

Inhibition of Alpha Interferon Signaling by Hepatitis B Virus[∇]

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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), the binding of activated STAT1 to interferon-stimulated response elements (via electrophoretic mobility shift assays), and the induction of interferon target 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 few nucleos(t)ide analogues, such as lamivudine and adefovir, or alpha interferon (IFN- α), recently in pegylated form (pegIFN- α) (19). pegIFN- α 2a 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- α 2a 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).

IFN- α/β binding to its receptor activates members of the Jak family of tyrosine kinases, which then phosphorylate STAT1, STAT2, and STAT3 on a single tyrosine residue. Phosphorylated STATs form dimers, translocate into the nucleus, bind to promoter elements of interferon-stimulated genes (ISGs), and activate the transcription of ISGs (4). This activation cycle is terminated by the tyrosine dephosphorylation in the nucleus, followed by the decay of dimers and the nuclear export of STATs (5, 30). The pathway is tightly controlled by a number of inhibitory proteins (18, 27), among them the protein inhibitor of activated STAT1 (PIAS1) (22). PIAS1 inhibits the last step in the Jak-STAT pathway, i.e., DNA binding. Complex formation between STAT1 and PIAS1 is regulated by an important posttranslational modification of STAT1, arginine methylation (25). The methylation of STAT1 is catalyzed by protein arginine methyltransferase 1 (PRMT1) and protects STAT1 from binding and inactivation by PIAS1 (25).

We have previously reported that HCV inhibits IFN- α -induced signaling at the level of STAT DNA binding (3, 15). The expression of HCV proteins in cells induces an increased expression of protein phosphatase 2Ac (PP2Ac) (8). PP2Ac was also overexpressed in extracts from liver cells of HCV transgenic mice and in liver biopsies from patients with CHC (8). PP2A is a heterotrimeric protein phosphatase consisting of a 36-kDa catalytic C subunit (PP2Ac), a 65-kDa structural A subunit, and a variable regulatory B subunit. PP2A is expressed in all cell types, is primarily a serine/threonine phosphatase, and is involved in a wide range of cellular processes, including cell cycle regulation, cell morphology, development, signal transduction, translation, apoptosis, and stress response (17, 24). PP2A regulates IFN- α signaling through a strong inhibi-

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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 (Lucerna-Chem, Lucerne, Switzerland). Anti-phospho-STAT1 (Tyr 701) was purchased from Cell Signaling Technology (BioConcept, Allschwil, Switzerland). Anti-STAT1 was from Santa Cruz (Santa Cruz Biotechnology, Inc., Heidelberg, Germany). Monoclonal antibody to methyl- and dimethylarginine was purchased from Abcam (Abcam Limited, Cambridge, United Kingdom). ^{14}C -AdoMet (specific activity, 53 mCi/mmol) was obtained from Amersham Biosciences (Amersham Pharmacia Biotech Europe GmbH, Dübendorf, Switzerland). C-terminally truncated recombinant HBV core protein (amino acids 1 to 149), used as an immunoblot standard, was purified as previously described (1). For detection, the anti-HBV core protein mouse monoclonal antibodies mc312 and mc158 (26) were used. H7TA-61 cells (a gift of Darius Moradpour) are Huh7 cells that constitutively express the tetracycline-controlled transactivator tTA (13). H7TA-61 cells were then transfected with the HBV-expressing plasmid pTRE-HBVT, and stable clones were selected as described elsewhere (29). In brief, pTRE-HBVT contains a slightly overlength HBV genome (subtype ayw [11]) fused behind a Tet-response element-controlled minimal promoter. In the absence of tetracycline or its analogue doxycycline (Dox), tTA binds to and activates the promoter, generating authentic HBV pregenomic RNA (pgRNA), the template for the translation of the viral core and polymerase proteins and the substrate for packaging into, and reverse transcription inside, viral core particles. In the presence of Dox, pregenomic RNA transcription is suppressed to below detectability. Because the mRNAs for the surface proteins (L, M, and S) and X protein are transcribed from the endogenous HBV promoters, they are not subject to Dox control, allowing for an essentially constitutive expression of the gene products. From one of the clones displaying tight control by Dox, the stable cell line Huh7.93 was established and used in this study.

Patients and biopsies. From August 2002 to April, 2005, all patients with chronic hepatitis B who were referred to the outpatient liver clinic of the University Hospital Basel and who had liver biopsies were asked for their permission to use part of the biopsy for this study. The protocol was approved by the ethical commission of Basel. Written informed consent was obtained from all patients who agreed to participate in the study. A semiquantitative grading and staging of all biopsies of patients with chronic hepatitis B was performed according to the method of Ishak et al. (16). The expressions of HBsAg and HBeAg were analyzed by routine immunohistochemistry methods in the Institute of Pathology of the University of Basel, and the expression levels were semiquantitatively determined as a percentage of hepatocytes with positive stainings. Control samples were from patients who underwent ultrasound-guided liver biopsies of focal lesions (mostly metastasis of carcinomas) and who were asked for their permission to obtain a biopsy from the normal liver tissue outside the focal lesion. Only samples with a histologically confirmed absence of liver disease were used as controls.

Preparation of extracts from cells and liver biopsies. Whole-cell lysates and nuclear extracts were prepared as previously described (8). The liver biopsies were homogenized in 100 μl of lysis buffer (100 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate), and the lysates were then centrifuged at 14,000 rpm for 5 min. Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad Laboratories AG, Reinach, Switzerland).

