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The Level of Hepatitis B Virus Replication Is Not Affected by Protein ISG15 Modification but Is Reduced by Inhibition of UBP43 (USP18) Expression

Jung-Hwan Kim,2* Jiann-Kae Luo,3* and Dong-Er Zhang4*†

Hepatitis B virus (HBV) causes both acute and chronic infection of the human liver and is associated with the development of liver cirrhosis and hepatocellular carcinoma. UBP43 (USP18) is known as an ISG15-deconjugating enzyme and an inhibitor of type I IFN signaling independent of its enzyme activity. In this study, we examined the role of these two previously identified functions of UBP43 in the innate immune response to HBV viral infection. As an in vivo HBV replication model system, a replication-competent DNA construct was injected hydrodynamically into the tail veins of mice. Although the lack of ISG15 conjugation in the absence of ISG15-activating enzyme UBE1L (UBA7) did not affect the level of HBV replication, the steady-state level of HBV DNA was substantially reduced in the UBP43-deficient mice in comparison to the wild-type controls. In addition, introduction of short hairpin RNA against UBP43 resulted in substantially lower levels of HBV DNA at day 4 postinjection and higher levels of ISG mRNAs. These results suggest that HBV infection is more rapidly cleared if UBP43 expression is reduced. Furthermore, these results illustrate the therapeutic potential of modulating UBP43 levels in treating viral infection, especially for viruses sensitive to IFN signaling. The Journal of Immunology, 2008, 181: 6467–6472.

Hepatitis B virus (HBV)5 causes both acute and chronic infection of the human liver. Although a vaccine is available, hepatitis B remains a major health problem in many countries. The number of HBV carriers worldwide is estimated to be 400 million. HBV is an enveloped, partially double stranded DNA virus. The replication of the viral genome occurs through an RNA intermediate that requires its own reverse transcriptase activity (1). Chronic HBV infection can lead to the development of liver cirrhosis and hepatocellular carcinoma (2, 3). Although HBV infection itself is not cytopathic to the hepatocyte, hepatitis is thought to result from the immune response to the viral infection.

Host response during the early phases of viral infections are characterized by the production of type I IFN (IFN-α/β) and activation of NK cells (4). HBV replication can be efficiently inhibited by IFN treatment (5–7). IFN regulate diverse biological functions, including induction of the antiviral response, inhibition of cell proliferation, and immunomodulatory activities (8–12). IFN-α/β stimulation leads to the up-regulation of IFN-stimulated genes (ISG). One of these ISG is ISG15, an ubiquitin-like protein that conjugates to cellular substrates to form ISGylated proteins (13, 14). The conjugation is executed by an enzymatic cascade that includes an E1-activating enzyme (UBE1L, also known as UBA7; Ref. 15), an E2-conjugating enzyme (UbcH8; Refs. 16, 17), and E3 ligases (18–22). The conjugation can be reversed by ubiquitin protease 43 (UBP43, also known as USP18), which is an IFN-inducible cysteine protease (23). As expected, UBP43-deficient cells show high levels of ISG15-modified proteins (24). Importantly, UBP43-deficient cells are hypersensitive to type I IFN and undergo apoptosis upon IFN stimulation (25). Furthermore, lack of UBP43 results in enhanced and prolonged STAT1 phosphorylation and increased induction of hundreds of ISG, as confirmed by gene expression microarray studies (26). Loss of UBP43 in mice results in resistance to the cytopathic effects caused by a number of viruses including lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), and Sindbis virus (27). Interestingly, UBP43 has two independent roles in the innate immune response. First, the isopeptidase activity of UBP43 functions to remove ISG15 from ISG15-conjugated proteins (23). Second, independent of its role in protein ISGylation, UBP43 competes with JAK1 for binding to the IFNAR2 subunit of the IFN receptor and inhibits IFN-induced JAK/STAT signal transduction (28).

