Inhibition of Alpha Interferon Signaling by Hepatitis B Virus

Verena Christen,1 Francois Duong,1 Christine Bernsmeier,1 Dianxing Sun,2 Michael Nassal,2 and Markus H. Heim1*

Department of Research, University Hospital Basel, CH-4031 Basel, Switzerland,1 and Department of Medicine II, University Hospital Freiburg, D-79106 Freiburg, Germany2

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Alpha interferon (IFN-α) and pegylated IFN-α (pegIFN-α) are used for the treatment of chronic hepatitis B (CHB). Unfortunately, only a minority of patients can be cured. The mechanisms responsible for hepatitis B virus (HBV) resistance to pegIFN-α treatment are not known. PegIFN-α is also used to treat patients with chronic hepatitis C (CHC). As with chronic hepatitis B, many patients with chronic hepatitis C cannot be cured. In CHC, IFN-α signaling has been found to be inhibited by an upregulation of protein phosphatase 2A (PP2A). PP2A inhibits protein arginine methyltransferase 1 (PRMT1), the enzyme that catalyzes the methylation of the important IFN-α signal transducer STAT1. Hypomethylated STAT1 is less active because it is bound by its inhibitor, PIAS1. In the present work, we investigated whether similar molecular mechanisms are also responsible for the IFN-α resistance found in many patients with chronic hepatitis B. We analyzed the expression of PP2A, the enzymatic activity of PRMT1 (methylation assays), the phosphorylation and methylation of STAT1, the association of STAT1 with PIAS1 (via coimmunoprecipitation assays), and the expression of interferon-stimulated genes (via real-time RT-PCR) in human hepatoma cells expressing HBV proteins as well as in liver biopsies from patients with chronic hepatitis B and from controls. We found an increased expression of PP2A and an inhibition of IFN-α signaling in cells expressing HBV proteins and in liver biopsies of patients with CHB. The molecular mechanisms involved are similar to those found in chronic hepatitis C.

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV) (19, 21). Chronic hepatitis B (CHB) can progress to cirrhosis and hepatocellular carcinoma. Approved treatments for CHB include a number of nucleos(t)ide analogues, such as lamivudine and adefovir, or alpha interferon (IFN-α), recently in pegylated form (pegIFN-α) (19). PegIFN-α2a given for 48 weeks can induce the seroconversion of hepatitis B e antigen (HBeAg) in 32% of patients (20). However, over 60% of patients will continue to suffer from chronic active hepatitis B despite pegIFN-α2a therapy. The molecular mechanisms responsible for the ineffectiveness of IFN-α treatments in CHB are not known. PegIFN-α (in combination with ribavirin) is also the current standard therapy for chronic hepatitis C (CHC). Interestingly, as with CHB, pegIFN-α is not effective in many patients with CHC. Over the last years, several molecular mechanisms responsible for viral evasion of the type I IFN system have been studied (10, 14). One of these mechanisms involved in the evasion of hepatitis C virus (HCV) has been elucidated in our laboratory over the last years: HCV proteins interfere with IFN-α-induced signaling through the Jak-STAT pathway (3, 8, 15).

The interferon system is an important component of the host response against viruses, and mice with deficiencies of IFN receptors or of signal transducer and activator of transcription 1 (STAT1) are highly susceptible to viral infections (2, 9, 23).

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tion of PRMT1 (6). The inhibition of PRMT1 results in a reduced level of STAT1 methylation and an increased binding of STAT1 by its inhibitor Pias1 not only in cultured cells either expressing HCV proteins or overexpressing PP2Ac but also in liver extracts of HCV transgenic mice and in liver biopsies from patients with CHC (8).

In the present study, we have used a cell line that allows the controlled expression of hepatitis B virus and liver biopsies from patients with CHB to analyze PP2Ac expression and IFN-α signaling through the Jak-STAT pathway. Although HCV and HBV are completely unrelated viruses, we found very similar molecular mechanisms of viral interference with IFN-α signaling.

MATERIALS AND METHODS

Reagents, antibodies, and cells. Human IFN-α (Roferon) was obtained from Hoffmann La Roche (Basel, Switzerland). Purified PP2A and anti-PP2Ac were purchased from Upstate (L various HBV core proteins monoclonal antibodies mc312 and mc158 (26) were run in duplicate by using an ABI 7000 sequence detection system (Applied Biosystems), mRNA expression levels of IP10 were expressed as asefford increase according to the formula ΔACT (phosphate-buffered saline) = ΔCT (interferon stimulation).

