The Hepatitis B Virus X Protein Disrupts Innate Immunity by Downregulating Mitochondrial Antiviral Signaling Protein

Congwen Wei, Caifei Ni, Ting Song, Yu Liu, XiaoLi Yang, Zirui Zheng, Yongxia Jia, Yuan Yuan, Kai Guan, Yang Xu, Xiaozhong Cheng, Yanhong Zhang, Xiao Yang, Youliang Wang, Chaoyang Wen, Qing Wu, Wei Shi and Hui Zhong

*J Immunol* published online 16 June 2010
http://www.jimmunol.org/content/early/2010/06/16/jimmunol.0903874

---

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/06/16/jimmunol.0903874.4.DC1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Hepatitis B Virus X Protein Disrupts Innate Immunity by Downregulating Mitochondrial Antiviral Signaling Protein

Congwen Wei,*1 Caifei Ni,*1 Ting Song,*1 Yu Liu,† Xiaoli Yang,† Zirui Zheng,* Yongxia Jia,* Yuan Yuan,* Kai Guan,* Yang Xu,‡ Xiaozhong Cheng,* Yanhong Zhang,* Xiao Yang,* Youliang Wang,* Chaoyang Wen,§ Qing Wu,† Wei Shi,‡ and Hui Zhong*

Previous studies have shown that both hepatitis A virus and hepatitis C virus inhibit innate immunity by cleaving the mitochondrial antiviral signaling (MAVS) protein, an essential component of the virus-activated signaling pathway that activates NF-κB and IFN regulatory factor-3 to induce the production of type I IFN. For human hepatitis B virus (HBV), hepatitis B s-Ag, hepatitis B e-Ag, or HBV virions have been shown to suppress TLR-induced antiviral activity with reduced IFN-β production and subsequent induction of IFN-stimulated genes. However, HBV-mediated suppression of the RIG-I–MDA5 pathway is unknown. In this study, we found that HBV suppressed poly(deoxyadenylate-thymidylate)-activated IFN-β production in hepatocytes. Specifically, hepatitis B virus X (HBX) interacted with MAVS and promoted the degradation of MAVS through Lys136 ubiquitin in MAVS protein, thus preventing the induction of IFN-β. Further analysis of clinical samples revealed that MAVS protein was downregulated in hepatocellular carcinomas of HBV origin, which correlated with increased sensitivities of primary murine hepatocytes isolated from HBX knock-in transgenic mice upon vesicular stomatitis virus infections. By establishing a link between MAVS and HBX, this study suggests that HBV can target the RIG-I signaling by HBX-mediated MAVS downregulation, thereby attenuating the antiviral response of the innate immune system. 

The online version of this article contains supplemental material.

*Beijing Institute of Biotechnology and †The General Hospital of Chinese People’s Liberation Army, Beijing; and ‡Key Laboratory for Molecular Enzymology and Engineering, Jilin University, Changchun, China

1C.W., C.N., and T.S. contributed equally to this work.

Received for publication December 8, 2009. Accepted for publication May 16, 2010.

This work was supported in part by National Natural Science Foundation of China Grants 30772605, 30700413, 30870500, and 30871276 and in part by Beijing National Science Foundation Grant 7092081.

Address correspondence and reprint requests to Drs. Hui Zhong and Wei Shi, Beijing Institute of Biotechnology, Beijing, China. E-mail addresses: towall@yahoo.com and shiw@jlu.edu.cn

The online version of this article contains supplemental material.

Abbreviations used in this paper: CARD, caspase recruitment domain; HA, hemagglutinin; HAV, hepatitis A virus; HBsAg, hepatitis B e-Ag; HBsAg, hepatitis B s-Ag; HBV, hepatitis B virus; HBX, hepatitis B virus X; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IB, immunoblotting; IKK, IκB kinase; IRF, IFN regulatory factor; MAVS, mitochondrial antiviral signaling; N–RIG-I, RIG-I N-terminal CARD-like domain mutant; poly(dAT:dAT), poly(deoxyadenylate-thymidylate); TM, transmembrane; VSV, vesicular stomatitis virus; WT, wild-type.

Copyright © 2010 by The American Association of Immunologists, Inc. 0002-1767/10S16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0903874

The Journal of Immunology
expression by cytosolic dsDNA also requires the intracellular dsRNA sensor RIG-I and its adaptor molecule MAVS (22, 23), raising the possibility that HBV might have also evolved strategies to interrupt the intracellular RIG-I–MAVS signaling pathway.

Among the proteins encoded by HBV, the hepatitis B virus X (HBX) protein is essential for viral replication in vivo (24) and is thought to contribute to hepatocarcinogenesis (25–27). HBX exerts most of its activities through direct interaction with TATA-binding proteins, leucine-zipper proteins, and DNA repair proteins (28–32). In this study, we report that MAVS interacts with HBX and is another new target of HBX protein. We found that HBX promoted the degradation of MAVS, thus preventing the induction of IFN-β. Further analysis of clinical samples revealed that MAVS protein was downregulated in hepatocellular carcinomas (HCCs) of HBV origin, which correlated with increased sensitivities of primary murine hepatocytes isolated from HBX knock-in transgenic mice upon vesicular stomatitis virus (VSV) infections.