HBV infection is restricted to humans and chimpanzees, and the lack of a small animal model hampers the understanding of HBV biology. Although several lines of transgenic mice have been established, these may not fully represent the natural course of infection because the production of HBV comes from an integrated viral genome and the mice are immunologically tolerant to viral Ags (29, 30). Recently, it was found that i.v. injection of plasmid DNA with acute circulatory overload leads to high levels of gene expression in mouse liver (31, 32). A mouse model of acute HBV infection was established using this hydrodynamics-based gene delivery system (33). To evaluate ISG15 conjugation-dependent and
Materials and Methods

**Plasmid constructs**

pSP65-ayw1.3 containing an over-length copy of HBV was provided by Dr. F. V. Chisari (The Scripps Research Institute, La Jolla, CA). The pGEM-ayw1.0 construct was made by excising one copy of the HBV genome from pSP65-ayw1.3 with Spol enzyme and ligation into the Spol site of the pGEM-TZf(−) vector (Promega; see Fig. 1A). Control shRNA and mouse-specific shRNA were generated with minor modifications from previously established protocols (38). In brief, primers 5′-ggggatcc aag gtcgcc aagggaggg cctattc-3′ and 5′-ggggaattc aagggcctc tcgggtg tctcttgaac-3′ (for control shRNA) and primers 5′-gggaattc aagggcctc tcgggtg tctcttgaac-3′ and 5′-ggggaattc aagggcctc tcgggtg tctcttgaac-3′ (for UBP43 shRNA12) were used to PCR-amplify shRNA constructs under the control of the U6 promoter. The PCR products were digested with EcoRI/BglII and ligated into the EcoRI/BamHI site of pBluescript KS (−) (Stratagene). The excised construct was then religated with T4 DNA polymerase, then with EcoRI. The excised construct was then ligated into the HpaII/EcoRI site of pMSCVpuro (BD Clontech) and confirmed by sequencing. Plasmid DNAs were prepared by using an EndoFree plasmid maxi kit (Qiagen) according to manufacturer’s instructions.

**In vivo studies**

The generation of UBP43−/− and UBE1L−/− mice was described previously (24, 34). All animals used in the studies were handled in accordance with guidelines of The Scripps Research Institute, and procedures were approved by the Institutional Animal Care and Use Committee of the institute. The genetic background of mice used in this study was C57BL/6, 129Sv, FVB, or mixed background of C57BL/6 and 129Sv. The pair was then excised by a two-step digestion, first with XhoI and blunt-ted with T4 DNA polymerase, then with EcoRI. The excised construct was then ligated into the HpaII/EcoRI site of pMSCVpuro (BD Clontech) and confirmed by sequencing. Plasmid DNAs were prepared by using an EndoFree plasmid maxi kit (Qiagen) according to manufacturer’s instructions.

**Hydrodynamic injection of naked plasmid DNA**

Twenty micrograms of plasmid DNA was diluted with 2.0 ml of saline and injected via the tail vein of 6–9-wk-old mice within 10 s. The livers of the mice were dissected into pieces and frozen immediately in liquid nitrogen 4 days after injection.

**Analysis of HBV replication by Southern blotting**

A piece of liver tissue was digested overnight in lysis buffer (50 mM Tris-Cl (pH 7.4), 20 mM EDTA (pH 8.0), 100 mM NaCl, 0.5% SDS, 0.5 mg/ml proteinase K) at 55°C, and the DNA was phenol-chloroform extracted. Twenty micrograms of DNA was digested with Hinfl restriction enzyme, and analyzed by Southern blotting with32P-labeled HBV probe. RC, relaxed circular; SS, single-strand. Numbers on the left show DNA m.w. size markers in kilobases.

**Detection of ISGylated proteins**

Liver tissue was homogenized in radioimmune precipitation assay buffer (150 mM NaCl, 10 mM Tris-Cl (pH 7.2), 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA), sonicated briefly, then resolved in 8–18% discontinuous gradient SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane (Hybond-ECL; Amersham Biosciences) and detected with rabbit anti-mouse ISG15 polyclonal Ab as described previously (39).

