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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)5 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 mouse 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
-independent functions of UBP43 in the innate immune response to HBV infection, we used the HBV DNA hydrodynamic injection approach and examined HBV replication in wild-type controls, UBE1L−/− mice that lack ISG15 conjugation upon IFN stimulation, and UBP43−/− mice (24, 34). Furthermore, we also tested the therapeutic potential of UBP43 short hairpin (sh) RNA for the treatment of HBV infection. Although there are several reports that indicate the antiviral role of ISG15 modification (35–37), ISGylation does not affect the replication of HBV in this model. However, the steady-state level of HBV DNA is substantially reduced in the absence of UBP43 or in the presence of UBP43-silencing shRNA, indicating that the anti-HBV effect of UBP43 is related to its role in modulating IFN signaling and is independent of its role in ISG15 conjugation. These results suggest UBP43 as an attractive therapeutic target for the treatment of HBV infection.

**Materials and Methods**

**Plasmid constructs**

pSP65-ayw.1.3 containing an over-length copy of HBV was provided by Dr. F. V. Chisari (The Scripps Research Institute, La Jolla, CA). The pGEM-ayw.1.0 construct was made by excising one copy of the HBV genome from pSp65-ayw.1.3 with SpH1 enzyme and ligation into the SpH1 site of the pGEM-TZf(−) vector (Promega; see Fig. IA). Control shRNA and mouse-specific shRNA were generated with minor modifications from previously established protocols (38). In brief, primers 5′-gggagagcc aagtgaggggg-3′ and 5′-gggagagcc aagtgaggggg cctatttcc-3′ (for control shRNA) and primers 5′-gggagagcc aagtgaggggg cctatttcc-3′ (for SpH1 shRNA) and primers 5′-gggagagcc aagtgaggggg cctatttcc-3′ (for UBP43 shRNA) 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 plBmisscript KS (−) (Stratagene). The insert was then excised by a two-step digestion, first with XhoI and blunted 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 comparison is always with mice in the same strain background.

**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 that lack ISG15 conjugation upon IFN stimulation and UBP43−/− mice that lack ISG15 conjugation upon IFN stimulation, and UBP43−/− mice (24, 34). Formerly, we also tested the therapeutic potential of UBP43 short hairpin (sh) RNA for the treatment of HBV infection. Although there are several reports that indicate the antiviral role of ISG15 modification (35–37), ISGylation does not affect the replication of HBV in this model. However, the steady-state level of HBV DNA is substantially reduced in the absence of UBP43 or in the presence of UBP43-silencing shRNA, indicating that the anti-HBV effect of UBP43 is related to its role in modulating IFN signaling and is independent of its role in ISG15 conjugation. These results suggest UBP43 as an attractive therapeutic target for the treatment of HBV infection.

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brane was hybridized with 32P-labeled DNA probe generated by the Prime-it II Random Primer Labeling Kit (Stratagene) using partial cDNA fragments of IFN-inducible genes as the template. The partial cDNA fragments were obtained by PCR amplification using a cDNA library from IFN-treated mouse bone marrow macrophages as a template. Primers 5′-atggctctgagcttttc-3′ and 5′-ccatggagcttttc-3′ for Gbp1, 5′-atggctctgagcttttc-3′ and 5′-ccatggagcttttc-3′ for IRF1, 5′-atggctctgagcttttc-3′ and 5′-ccatggagcttttc-3′ for IRF7, 5′-atggctctgagcttttc-3′ and 5′-ccatggagcttttc-3′ for Cxcl9, and 5′-atggctctgagcttttc-3′ and 5′-ccatggagcttttc-3′ for Mx2 were used in amplification.

**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).

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.

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

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.
UBP43 from eight comparable pairs was 4.01 ± 2.69 (%) compared with UBP43/mice (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 specifically binds to the IFNAR2 subunit and inhibits the activity of receptor-associated JAK1 by blocking the interaction between JAK and IFN receptor (28). Considering that the level of HBV replication was not affected by ISGylation (Figs. 2 and 3), this result indicates that the isopeptidase-independent activity of UBP43 affects the level of HBV replication. As further proof of the effect of UBP43 on HBV, total liver RNA was analyzed by Northern blotting with radiolabeled HBV (Fig. 4C, 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).

Coinjection of shRNA expression constructs with ayw 1.3 construct hydrodynamically into the tail vein of wild-type mice. 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 mice results in greater resistance to viral challenge (14). It was reported previously that UBP43-deficient cells were 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 (UBE1LΔ/Δ) 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-mutant 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
The authors have no financial conflict of interest.

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