Materials and Methods

Cell culture and transfections

HEK293, HepG2, HepG2215, and HepG2-1117 cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT). Cells were treated with MG132 (Sigma-Aldrich, St. Louis, MO) as noted in the text. HepG2-1117 cells contain 1.05-fold HBV genome (subtype ayw) under inducible Tet-off control (33). Myc-tagged HBV and their mutants, Myc-tagged HBX and HBV X, S, S1, and C, were expressed by cloning the genes into the pcDNA3-based vector (Invitrogen). HBV DNA genome head-to-tail dimer was provided by Prof. Y. Wang in our laboratory. HBX-deleted HBV-2 DNA was generated by two-step PCR using a QuikChange kit (Stratagene, La Jolla, CA). GST fusion proteins were generated by expression in pGEX4T-2-based vectors (Amersham Biosciences, Piscataway, NJ) in Escherichia coli BL21 (DE3). Flag-MAVS plasmid was provided by Zhijian Chen (University of Texas Southwestern Medical Center, Dallas, TX), and NF-κB–Luc, IFN-β–Luc, or IRF-3–Luc reporter gene constructs were provided by Li Li (Biotechnology Institute of China, Beijing, China). MAVS mutants were cloned into pcDNA3 using overlap extension PCR.

Immunoprecipitation and immunoblot analysis

Immunoprecipitation and immunoblot analysis were performed as previously described (34). Anti-MAVS (Abcam, Cambridge, MA), anti-Myc, HRP-conjugated anti-Flag (Sigma-Aldrich), anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-β-tubulin (Sigma-Aldrich) Abs were used.

Subcellular fractionation

Cells were washed 36 h after transfection in hypotonic buffer (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 1.5 mM MgCl2, protease inhibitors) and then homogenized in the same buffer by bouncing 20 times. The homogenate was centrifuged again at 5000 × g for 5 min to remove nuclei and unbroken cells. The supernatant was centrifuged again at 5000 × g for 10 min to generate membrane pellets containing mostly mitochondria and cytosolic supernatant.

Protein-binding assays

In GST pull-down experiments, cell lysates were incubated for 2 h at 4°C with 5 μg purified GST or GST fusion proteins bound to glutathione beads. The absorbates were washed with lysis buffer and then subjected to SDS-PAGE and immunoblot analysis. An aliquot of the total lysates (5%, v/v) was included as a loading control on the SDS-PAGE.

Luciferase reporter assays

HepG2 cells were transfected with 0.2 μg of the luciferase reporter pNF-κB–Luc, IFN-β–Luc, or IRF-3–Luc plus 0.02 μg of the Renilla reporter pRL-TK, with or without various amounts of MAVS, RIG-I–NF-κB CARD-like domain mutant (N–RIG-I) expression vector, or poly(dexoxyadenylate-thymidylate) (poly[dA:dT]; AT). Transfected cells were collected and luciferase activity was assessed as previously described (34). All experiments were repeated at least three times.

RNA analysis

First-strand cDNA was generated from total RNA using random priming and Moloney murine leukemia virus reverse transcriptase (Invitrogen).

Real-time PCR was performed using Quantitect SYBR Green PCR Master Mix (Qiagen, Valencia, CA) in triplicate experiments and analyzed on an ABI Prism 7700 analyzer (Applied Biosystems, Foster City, CA). All real-time values were normalized to 18S RNA with IFN-β using the following primers: IFN-β sense, 5′-CAGCAGCTCTTCTCCATGA-3′; IFN-β antisense, 5′-AGCCAGTGCTGATGAACTT-3′.

In vivo ubiquitination assays

HEK293 cells were cotransfected with plasmids expressing Flag-MAVS, Myc-HBX, and hemagglutinin (HA)-tagged ubiquitin. Cells were treated with MG132 (20 μM, 6 h) at 48 h after transfection, and they were then immunoprecipitated with anti-Flag Ab.

Clinical samples

Liver tumor samples and adjacent noncancerous tissues were obtained from Shaanxi Chao Yang Biotechnology (Xi’an, Shaanxi, China). Immunohistochemistry was performed as previously described (35). Rabbit anti-MAVS (Abcam) were used as primary Abs.

Mice and cells

HBX knock-in mice were generated as previously described (36). Primary murine hepatocytes from wild-type (WT) and HBX transgenic mice were isolated and cultured as described previously (19).

Viral infections

VSV, originally obtained from Dr. W. Chen (Institute of Pathology, Beijing, China), was harvested from cell culture supernatants of BHK-21 cells, and virus titers were determined by plaque formation on Vero cells. Primary murine hepatocytes were infected with the equivalent of 10 ml of VSV stock in serum-free medium (1 ml/well) for 1 h at 37°C. The infecting medium was then removed and replaced with 1 ml of normal growth medium. Cell supernatants were recovered 24 h postinfection, and virus titers were determined by plaque formation on Vero cells. HepG2-1117 cells were infected for 20 h with VSV (multiplicity of infection of 0.002) but were not killed; cells were fixed and then stained with amino black.