**Results**

**Replication of HBV in hydrodynamically injected mice**

An ayw 1.3 super genomic DNA construct that has been shown to support HBV gene expression and replication in the livers of transgenic mice (30) was injected i.v. into mice. At day 4 postinfection, mice were sacrificed and total genomic DNA from liver tissue was analyzed by Southern blotting with a 32P-labeled HBV DNA probe after HindIII digestion. As a negative control, a construct containing one copy of the HBV genome (ayw 1.0), which cannot provide the appropriate transcripts of HBV, was used (Fig. 1A). As shown in Fig. 1B, only the DNA sample injected with ayw 1.3, but not with ayw 1.0, showed the conventional HBV replication signal.

**ISGylation does not affect the level of HBV replication**

IFN are used to treat HBV infections, and IFN stimulate the expression of hundreds of ISG, including ISG15. Previous studies...
suggested that ISG15 modification plays a role in certain antiviral responses. ISG15 was linked to IFN-mediated inhibition of HIV-1 replication (40) and the NS1B protein of influenza B virus was found to bind free ISG15 and possibly to inhibit its conjugation (15). A more recent study suggests that ISG15 inhibits Sindbis virus infection in mice (35). Furthermore, ISG15 knockout mice showed increased susceptibility to Sindbis, herpes, and influenza viruses (36). However, ISG15- and ISGylation-defective mice did not show any difference in response to VSV and LCMV infection (34, 41). ISG15-deconjugating enzyme UBP43 regulates cellular levels of ISGylated protein via its deconjugating enzyme activity and also functions as a potent inhibitor of type I IFN signaling (23, 28). UBP43 plays an important role during innate immune responses (27, 42, 43). Because HBV is currently one of the major threats to human health, the two different roles of UBP43 in innate immune responses were analyzed for effects on HBV. We first address the ISG15 modification-related function of UBP43 using ISG15-activating enzyme UBE1L knockout mice (which lack protein ISGylation) and the HBV DNA hydrodynamic injection approach.

As a preliminary experiment, the effect of hydrodynamic injection on ISGylation was checked. C57BL/6 wild-type mice were injected with saline and 50 μg of either ayw1.0 or ayw1.3 constructs. Mice were sacrificed 2 days later and total liver lysate was analyzed by immunoblotting with an anti-mouse ISG15 Ab. Interestingly, injection of any DNA construct, regardless of HBV replication competence (Fig. 2A, lanes 3 and 4), results in an induction of ISGylation compared with noninjected mice (Fig. 2A, lane 1) or mice injected with an equivalent volume of saline (Fig. 2A, lane 2). Furthermore, hydrodynamic injection of the ayw1.3 construct showed significant differences between UBE1L+/+ and UBE1L−/− mice in their ISGylation without any other treatment to induce the IFN response (Fig. 2B).

To check the effect of ISGylation on HBV replication, UBE1L+/+ or UBE1L−/− mice were hydrodynamically injected with 20 μg of the ayw1.3 construct. Mice were sacrificed 4 days later and total DNA from liver tissue was analyzed after HindIII digestion by Southern blotting using a 32P-labeled HBV DNA probe. It is known that the transfection efficiency of hepatocytes in the liver by hydrodynamic injection ranges on average from 5 to 10% within a given experiment, depending on the efficiency of the plasmid injection as well as the genetic background, and replication levels can vary by as much as 5-fold between given experiments (33). Due to this variability within and between batches of experiments, measurement of the differences in HBV replication levels between UBE1L+/+ and UBE1L−/− mice was done by comparing, within each batch of experiments, pairs of mice of each genotype that showed similar amounts of input DNA. For each pair of UBE1L+/+ and UBE1L−/− mice, signal intensity of the replication intermediates was divided by that of the input DNA, and the ratio for the UBE1L−/− mouse is expressed as a percentage of that observed in the wild type. Four independent pairs are shown in Fig. 3A. The relative level of HBV replication in UBE1L−/− mice was 98.87 ± 13.87% (n = 4 pairs) of that in UBE1L+/+ mice.