Methylation assay. To compare methyltransferase activity between Huh7 cells and Huh7.93 cells, 20 μg of whole-cell lysate from each cell line was incubated in the presence of 3 μL of 3′-AdoMet for 2 h at 37°C. The reactions were then stopped by adding 5 μL of sample loading buffer, and the reaction volumes were boiled for 5 min and separated on an 8% sodium dodecyl sulfate-polyacrylamide gel. The upper part of the gel was dried and then exposed to a phosphorimager plate for 3 days. The lower part of the gel was cut out and stained with Coomassie blue to check for equal loading.

Quantification of HBs antigen in the culture medium of Huh7.93 cells. Huh7.93 cells were grown for up to 7 days in Dulbeccco’s modified Eagle’s medium containing doxycycline. A total of 500 μL of the culture medium was sampled daily, and the amount of HBsAg was quantified using the automated test system Elecsys 2010 from Hoffmann La Roche (Basel, Switzerland) (32). As a negative control sample, culture medium was used. In short, Elecsys 2010 is a two-step sandwich assay developed and routinely used for the quantitative detection of HBsAg in human serum or plasma.

In the first incubation step, biotinylated and ruthenylated monoclonal antibodies directed against HBsAg are used to detect HBsAg in the sample. Streptavidin–coated magnetic microparticles are then added to the mixture. In the measuring cell of the Elecsys system, the microparticles are magnetically captured on the surface of the electrode. Unbound substances are removed in the presence of ProCell. The application of voltage to the electrode induces chemiluminescence, which is measured with a photomultiplier.

Results were calculated with the Elecsys software by comparing the chemiluminescence signal obtained from the sample with the cutoff value previously obtained by HBsAg calibration. The amount of HBsAg in the samples was then expressed as the signal/cutoff ratio.

Immunofluorescence with Huh7.93 and HTTA61 cells. Huh7.93 and HTTA61 cells were grown in a six-well plate until 90% confluence. After aspirating out the culture medium, the wells were washed once with phosphate-buffered saline. To fix the cells, 100% methanol (−20°C) was added for 10 min at −20°C. After another three washes for 5 min at room temperature with Tris-buffered saline−Tween 20 (TBST), the cells were incubated with blocking solution (TBST with 5% bovine serum albumin) for 1 h at room temperature. After that, the cells were washed once with TBST before incubation with anti-hepatitis B virus surface antibody (catalog no. MU364-UCE; BioGenex, San Ramon, CA) over night at 37°C. After three washes with TBST, they were incubated with Cy3-conjugated secondary antibody (Amersham, Dübendorf, Switzerland) for 1 h 30 min at room temperature. Nuclear staining was performed with Hoechst (Amersham) for 5 min at room temperature. After washing, a coverslip was
RESULTS

Expression of hepatitis B virus antigens in Huh7 cells induces expression of PP2Ac.

To investigate whether HBV interferes with IFN-α signaling, we used the controllably HBV-producing cell line Huh7.93 (29) and compared it to unmodified Huh7 cells and, selectively, to the parental tTA-containing H7TA-61 cells. The Huh7.93 cell line is one of several lines established by stably transfecting the HBV expression plasmid pTRE-HBVVT (Fig. 1A) (see Materials and Methods) into the Huh7-derived cell line H7TA-61, which contains the Tet-controlled transactivator tTA. In the absence of tetracycline or its analog Dox, tTA activates the Tet response element promoter, leading to the transcription of HBV pgRNA and consequently core and polymerase protein translation; under these conditions, viral replication occurs and complete virions are generated. In the presence of Dox, the transcription of pgRNA is suppressed such that no viral replication can be detected by Southern blotting (29). In contrast, the surface proteins, and likely the hepatitis B virus X protein (HBx), are constitutively expressed from the original endogenous viral promoters, which are not Dox responsive. These data were corroborated here in that HBsAg was easily detected by immunostaining in the perinuclear cytoplasm of Huh7.93 cells (Fig. 1B) and in the supernatants of Huh7.93 cells cultured with Dox (Fig. 1C), whereas core protein became detectable only when the cells were cultured in medium without Dox (Fig. 1D).

Because of its role in regulating the IFN response, we first measured the protein expression levels of PP2Ac in Huh7.93 versus Huh7 or H7TA-61 cells by using purified PP2Ac for calibration (Fig. 2A). In Huh7.93 cell lysates, the mean PP2Ac expression level was 30.2 ng/μg total protein with a standard error of 3.5 ng/μg (Fig. 2B). This level was significantly higher than that in Huh7 cell lysates, where the mean PP2Ac expression level was 16.1 ng/μg total protein (standard error, 2.2 ng/μg). Inducing HBV replication by cultur-
level in Huh7.93 cells is most likely due to the expression of the HBV surface and/or X proteins.