Results

Activation of IFN-β expression by cytosolic dsDNA is blocked by HBV

Recent reports suggest that HAV or HCV infection blocks the induction of IFN-β synthesis by dislodging MAVS from the mitochondria. To determine whether RIG-I–MDA5 signaling was disrupted by HBV infection, we ectopically expressed N–RIG-I or MAVS together with a luciferase reporter driven by the IFN-β promoter (IFN-β–Luc) in HepG2 cells transfected with a head-to-tail dimer of HBV genome (HBV-2 DNA). Previous studies have reported that HBV-2 DNA transfection supports the production of HBV particles in HepG2 cells. When using HepG2 cells, we also found that cells transfected with HBV-2 DNA continuously secreted HBsAg into the supernatant (Supplemental Fig. 1), suggesting that the transfected HBV DNA could replicate and produce virus particles. We thus analyzed whether HBV could affect type I IFN signaling using the HBV DNA-transfected HepG2 cells. As shown in Fig. 1A, both MAVS and N–RIG-I activated the IFN-β promoter in HepG2 cells transfected with empty vector, whereas these responses were substantially reduced in HepG2 cells transfected with the HBV-2 DNA. These results suggest that HBV DNA blocks RIG-I–MDA5 signaling. Poly(dAT:dAT) is a synthetic dsDNA that mimics dsDNA virus, which has previously been shown to induce IFN-β through RIG-I–MDA5 (11). In agreement with this observation, we also demonstrated that IFN-β response to the transfected poly(dAT:dAT) or VSV infection was reduced by HBV DNA (Fig. 1A). Furthermore, we observed that HBV DNA suppressed the activation of NF-κB and IRF-3 reporters through MAVS, RIG-I, poly(dAT:dAT), and VSV infection (Fig. 1A). To better understand the interference of IFN-β signaling by HBV, 105 HepG2 monolayer cells in a 6-cm plate were infected with HBV-positive serum containing 105 copies/ml HBVs and with control serum from uninfected individuals. Cells were washed eight times to remove the excess of viral inputs 2 h postinfection.

Downloaded from http://www.jimmunol.org/ by guest on April 20, 2017
HBV DNA blocks IFN-β induction by MAVS. A, HepG2 cells were transfected with plasmid-expressing HBV-2 DNA. After 36 h, cells were transfected with N–RIG-I, MAVS, or poly(dAT:dAT) together with IFN-β–Luc, NF-κB–Luc, or IRF-3–Luc. Cells transfected with HBV-2 DNA together with IFN-β–Luc, NF-κB–Luc, or IRF-3–Luc were infected with VSV for 20 h. The Luc activity was measured 24 h later and normalized for transfection efficiency. B, HepG2 cells were infected with HBV-positive serum containing 10^7 copies/ml HBV. Cells were washed eight times to remove excess viral inputs 2 h postinfection and were then transfected with poly(dAT:dAT) together with IFN-β–Luc, NF-κB–Luc, or IRF-3–Luc. Serum from uninfected individuals was used as a control. The Luc activity was measured 24 h later and normalized for transfection efficiency. C, HepG2-1117 cells were infected with VSV with or without doxycycline. Protein extracts were then resolved by SDS-PAGE (upper panel) or native gel electrophoresis (lower panel). Phosphorylation or dimerization of IRF-3 was detected by IB with an IRF-3 Ab. D, The experiments were carried out as in B, except that HBV X, S, S1, and C gene expression plasmid was transfected together with poly(dAT:dAT). Cell lysates were immunoblotted with anti-Flag Ab. The Luc activity was measured 24 h later and normalized for transfection efficiency. E, HepG2 cells were transfected with HBV-2 DNA or HBV-2 DNA–ΔHBX. After 36 h, cells were transfected with poly(dAT:dAT) together with IFN-β–Luc. The Luc activity was measured 24 h later and normalized for transfection efficiency. Cell lysates were immunoblotted with anti-HBX Ab. F, HepG2 cells were transfected with plasmids expressing HBX together with IFN-β–Luc, NF-κB–Luc, or IRF-3–Luc and were then infected with VSV. The Luc activity was measured 24 h later and normalized for transfection efficiency. G, HepG2 cells were transfected with the HBV-2 DNA or HBX expression plasmid together with the expression vectors for RIG-I, DAI, MAVS, TBK1, or IKKε. Cell lysates were immunoblotted with anti-Flag or anti-Myc Ab. β-Tubulin was used as an equal loading control. For A, B, and D–F, the Luc activity was measured 24 h later and normalized for transfection efficiency. The error bar represents SD from the mean value of duplicated experiments. IB, immunoblotting.
and were then transfected with poly(dAT:dAT) together with IFN-β-luc, NF-κB-Luc, or IRF-3-Luc. We found that HBV also inhibited the induction of IFN-β, IRF-3, and NF-κB by cytosolic poly(dAT:dAT) (Fig. 1B). HepG2-1117 cells contain Tet-off HBV-1.05 genome. Supplemental Fig. 1C shows that IFN-β, IRF-3, and NF-κB levels induced by VSV were lower in HepG2-1117 cells without doxycycline. In agreement with these data, immunoblotting experiments also showed that VSV-activated IRF-3 phosphorylation was inhibited by HBV and in HepG2-1117 cells without doxycycline (Supplemental Fig. 1B, 1C). To delineate the mechanism underlying the