FIGURE 2. Hydrodynamic injection of naked DNA results in induction of ISGylation in the mouse liver. Liver lysate was analyzed 2 days after injection by immunoblotting using anti-mouse ISG15 Ab. A, Wild-type mice injected with ayw 1.0 and ayw 1.3 constructs. NI, Not injected; S, saline injected; 1.0, ayw 1.0; 1.3, ayw 1.3. B, UBE1L+/+ or UBE1L−/− mice were injected with ayw 1.3 construct. Asterisks indicate nonspecific cross-reactive bands. Ponceau staining indicates the relative amount of protein loading.

FIGURE 3. Absence of UBE1L does not affect the level of HBV replication. A, UBE1L+/+ (lanes 1, 3, 5, and 7) or UBE1L−/− (lanes 2, 4, 6, and 8) mice were injected with the ayw 1.3 construct, and the total DNA was analyzed by Southern blotting with an HBV probe. Relative replication was measured (see Results for details) and indicated as a percentage below each lane. B, The relative replication is summarized as a graph. The average HBV replication in UBE1L−/− mice was 98.87 ± 13.87% (n = 4 pairs) of that in UBE1L+/+ mice.

The level of HBV replication is reduced in the absence of UBP43

We next examined the effect of the absence of UBP43 on the level of HBV replication. UBP43+/+ or UBP43−/− mice were hydrodynamically injected with 20 μg of ayw1.3 construct and analyzed as described above. We had eight comparable pairs from four independent injections, and four representative Southern blot results are shown (Fig. 4A). The relative level of HBV replication in
UBP43 from eight comparable pairs was $4.01 \pm 2.69$ (%) compared with UBP43 wild-type (Fig. 4B). In conclusion, the level of HBV replication was reduced significantly in the absence of UBP43.

We observed previously that the lack of UBP43 results in enhanced and prolonged STAT1 phosphorylation (25) and increased induction of hundreds of ISG (26). More recently, we reported that UBP43 affects the level of HBV replication. Together with a previous report (28), our results strongly suggest that UBP43 negatively regulates JAK/STAT signaling by a mechanism independent of its isopeptidase activity. To further elucidate the enzyme activity-independent function of UBP43, we are in the process of generating a UBP43-deficient cell line to test the hypothesis that UBP43 regulates JAK/STAT signaling by a mechanism independent of its isopeptidase activity.

Coinjection of shRNA against mouse UBP43 results in a significant decrease of HBV replication levels compared with that of a nonspecific control shRNA (lane 2).

The shRNAs expression vectors were coinjected with the aly virus construct. Mouse livers were taken 4 days after injection and total DNA was isolated. The level of HBV replication was analyzed by Southern blotting. As shown in Fig. 5B, coinjection of UBP43 shRNA results in a significant decrease of HBV replication levels compared with that of control shRNA.

Discussion

In UBP43 knockout mice, the steady-state level of HBV DNA was reduced significantly, and this result is consistent with previous reports that loss of UBP43 in mouse cells was hypersensitive to type I IFN, indicating its negative regulatory role on IFN signaling (25). Because UBP43 is an ISG15-specific protease that removes ISG15 from modified target proteins (23), it was thought that the negative regulation of JAK/STAT signaling might be achieved through controlling ISG15 conjugation levels. However, loss of ISGylation (UBE1L) did not affect the level of HBV replication. Together with a previous report (28), our results strongly suggest that UBP43 negatively regulates JAK/STAT signaling by a mechanism independent of its isopeptidase activity. To further elucidate the enzyme activity-independent function of UBP43, we are in the process of generating mice that only express an isopeptidase inactive form of UBP43 via knockin strategy. By comparing wild-type and UBP43-deficient mice, the two important roles of UBP43 will be further clarified.