Because PP2Ac can inhibit the enzymatic activity of PRMT1 (6), the up-regulation of PP2Ac expression in Huh7.93 cells should inhibit PRMT1 in these cells. We tested PRMT1 activity in cell extracts from Huh7 and from Huh7.93-1 cells by using an in vitro methylation assay and found a strong inhibition of PRMT1 in Huh7.93 cells (Fig. 2D).

**IFN-α signal transduction is inhibited in HBV antigen-expressing cells.** We then tested whether HBV protein expression and PP2Ac overexpression inhibits IFN-α signaling. Activated STAT1 forms dimers, translocates into the nucleus, and binds to promoter elements of ISGs. We analyzed the binding of activated STAT1 dimers by EMSA with the m67-SIE oligonucleotide probe. A strong gel shift signal was induced by IFN-α in Huh7 cells and in H7TA-61 cells, whereas the signal was weak in Huh7.93 cells (Fig. 3A). The derepression of Huh7.93 cells by removing Dox from the culture medium did not further decrease the gel shift signal, indicating that the expression of the surface proteins and/or the X protein is sufficient to impede IFN-α signaling (Fig. 3B). This inhibition of STAT1-DNA binding was not caused by a reduced STAT1 expression level because STAT1-specific bands in Western blots were equally strong in extracts from Huh7 and Huh7.93 cells (Fig. 3C). We also observed no difference in IFN-α-induced phosphorylation of STAT1 on tyrosine 701 (Fig. 3C). Therefore, we conclude that the inhibition of STAT1 signaling has to be downstream of STAT1 activation at the receptor kinase complex.

We next looked at the methylation status of STAT1 and indeed found a strong reduction in Huh7.93 cells (Fig. 4A). Since STAT1 methylation regulates the association of STAT1 and PIAS1 (25), we carried out coimmunoprecipitation experiments with STAT1 and PIAS1 antibodies. In accordance with the reduced STAT1 methylation, we detected an increased binding of PIAS1 to STAT1 in Huh7.93 cells (Fig. 4B). Finally, the induction of the interferon-stimulated gene IP10 was quantified by measuring the IP10 mRNA concentration after a 6-h incubation of activated STAT1 dimers, translocates into the nucleus, and binds to promoter elements of ISGs. We analyzed the binding of activated STAT1 dimers by EMSA with the m67-SIE oligonucleotide probe. A strong gel shift signal was induced by IFN-α in Huh7 cells and in H7TA-61 cells, whereas the signal was weak in Huh7.93 cells (Fig. 3A). The derepression of Huh7.93 cells by removing Dox from the culture medium did not further decrease the gel shift signal, indicating that the expression of the surface proteins and/or the X protein is sufficient to impede IFN-α signaling (Fig. 3B). This inhibition of STAT1-DNA binding was not caused by a reduced STAT1 expression level because STAT1-specific bands in Western blots were equally strong in extracts from Huh7 and Huh7.93 cells (Fig. 3C). We also observed no difference in IFN-α-induced phosphorylation of STAT1 on tyrosine 701 (Fig. 3C). Therefore, we conclude that the inhibition of STAT1 signaling has to be downstream of STAT1 activation at the receptor kinase complex.

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**Uregulation of PP2Ac in chronic hepatitis B.** To corroborate the physiological relevance of the cell line-derived data described above, we next measured the PP2Ac expression levels in liver biopsy extracts from patients with chronic hepatitis B and in noninfected control samples by using a semiquantitative Western blot method (7). The controls were from patients who had ultrasound-guided biopsies of a focal lesion in the liver. The liver parenchyma outside the lesion was biopsied as well, and if the histological evaluation did not show any liver pathology, the samples were included as controls for the present study. The median PP2Ac concentrations were 11.9 ng/µg total protein in controls and 17.5 ng/µg total protein in biopsies from patients with chronic hepatitis B, a difference that was statistically significant when tested with the Mann-Whitney U test (P = 0.0003). Interestingly, the PP2Ac expression level correlated positively and significantly with the percentage of hepatocytes that stained positively for HBsAg (Fig. 5B) (r = 0.565; P = 0.021, Fisher’s Z test). HbcAg

![Figure 2](image_url)
FIG. 3. (A) IFN-α-induced binding of activated STAT1 is impaired in Huh7.93 cells compared to that in the parental cell lines Huh7 and H7TA-61. Shown are EMSA results using the SIE-m67 oligonucleotide probe. Cells were left untreated (–) (lanes 1, 3, 6, and 8) or they were treated (+) for 20 min with 1,000 U/ml human IFN-α (hIFN-α) (lanes 2, 4, 5, 7, and 9). In lane 5, the nuclear extract of Huh7 (hIFN-α treated) was incubated with anti-STAT1 antibody prior to the binding reaction to perform a supershift (asterisk). (B) Huh7.93 cells were cultured in medium with (++) or without (–) Dox as indicated. Cells were then stimulated for 20 min with 1,000 U/ml hIFN-α. Nuclear extracts were analyzed with EMSA with SIE-m67. No further decrease in signal intensity was observed in derepressed cells. (C) Cytoplasmatic extracts were used to perform Western blot analysis. There is no difference in the phosphorylation of STAT1 on tyrosine 701 between the control cells and the HBV-expressing cells (upper part). The membrane was rebotted for STAT 1 as a loading control (lower part). +, with; –, without.