FIGURE 2. HBX associates with MAVS. A. HEK293 cells were transfected with Flag-MAVS-expressing plasmid. The GST-fusion protein absorbates from cell lysates were analyzed by immunoblotting with anti-Flag Ab (top panel). Loading of the GST proteins was assessed by Coomassie blue staining (bottom panel). B. HEK293 cells were cotransfected with Flag-MAVS and Myc-HBX expression plasmid or Flag-vector, and anti-Flag or IgG immunoprecipitates were analyzed by immunoblotting with anti-Myc or anti-Flag Ab. C. HEK293 cells were transfected with Flag-MAVS expression plasmid or Flag-vector, and anti-Flag immunoprecipitates were analyzed by immunoblotting with anti-MAVS or anti-Flag Ab. D. HEK293 cells were cotransfected with Flag-MAVS expression plasmid and Myc-HBX (73–154) or Myc-HBX (73–154 C115A) plasmid, and mitochondria and nuclear and cytosolic fractions were analyzed by immunoblotting with anti-Myc, cytochrome c, or lamin B1 Ab. E. HEK293 cells were cotransfected with Flag-MAVS expression plasmid and Myc-HBX (73–154) or with Myc-HBX (73–154 C115A) plasmid, and anti-Flag immunoprecipitates were analyzed by immunoblotting with anti-Myc or anti-Flag Ab.
reduced IFN-β signaling, expression constructs for four HBV-encoded proteins (X, S, S1, and C) were generated, and one of these four constructs was used to cotransfect HepG2 cells together with poly(dAT:dAT). As shown in Fig. 1D, the induction of IFN-β by poly(dAT:dAT) was only inhibited by HBX. To confirm the inhibitory role of HBX in IFN-β signaling, we deleted the HBX gene in HBV-2 DNA. We found substantially higher levels of IFN-β induction in the cells thus transfected compared with those transfected with HBV-2 DNA (Fig. 1E). HBX introduction also blocked IFN-β promoter activation by VSV induction (Fig. 1F), suggesting that HBX was able to independently inhibit IFN-β activation.

To determine the modulation of protein abundance of the RIG-I–MAVS pathway by HBV DNA and HBX, immunoblotting was used to analyze the levels of RIG-I, MAVS, DAI, IKKe, and TBK1 proteins from HEK293 cells transfected with either empty vector, HBV-2 DNA, or HBX expression plasmid. As shown in Fig. 1G, MAVS protein abundance was substantially reduced by HBV-2 DNA or HBX, suggesting that MAVS is a target of HBV and that HBX is a potential factor that may interfere with the stability of MAVS and thus IFN-β signaling.

**HBX interacts with MAVS**

Our observation that HBX could downregulate MAVS, as well as the fact that both MAVS and some portion of HBX reside in the mitochondria compartment, raised the possibility that HBX might physically interact with MAVS. To test this possibility, lysates from HEK293 cells were incubated with GST or GST-HBX fusion protein. We found that MAVS bound to GST-HBX but not to GST (Fig. 2A), demonstrating an in vitro interaction between HBX and MAVS.

**FIGURE 3.** HBX blocks IFN-β induction by MAVS. **A,** HEK293 cells were transfected with increasing amounts of HBX expression vector. After 36 h, cells were transfected with MAVS together with IFN-β–Luc, NF-κB–Luc, or IRF-3–Luc. The Luc activity was measured 24 h later and normalized for transfection efficiency. The error bar represents SD from the mean value of duplicated experiments. **B,** The experiments were carried out as in **A,** except that N–RIG-I plasmid was transfected in lieu of MAVS. **C,** HEK293 cells were transfected with plasmid-expressing HBX. After 36 h, cells were transfected with poly(dAT:dAT). RNA was extracted and IFN-β mRNA was analyzed by quantitative RT-PCR. **D,** The experiments were carried out as in **A,** except that IKKe plasmid was transfected in lieu of MAVS.
HBX destabilizes MAVS to evade innate immunity.

**FIGURE 4.** HBX downregulates MAVS. A, HEK293 cells were transfected with plasmids expressing increasing amount of Myc-HBX (0.25, 0.5, and 0.75 μg). Whole-cell lysates were analyzed by immunoblotting with anti-MAVS or anti-Myc Ab. β-Tubulin was used as an equal loading control. B, Myc-HBX or Myc-HBX 73–154 C115A plasmid was transfected with the expression vector encoding HA-tagged ubiquitin. Cells were grown in DMEM containing MG132 (20 μM) for 6 h. Anti-MAVS immunoprecipitates were analyzed by immunoblotting with anti-HA Ab, whole-cell lysates were subjected to immunoblotting with anti-Myc and anti-MAVS Ab, and β-tubulin was used as an equal loading control. C, Myc-HBX or empty vectors were cotransfected with plasmids encoding Flag-MAVS or its mutants. Whole-cell lysates were analyzed by immunoblotting with anti-Flag or anti-Myc Ab. Ten nanograms of plasmid encoding GFP was transfected as a loading control, and whole-cell lysates were analyzed by immunoblotting with anti-GFP Ab. D, Myc-HBX or empty vectors were cotransfected with plasmids encoding Flag-MAVS or its mutants together with IFN-β–Luc. The Luc activity was measured 24 h later and normalized for transfection efficiency. The error bar represents SD from the mean value of duplicated experiments. E, Myc-HBX was transfected with the expression vector encoding Flag-MAVS or Flag-MAVS K136R mutant together with HA-tagged ubiquitin. Cells were grown in DMEM containing MG132 (20 μM) for 6 h. Anti-Flag immunoprecipitates were analyzed by immunoblotting with anti-HA Ab, whole-cell lysates were subjected to immunoblotting with anti-Myc and anti-Flag Ab, and β-tubulin was used as an equal loading control. F, HEK293 cells were cotransfected with plasmids expressing Flag-MAVS and Myc-HBX or its mutants. Whole-cell lysates were analyzed by immunoblotting with anti-Flag or anti-Myc Ab, and β-tubulin was used as an equal loading control. G, HEK293 cells were cotransfected with Flag-MAVS, Myc-HBX, or HBX mutants together with IFN-β–Luc. The Luc activity was measured 24 h later and normalized for transfection efficiency. The error bar represents SD from the mean value of duplicated experiments.
To test if HBX binds to MAVS in mammalian cells, HEK293 cells were transfected with Myc-tagged HBX and Flag-tagged MAVS. Immunoblotting analysis of anti-Flag immunoprecipitates with anti-Myc Ab showed a significant association between Flag-MAVS and Myc-HBX (Fig. 2B). To further characterize the endogenous MAVS interaction with HBX, lysates from HEK293 cells transfected with Flag-HBX were subjected to immunoprecipitation with anti-Flag Ab by immunoblotting with an anti-MAVS Ab, and MAVS was found in Flag-HBX–transfected immunoprecipitates but not in the nontransfected control immunoprecipitations (Fig. 2C). Additionally, lysates from HEK293 cells transfected with HBV-2 DNA were subjected to immunoprecipitation. HBX was shown to interact with endogenous MAVS in the HBV-2 DNA-transfected cells (Fig. 2C), suggesting that HBX interacts with MAVS in vivo.