It was reported that ISG15 knockout mice had increased susceptibility to influenza, herpes, and Sindbis viruses (36). Similar to

FIGURE 4. The level of HBV replication is inhibited in the absence of UBP43. A, UBP43+/+ (lanes 1, 3, 5, and 7) or UBP43−/− (lanes 2, 4, 6, and 8) mice were injected with the aly virus construct, and total DNA was analyzed by Southern blotting with an HBV probe. Relative replication was measured as for Fig. 3 and indicated as a percentage below each lane. Four representative experiments are shown in the figure. B, The relative replication is summarized as a graph. The average HBV replication in UBP43−/− mice was $4.01 \pm 2.69$ (%) ($\alpha = 8$ pairs) of that in UBP43+/+ mice. C, Levels of IFN-inducible genes in UBP43−/− mice. Total RNA from the liver was isolated and analyzed by Northern blotting with radiolabeled HBV (top panel), Cxcl9, Gbp1, IRF1, IRF7, and Mx2 probes as indicated. Equal RNA loading was confirmed by the relative amount of 18S and 28S rRNA (bottom panel).

FIGURE 5. Coinjection of UBP43 shRNA with aly virus results in the inhibition of HBV replication. A, Activity of shRNA. 293T cells were cotransfected with V5-tagged mouse UBP43 and control or UBP43 shRNA constructs. B, Coinjection of UBP43 shRNA expression constructs with aly virus. 10 μg of aly virus construct, and 10 μg of shRNA constructs were coinjected into 129sv mouse tail veins hydrodynamically. Total DNA of mouse livers were analyzed by Southern blotting as described above.
ISG15 knockout mice, UBE1L knockout mice also had increased susceptibility to influenza B virus infection (D. Lenschow, manuscript in preparation). However, we could not observe any effect on HBV replication in UBE1L knockout mice. This correlates with our recent study that demonstrated that UBE1L knockout mice also exhibited normal antiviral responses against VSV and LCMV infections (34). It seems likely that the antiviral activity of ISG15 or ISGylation might be restricted to specific viruses.

Changes caused by hydrodynamic injection with naked DNA within mice were reported previously (31). Serum biochemistry including major ion concentration (Na+, K+, and Cl–), major protein concentration (albumin and the total protein), and the concentration of liver-specific enzymes including alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase and total bilirubin were checked. All the biochemical parameters evaluated were in the normal range with the exception of alanine aminotransferase on day 1. In this study, we could observe induction of ISGylation as shown in Fig. 2, A and B. These results indicate that hydrodynamic injection of DNA itself, regardless of HBV replication, may induce the IFN response, although further research is required to confirm this.

In this study, by coinjecting shRNA against UBP43 together with ayw 1.3 into wild-type mice, we showed that UBP43 plays an important role in regulating HBV replication. In a stable transfection study, inhibition of UBP43 expression by shRNA resulted in prolonged STAT1 activation (phosphorylation) (28). Consistent with this fact, expression of several IFN-inducible genes including Cxcl9, Gbp1, IRF1, IRF7, and Mx2 are up-regulated in the liver tissue of UBP43 KO mice (Fig. 4C). The inhibition of UBP43 expression by shRNA results in hypersensitivity to IFN. In other words, the decrease in UBP43 levels results in a strengthened immune response. As further evidence, it has been recently shown that UBP43 expression is up-regulated in patients that are nonresponsive to IFN treatment (44). Most recently, Randall et al. reported that knockdown of UBP43 was effective in inhibiting HCV replication and viral production in an in vitro cell line model (45). Together with the in vivo data of our current report, these results provide strong evidence that UBP43 shRNA as a therapeutic tool can essentially be applied to every viral or bacterial infection that is known to be sensitive to IFN treatment.

Hydrodynamic injection of HBV DNA is a mouse model of acute HBV infection. Although the current data indicate the potential use of UBP43 shRNA as an anti-HBV therapy, we have not yet provided evidence for effects on chronic HBV infection. Additional studies, such as hydrodynamic injection of UBP43 shRNA into HBV transgenic mice, would be a good approach to address this issue. Furthermore, mouse models for various infectious diseases could be examined with UBP43 shRNA to provide further evidences of the therapeutic value of targeting UBP43 expression.

Acknowledgments
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Disclosures
The authors have no financial conflict of interest.

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