential role in the antiviral innate immune response leading to the activation of IFN-β (44). The present study sought to identify HBx-interacting cellular proteins from mouse liver by using IP/MS, and four novel cytoplasmic binding partners of HBx, including IPS-1, were identified. The interaction of IPS-1 and HBx was confirmed by reciprocal co-IP and Western blotting experiments in transfected human liver HepG2 cells. IPS-1 is important in both dsRNA- and dsDNA-induced activation of IFN-β and is targeted for inactivation by several viruses as part of the viral strategy to benefit replication. Since HBV is a DNA virus that replicates through an RNA intermediate, we examined the effect of HBx on both pathways. We show that HBx expressed alone or in the context of HBV replication specifically inhibits the dsDNA pathway of IFN-β activation in a dose-dependent manner. Consistent with the cytoplasmic localization of IPS-1, we show that HBx localized to the cytoplasm by virtue of an in-frame nuclear export signal dampens the activation of dsDNA-induced IFN-β, while HBx targeted to the nucleus by a nuclear localization signal does not. These results may explain, in part, how HBV is able to avoid activating the innate immune response during virus infection. Our findings are significant in that HBV now joins HAV and HCV in targeting the same innate immunity pathway for inactivation.

It is established that HBV replication is susceptible to control by an activated innate immune response. TLR agonists inhibit HBV replication in transgenic mice (18) and in HBV-Met immortalized mouse hepatocytes (56). Activation of the RIG-I pathway by overexpression of key adaptor molecules, including IPS-1, suppresses HBV replication in plasmid-transfected HepG2 and Huh7 cells (15). Treatment of HBV-infected HepaRG cells with IFN receptor 1 silencing RNA or with antibody against IFN-β leads to reduced HBV replication (31). Together, these studies demonstrate that HBV replication can be controlled by the innate immune response and establish the need for HBV to have a mechanism to circumvent this inhibition. Indeed, studies of the related woodchuck and duck hepadnavirus replication show that following viral entry, the virus spreads to the vast majority of hepatocytes (19, 38), indicating that the virus can overcome the innate immune response. Other studies in chimpanzees (52), primary human hepatocytes exposed to HBV-positive serum (17), and acutely infected humans (10) reveal that early following HBV infection, there is no activation of IFN-α/β. These observations demonstrate that HBV has evolved a strategy(ies) to block the innate immune response. In this context, our finding that HBx binds to the IPS-1 adaptor protein and diminishes IFN-β signaling is of significant importance in the HBV field.

A model of HBV-induced inhibition of innate immunity needs to be considered within the context of what is known of HBV replication (reviewed in reference 39). HBx does not reside within the virus particle, and so an alternative strategy must be in place to block the earliest steps in innate immunity. While our study was in progress, two laboratories reported that the HBV polymerase (Pol) protein (which is present in the virus particle), but not HBx, has the ability to block the activation of IFN-β induced by poly(I:C) (48, 61) (Table 2). Those studies did not investigate the impact of Pol or HBx on the poly(dAT:dAT)-induced IFN-β pathway.

TABLE 2. Inactivation of RIG-I signaling by HBV proteins

HBV protein	Inhibition of IFN-β induced by:			Reference or source
	Poly(I:C)[a]	RNA viruses	Poly(dAT:dAT)[a]	
Pol	Yes	Yes[b]	—[c]	61
	Yes	Yes[d]	—	48
HBx	No	No[b]	—	61
	No	—	—	48
	—	Yes[e]	Yes	51
	Yes	Yes[e]	—	49
	No	—	Yes	Present study

[a] Poly(I:C)- and poly(dAT:dAT)-induced IFN-β, as described in Materials and Methods.
[b] Inhibited IFN-β induced by Newcastle disease virus and Sendai virus.
[c] —, not tested.
[d] Inhibited IFN-β induced by Sendai virus.
[e] Inhibited IFN-β induced by vesicular stomatitis virus.

Available data suggest that HBx may impact multiple sites within the RIG-I pathway (Table 2). While we and others found that HBx has no effect on poly(I:C)-induced IFN-β signaling (Fig. 4C) (48), other recent reports suggest that HBx can inhibit poly(I:C)- and vesicular stomatitis virus (VSV)-induced IFN-β (49, 51). Additional studies are needed to sort out these discrepancies. The finding that at least two HBV proteins (HBx and polymerase) act to block the innate immune response supports the idea that HBV has evolved multiple strategies to overcome the host immune response during the entry/expansion phases of disease. For example, the presence of HBV polymerase in the virus particle ensures that infected cells do not mediate a robust innate response, while HBx may be involved at a later point in the HBV replication cycle.

The mechanism by which HBx inhibits RIG-I signaling is not yet established, but both IPS-1-dependent and -independent pathways may be considered. IPS-1-dependent pathways are suggested by the interaction of HBx with IPS-1 reported in this study (Fig. 2) and in two studies published while the manuscript was in preparation (48, 51). By analogy to other hepatitis viruses, it is possible that the HBx–IPS-1 interaction directly alters IPS-1 stability or subcellular localization. The HCV nonstructural NS3-4A protein binds IPS-1 and mediates its cleavage, thereby diminishing its ability to signal downstream targets (6, 28, 30). Similarly, the HAV nonstructural 2B/3ABC proteins bind IPS-1 and mediate its cleavage to subsequently inactivate IPS-1 signaling (37, 60). Of interest, several laboratories have independently demonstrated that HBx localizes to the outer mitochondrial membrane (3, 45, 50), and one study revealed lower steady-state levels of IPS-1 in HBx-transfected cells (51). Recently, IPS-1 was discovered to also associate with peroxisomes (9, 41), raising the possibility that HBx–IPS-1 acts at that subcellular localization. Alternatively, HBx may inhibit innate immunity through IPS-1-independent pathways, such as TLR signaling (43). Consistent with this idea, preincubation of HepaRG cells with HBV-positive serum led to a suppression of TLR-mediated innate immunity (57) and a resistance to VSV-mediated RIG-I signaling (51). Additional studies are

needed to firmly establish the molecular basis by which HBx inhibits RIG-I signaling.

In summary, our study provides evidence that the HBV regulatory protein HBx interferes with the activation of the innate immune response. The strengths of this study include the fact that HBx binding partners were identified from liver tissue, which supports high levels of HBV replication (22). None of the four cytoplasmic binding partners of HBx identified in this study have previously been reported using yeast 2-hybrid screens, a finding that may suggest that mouse and human liver cells contain a factor(s) that facilitates the interaction of IPS-1 with HBx. Our discovery that HBx binds to IPS-1 led us to identify HBx inhibition of dsDNA-activated IFN-β signaling, an observation that is consistent with the failure of HBV to activate IFN-β during the entry/expansion phases of infection. Our results validate the IP/MS approach as a sensitive and useful tool for identifying biologically relevant binding partners of HBx. However, the lack of a convenient HBV infection model slows progress toward identifying a precise molecular pathway through which HBx acts. Our findings are significant in providing a possible explanation for how HBV is able to efficiently infect and spread through the liver without activating type I interferons. Importantly, HBV now joins HCV and HAV in targeting the same innate immune response pathway through the IPS-1 adaptor protein, presumably as a strategy for virus replication in the liver.

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