To map the domains of MAVS responsible for the interaction with HBX, Flag-tagged proteins containing a deletion of various regions of MAVS (CARD, pro, and transmembrane [TM] domain) were prepared, and the ability of each of these mutants to interact with HBX was analyzed by immunoprecipitation. Fig. 2D shows that Myc-tagged HBX interacted with full-length and pro mutant MAVS but not with the TM mutant MAVS. The CARD MAVS mutant also interacted with HBX with lower affinity. Thus, the interaction with HBX is specific for the CARD and TM domains of MAVS.

To define the interacting regions of HBX on MAVS, Myc-tagged proteins containing N-terminal and C-terminal HBX were prepared, and the ability of each of these proteins to interact with MAVS was analyzed by immunoprecipitation. The C-terminal 73–154 residues of HBX did not affect its ability to interact with MAVS, whereas the N-terminal HBX 1–72 domain totally eliminated its ability to bind MAVS (Fig. 2E). Thus, the interaction with MAVS requires the C-terminal domain of HBX. A previous report revealed the key amino acid for mitochondrial targeting was mapped to be HBX C terminus 111–116 aas (37); when Cys 115 of HBX was mutated to alanine (HBX C115A), the mitochondrial targeting property of HBX was abrogated. Consistent with this report, we found that when Cys115 of HBX was mutated to alanine (HBX C115A), the mitochondrial targeting property of HBX was abrogated. Consistent with this report, we found that when Cys115 of HBX was mutated to alanine (HBX C115A), its mitochondrial localization was partially abrogated and its interaction with MAVS was much weaker than for the intact HBX C-terminal (HBX 73–154) (Fig. 2F, 2G).

**HBX blocks MAVS-mediated IFN-β induction**

To better understand how HBX blocks IFN-β signaling through MAVS, increasing amounts of HBX expression vector and 0.5 μg of MAVS expression construct were transfected into HEK293 cells together with IFN-β–Luc. As was shown in Fig. 3A, as low as 0.5 μg of HBX expression construct was sufficient to exert a potent repression of IFN-β response, and the extent of repression increased with increasing amounts of HBX expression, suggesting that HBX inhibited the induction of IFN-β by MAVS in a dose-dependent manner. We also observed similar repression of MAVS–induced activation of NF-κB and IRF-3 reporters by HBX (Fig. 3A). We then transfected cells with poly(dAT:dAT) or with an expression vector encoding N–RIG-I together with HBX. Fig. 3B shows that the induction of IFN-β by RIG-I or poly(dAT:dAT) (data not shown) was also inhibited by HBX. As expected, IFN-β mRNA levels were reduced sharply in cells containing the HBX expression plasmid (Fig. 3C). In contrast, the induction of IFN-β by IFN-α was not affected (Fig. 3D). Collectively, these data suggest that HBX inhibits RIG-I–MAVS signaling at the level of MAVS or at a step downstream of MAVS, but upstream of IKKε.