FIG. 4. (A) STAT1 methylation (asterisk), as detected by an immunoprecipitation (ip) with antibodies to monomethyl- and dimethylarginine and Western blotting with STAT1 antibodies, is impaired in cells expressing viral proteins (lane 2) compared to that in Huh7 cells (lane 1). (B) Binding of PIAS1 to STAT1, as detected by immunoprecipitation (ip) with STAT1 antibodies and Western blotting with PIAS1 antibodies in Huh7.93 cells, is enhanced (lane 2) compared to that in Huh7 cells (lane 1). Densitometric analysis of the PIAS1 signals is shown in the lower panel. The values are the integrated densities measured with NIH Image software and expressed as arbitrary units. (C) Reduced IFN-α target gene induction in the presence of HBV proteins. Huh7 and Huh7.93 cells were stimulated with human IFN-α (1,000 U/ml) for 6 h. The amount of the interferon target gene IP10 was measured with real-time RT-PCR in three independent samples (each sample was measured in duplicate). The transcriptional induction of IP10 is inhibited in Huh7.93 cells (right bar) compared to that in the parental cell line Huh7 (left bar).
expression level and the degree of inflammation in these biopsies (data not shown).

**DISCUSSION**

We have previously shown that hepatitis C virus, a member of the Flaviviridae family, induces the upregulation of PP2Ac in vivo (liver biopsy studies of HCV transgenic mice) and in vitro (tetracycline-regulated HCV protein expression in HCV replicon cells) (3, 8). Here we report that HBV, a member of the hepadnaviruses, also induces a significant up-regulation of PP2Ac. PP2A is an important serine/threonine phosphatase involved in many cellular processes (17, 24), and the overexpression of its catalytic subunit PP2Ac is likely to have important consequences for the host cell. We have concentrated on the effect of PP2Ac up-regulation on IFN-\(\alpha\) signaling through the Jak-STAT pathway. The key finding of these studies was that PP2Ac physically interacts with and inhibits PRMT1 in cells and also in biochemical experiments using purified proteins (6, 8). Here we show that Huh7 cells that express HBV antigens (Huh7.93) have elevated expression levels of PP2Ac compared to those of generic Huh7 cells as well as those of the tTA-containing H7TA-61 parental cells and that the enzymatic activity of PRMT1 is strongly inhibited (Fig. 2). Most likely, the inhibition of PRMT1 is a consequence of PP2Ac overexpression. Like PP2A, PRMT1 is an important enzyme expressed in all cells and involved in arginine methylation of many proteins, among them histones (31) and RNA binding proteins, such as heterogeneous nuclear ribonucleoprotein, fibrillarin, nucleolin, and poly(A) binding protein II (12, 28). The arginine methylation of STAT1 by PRMT1 results in a reduced affinity of STAT1 to its response elements in IFN-\(\alpha\) target gene promoters, as shown by electrophoretic mobility shift assays (Fig. 3A). Interestingly, we did not observe any substantial differences between Huh7.93 cells grown in the presence of Dox versus those grown in the absence of Dox. Because the levels of HBV core protein and polymerase are drastically lowered by Dox, the surface proteins and/or the X protein are the most likely candidate antigens responsible for suppressing the IFN response. This question may be answered, for instance, by establishing comparable cell lines in which the production of the individual gene products is selectively knocked out. Importantly, the similarly increased PP2Ac levels in liver biopsies from chronic hepatitis B patients versus those from the noninfected controls strongly support the physiological relevance of our cell line data. In conclusion, we propose that the molecular mechanism by which HBV suppresses the IFN response involves an up-regulation of PP2Ac as the primary event, with an inhibition of PRMT1 and a reduced STAT1 methylation as its consequence. Unmethylated STAT1 is then bound by PIAS1 and has a reduced capacity to stimulate IFN-\(\alpha\) target genes. Our work identifies PP2Ac, PRMT1, and PIAS1 as potential therapeutic...
targets for strategies aimed at increasing the response rates of IFN-α-based treatments in chronic hepatitis B.

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