**HBX downregulates MAVS**

To further elucidate the mechanism for the inhibitory effect of HBX on antiviral signaling, we examined the effect of HBX on the endogenous MAVS protein level. Increasing amounts of the HBX expression vector were transfected into HEK293 cells. A striking reduction in the abundance of endogenous MAVS with overexpressed HBX was found, and this reduction of MAVS by HBX was also dose-dependent (Fig. 4A). As a control, increasing amounts of HBV S Ag expression vector did not change the level of endogenous MAVS (data not shown), indicating that this effect is specific to HBX. Similarly, the HepG2-1117 cell line, which contains an inducible Tet-off HBV-1.05 genome, showed reduced endogenous MAVS levels without doxycyline. Quantitative RT-PCR revealed that HBX overexpression did not change MAVS mRNA levels (data not shown), suggesting that HBX downregulates MAVS by posttranscriptional modification. Indeed, the estimated half-life of the MAVS was significantly longer than that of MAVS in the presence of HBX (data not shown). To further delineate the mechanisms responsible for the HBX-mediated MAVS degradation, HEK293 cells were cotransfected with plasmids expressing Myc-HBX or Myc-HBV 73–154 C115A together with HA-ubiquitin in the presence of proteasome inhibitors MG132 (20 μM for 6 h), and MAVS was then immunoprecipitated by anti-MAVS Ab and blotted with anti-HA Ab. As shown in Fig. 4B, WT HBX introduction led to an increased steady level of MAVS ubiquitination whereas HBX 73–154 C115A abolished this effect. These results indicate that HBX may have a major role in MAVS ubiquitination and its proteasome-mediated degradation.

To further dissect the possible HBX-mediated ubiquitination sites in MAVS, 14 mutants were generated by substituting the 14 lysine residues on the MAVS molecule individually with arginine (KRR) and then tested for their stability by introducing them with HBX into HEK293 cells. As shown in Fig. 4C, HBX had little effect on the cellular abundance of MAVS K136R mutations and modestly decreased the cellular abundance of MAVS K297R and K420R mutation. In contrast, HBX led to a sharp decrease on other MAVS KRR mutations and WT MAVS as well. Furthermore, MAVS K136R-induced IFN-β activation was only partially inhibited by HBX. The extent of HBX suppression on MAVS K297R and K420R mutation-induced IFN-β activation was much lower than that achieved with WT MAVS or other MAVS KRR mutations (Fig. 4D). In agreement with this observation, HBX induced increased ubiquitin levels of WT MAVS with less effect on the MAVS K136R mutant (Fig. 4E). These results suggest that Lys136 is one of the critical sites for HBX-mediated ubiquitination in MAVS proteins.

**FIGURE 5.** HBX affects the kinetics of RIG-I–MAVS signaling. HEK293 cells were cotransfected with Myc-MAVS and Flag–RIG-I expression plasmids together with the expression vector encoding GFP-HBX or empty vector. After 24 h, cells were treated with VSV for indicated times. Cells were then harvested at different times after VSV infection as indicated. Anti-Flag or IgG immunoprecipitates were analyzed by immunoblotting with anti-Myc or anti-Flag Ab. HBX expression was monitored by immunoblotting using GFP Ab. β-Tubulin was used as an equal loading control.
We also examined whether HBX 73–154 C115A could also affect the protein stability of MAVS. In contrast to the HBX 73–154 C-terminal domain, the cellular abundance of MAVS in 73–154 C115A-expressing cells was unchanged (Fig. 4F). Additionally, the extent of MAVS-induced IFN-β repression by HBX 73–154 C115A was lower than that achieved with WT HBX or with the HBX 73–154 C-terminal domain (Fig. 4G). These results indicate that interaction between HBX and MAVS is responsible for MAVS downregulation and MAVS-mediated antiviral signaling inhibition.

**HBX affects the kinetics of RIG-I–MAVS signaling**

Our studies have shown that HBX inhibits RIG-I–MAVS signaling by downregulating MAVS, indicating that HBX might affect the kinetics of the MAVS–RIG-I association upon virus infection. HEK293 cells were cotransfected with Myc-MAVS and Flag–RIG-I

---

**FIGURE 6.** Expressions of MAVS proteins in liver cancer patients and their association with HBV disease. A, HepG2, HepG2215, and primary murine hepatocytes isolated from HBX transgenic mice or control mice were cultured, and whole-cell lysates were analyzed by immunoblotting with anti-MAVS. β-Tubulin was used as an equal loading control. HepG2-1117 cells containing 1.05-fold HBV genome under Tet-off control were cultured with or without doxycycline for 7 d, whole-cell lysates were analyzed by immunoblotting with anti-MAVS, and β-tubulin was used as an equal loading control. B, Representative immunoblot of MAVS protein in HBV-induced HCC (C1–C8), HBV-irrelevant HCC (C5–C8), and in their matched controls (N1–N4). Immunoblot of HBX expression was also analyzed in C1–C8 samples. C, Representative immunohistochemical staining of MAVS expression in normal donor livers (1), HBV-irrelevant cirrhosis (2), HBV-irrelevant HCC (3), HBV-induced cirrhosis (4), and in HBV-induced HCC (5). 1–5, Original magnification ×200.
expression plasmids together with the expression vector encoding GFP-HBX or empty vector. After 24 h, cells were treated with VSV and then harvested at different times as indicated. As shown in Fig. 5, the interaction of MAVS and RIG-I was enhanced by VSV infection, whereas the introduction of HBX not only eliminated this enhancement but also reduced MAVS–RIG-I interaction. Because this RIG-I–MAVS interaction is required for RIG-I–mediated IFN-β signaling, the disruption of this interaction by HBX would squelch downstream signaling. Collectively, these observations solidify the negative regulatory role of HBX in MAVS-mediated antiviral responses, and they demonstrate that HBX exerts its inhibition of virus induced RIG-I–MAVS signaling through downregulation of MAVS.

Expression of MAVS proteins in liver cancer patients and their association with HBV disease

To examine the status of MAVS in the presence of HBV, we performed Western blotting analysis to examine the expression of MAVS protein levels in HepG2-derived, HBV DNA-transfected HepG2215 cells, HepG2-1117 cells that contain Tet-off HBV-1.05 genome, and in primary murine hepatocytes isolated from HBX transgenic mice. It is evident that MAVS was present at low levels in HepG2215 cells and in HepG2-1117 cells without doxycycline; additionally, transfected MAVS in HepG2-1117 cells showed increased ubiquitin modification when HBX expression was induced without doxycycline (Fig. 6A). Consistent with this finding, primary hepatocytes isolated from HBX knock-in transgenic mice also showed reduced endogenous MAVS level compared with WT littermate control mice (Fig. 6A).

We next examined the expression of MAVS proteins in HBV-induced HCC (n = 20) and matched healthy controls (n = 20), as well as in HBV-unrelated HCC (n = 20) and matched healthy controls (n = 20). Our results showed that 80% (16/20) of HBV-induced HCC expressed lower levels of MAVS (Fig. 6B) and that 10% (2/20) of HBV-unrelated HCC expressed lower levels of MAVS (Fig. 6B). The occurrence of reduced MAVS expression between the HBV-induced HCC and the HBV-unrelated HCC was significant. To further delineate the correlation between HBX and MAVS, HBX protein expression was also analyzed in those 20 HBV-induced HCC samples. Seventy-five percent (15/20) showed positive HBX expression (Fig. 6B); among the 15 HBX-positive HCC, 86% expressed lower levels of MAVS (13/15), suggesting a negative correlation between HBX expression and MAVS protein level.

MAVS protein levels were also measured by immunohistochemistry in normal donor livers (n = 14), in livers of HBV-unrelated cirrhosis and HCC (n = 30), in livers with cirrhosis caused by HBV infection (n = 22), and in HCC caused by HBV infections (n = 24). Results showed that 78.6% (11/14) of healthy livers, 76.6% (23/30) of HBV-unrelated liver diseases, and 30.4% (14/46) of HBV-related liver diseases stained strongly positive for MAVS (Fig. 6C). The overall differences in MAVS-positive staining between HBV-induced liver disease groups and the other three HBV-irrelevant groups including normal controls were significant (30.4 versus 77.6%). Taken together, these data suggest a negative correlation between MAVS protein and HBV liver disease.

**HBX regulates RIG-I-dependent antiviral cellular responses**

We next sought to determine whether HBX regulates replication of VSV virus replication, as RIG-I–mediated IFN-β signaling is critical in restricting replication of these RNA viruses. We therefore used VSV to infect HepG2-1117 cells with or without doxycycline. The results showed that HepG2-1117 without doxycycline increased the production of VSV ~50% (Fig. 7A). Furthermore, primary murine hepatocytes isolated from HBX knock-in mice or the WT littermate control mice were cultured and infected with VSV virus. Fig. 7B shows that exogenous expression of HBX increased the production of infectious VSV. Taken together, these results provide more evidence that HBX modulates the innate antiviral cellular response by acting as a negative regulator of RIG-I–mediated IFN-β signaling infection.

**Discussion**

The targeting of MAVS by both the cysteine protease of HAV and the serine protease of HCV represents a remarkable example of convergent virus evolution (38) and provides strong evidence for the importance of MAVS to host control of virus infections in the liver. In this study, we show that both HBV DNA or HBV suppressed poly(dAT:dAT)-activated IFN-β production in hepatocytes. Additionally, MAVS interacts with HBX and is indeed the target of HBX protein. Specifically, we found that HBX promoted the degradation

**FIGURE 7.** HBX regulates RIG-I–dependent antiviral cellular responses. A, HepG2-1117 cells with or without doxycycline were infected for 20 h with VSV (multiplicity of infection of 0.002) but not killed; cells were fixed and then stained with amino black. B, Primary murine hepatocytes were infected with the equivalent of 10 ml of VSV stock in serum-free medium (1 ml/well) for 1 h at 37°C. The infecting medium was then removed and replaced with 1 ml of normal growth medium. Cell supernatants were recovered 24 h postinfection, and virus titers were determined by plaque formation.
of MAVS through Lys\textsuperscript{136} ubiquitin in MAVS protein, thus preventing the induction of IFN-\(\beta\). We also show that the enhanced interaction of MAVS and RIG-I by VSV infection was eliminated in the presence of HBX. Meanwhile, MAVS protein abundance was reduced in patients with liver cancer that was caused by HBV infection, and primary hepatocytes isolated from HBX transgenic mice showed hypersensitivity to VSV infection. These studies indicate that HBV can target RIG-I–MAVS signaling by HBX-mediated MAVS downregulation and thereby attenuate the antiviral response of the innate immune system.

HBX, a virally encoded protein of 154 aas, has been shown to be essential for the establishment of HBV infection in vivo. Its gene product also activates a variety of viral and cellular promoters in diverse cell types. Although HBX does not bind to dsDNA, it does regulate transcription of a variety of cellular and viral genes by interacting with cellular proteins and/or components of signal transduction pathways (39, 40). The interaction of HBX with these proteins leads to activation of signal transduction pathways including Ras/Raf/MAPK, protein kinase C, and Jak1-STAT. HBX has been reported to activate TNF-mediated NF-\(\kappa\)B signaling by inducing the degradation of IkB-\(\alpha\). We show herein that HBX suppressed intracellular IFN-\(\beta\) signaling through MAVS degradation. Thus, it is very likely that HBX acts as a regulator of the antiviral pathway through different mechanisms under different stimuli.

MAVS contains an N-terminal CARD-like domain and a C-terminal transmembrane domain that target the protein to the outer mitochondrial membrane. The mitochondrial-targeting transmembrane domain is essential for MAVS signaling, thus implicating a new role for mitochondria in innate immunity. Previous reports have shown that HBX regulates fundamental aspects of mitochondrial physiology, including downregulating mitochondrial enzymes and increasing the cellular abundance of reactive oxygen species. We have shown herein that HBX regulates the stability of a mitochondrial protein that is essential for innate immunity, thus shedding new light on the physiological significance of HBX, which may contribute to liver disease associated with HBV-persistent infection. Previous reports have also shown that MAVS proteins undergo proteasomal degradation by ubiquitin E3 ligase RNF125-mediated ubiquitin conjugation (41). In this study, we demonstrate that HBX promotes MAVS ubiquitin to trigger its proteasome-mediated degradation through the Lys\textsuperscript{136} site, and that MAVS K136R elicits a higher level of IFN-\(\beta\) synthesis compared with WT MAVS, suggesting that the MAVS Lys\textsuperscript{136} site could be the ubiquitination site of RNF125.

The proteasome is involved in both ubiquitin-dependent and -independent proteolytic pathways. There have been several reports on cellular regulatory proteins and viral proteins that interact with subunits of the proteasome complex and participate in proteasome-dependent regulation (42, 43). PSMA7 is a subunit of proteasome that regulates the activity of this complex associated with HBX, suggesting that HBX may modulate the function of proteasome by interacting with PSMA7. Our previous results have shown that PSMA7 may regulate host innate immune signaling by destabilizing MAVS, raising the possibility that HBX may be potentially bridged by PSMA7 on the mitochondrial outer membrane to exert its inhibitory effect on innate immune response, rather than exert a direct effect.

Although the liver is a particularly important site of persistent viral infections in humans, very little is known about how innate immune signaling pathways function in hepatocytes. Regardless, there is strong, albeit indirect, evidence that type I IFN responses are important in the pathogenesis of chronic viral hepatitis. Innate host responses during the early phases of viral infections are mainly characterized by the production of type I IFN-\(\alpha\)-\(\beta\) cytokines and the activation of NK cells. HBV replication can be efficiently limited by \(\alpha\) and \(\beta\) IFN, but data on acutely infected chimpanzees suggest that such antiviral cytokines are not triggered by HBV replication (44). A further characteristic of HBV in relationship to early host defense mechanisms resides in the lack of IFN-\(\alpha\) and IFN-\(\beta\) production. HBV might have evolved strategies to escape the initial antiviral defense mechanisms activated by the TLR system or RIG-I–MDA5 pathway. Previous reports have shown that HBV almost completely abrogated TLR-induced antiviral activity (20). Cheng et al. (45) demonstrated that recombiant HBsAg inhibits LPS/TLR4-induced NF-\(\kappa\)B activation, leading to reduced COX-2, IL-18, and IFN-\(\gamma\) production in the human monocytic cell line THP-1. Other studies have shown lower TNF-\(\alpha\) levels in HBeAg-positive patients compared with HBeAg-negative patients (46, 47). In this study, we analyzed the involvement of MAVS in HBV infection by using liver samples from HBV-induced cirrhosis and from HCC patients. We demonstrated that MAVS was downregulated markedly in livers from these patients, with the decrease in MAVS having been correlated with HBV expression in HCC liver tumors. Several studies have demonstrated the pivotal position of RIG-I–MAVS signaling in host responses against a number of viruses (48, 49). Recent research has indicated that the induction of TLR- and RIG-I–MDA5-mediated host cellular immune responses are overexpressed of the three pattern recognition receptors adapters, IPS-1, TRIF, and MyD88, dramatically reduces the levels of HBV mRNA and DNA in both HepG2 and Huh7 cells. These observations provide more evidence that HBV might evolve multiple strategies to evade TLR-dependent and -independent signaling pathways; further understanding of the nature of these mechanisms should yield novel strategies for developing antivirals that evoke responses to eliminate HBV infection.

Acknowledgments
We thank Zhijian Chen (University of Texas Southwestern Medical Center, Dallas, TX) for providing the MAVS plasmid.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1

(A) HepG2 cells were transfected HBV 2-DNA, the level of the HBV S antigen in the culture supernatant was measured on 24, 36, 48, 72 and 96 h after transfection using ELISA. Mean and standard error are presented for 3 independent experiments. pDolmlO was used as a control transfected plasmid of HBV 2-DNA.

(B) HepG2 cells were infected with HBV, Cells were washed eight times to remove excess of viral inputs 2 h after infection and were then infected with VSV, protein extracts were then resolved by SDS-PAGE (upper panel) or native gel electrophoresis (lower panel). Phosphorylation or dimerization of IRF3 was detected by immunoblotting with an IRF3 antibody.

(C) HepG2-1117 cells were transfected with IFN-β-Luc, NF-κB Luc or IRF3-Luc and then were infected with VSV with or without doxycycline. The Luc activity was measured 24 h later and normalized for transfection efficiency.