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting were performed as previously described (8). To measure PP2Ac expression in human liver biopsies and in hepatoma cells, 50, 100, and 200 ng of purified PP2Ac was loaded on gels. These three samples allowed the calculation of a standard curve for each gel. The intensity of each band was measured by densitometry analysis using NIH Image software. The amount of PP2Ac in each liver biopsy and in each hepatoma cell sample was then calculated according to the standard curve.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays (EMSA) were performed as described previously (15) using 1- μg nuclear extract aliquots and the SIE-m67 oligonucleotide probe. STAT1 was supershifted with antibody sc-346 from Santa Cruz (LabForce AG, Nunningen, Switzerland).

RNA isolation, reverse transcription, and SYBR-PCR. Total RNA was isolated from the cells using a Perfect RNA eukaryotic mini kit (Vaudaux-Eppendorf, Basel, Switzerland) according to the manufacturer's instructions. RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega Biosciences, Inc., Wallisellen, Switzerland) in the presence of random hexamers (Promega) and deoxynucleoside triphosphate. The reaction mixture was incubated for 5 min at 70°C and then for 1 h at 37°C. The reaction was stopped by heating at 95°C for 5 min. SYBR-PCR was performed based on SYBR green fluorescence (SYBR green PCR master mix; Applied Biosystems, Foster City, CA). To prevent genomic DNA amplification, the primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and inducible protein 10 (IP10) were designed across exon-intron junctions. The primers for GAPDH were 5' GTCCTCTGTTCGACAGTCA 3' and 5' ACCTTCCCATGGTG TCTGA 3'. The primers for IP10 were 5' CGATTCTGATTTGCTGCCTTAT 3' and 5' GCAGGTACAGCGTACGGTTCT 3'. The difference in the cycle threshold (ΔC_T) value was derived by subtracting the C_T value for GAPDH, which served as an internal control, from the C_T value for IP10. All reactions were run in duplicate by using an ABI 7000 sequence detection system (Applied Biosystems). mRNA expression levels of IP10 were expressed as a severalfold increase according to the formula $2\Delta C_T$ (phosphate-buffered saline) - ΔC_T (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 ^{14}C -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 Dulbecco'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 2010 system, the microparticles are magnetically captured on the surface of the electrode. Unbound substances are removed with 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 H7TA61 cells. Huh7.93 and H7TA61 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 to the cells 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-UCF; BioGenex, San Ramon, CA) overnight 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

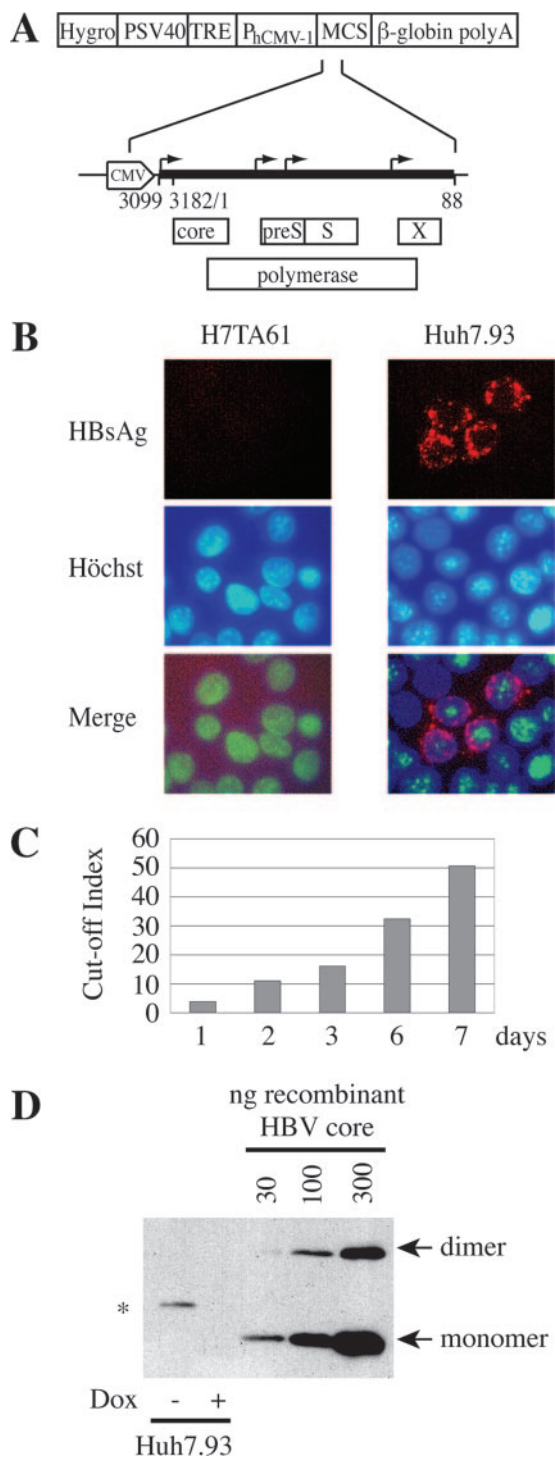


FIG. 1. (A) Schematic map of plasmid pTRE-HBVT used to establish the HBV-expressing Huh7.93 cell line. A slightly overlength ($\times 1.05$) HBV genome was cloned behind the Tet response element/minimal cytomegalovirus (CMV) promoter in plasmid pTRE2hyg (Clontech) such that the transcription of the full-length pregenomic RNA of HBV is controllable by the presence or absence of Dox. Without Dox, pregenomic RNA and its gene products core and polymerase are generated, leading to viral replication. With Dox, pregenomic RNA transcription is suppressed, whereas the subgenomic mRNAs for the surface proteins and X protein are transcribed from the Dox-independent endogenous viral promoters. Arrowheads denote approximate RNA start sites. For authentic polyadenylation, the

mounted in FluorSave reagent (Calbiochem), and pictures were taken under a microscope.

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-HBVT (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/ μ g total protein with a standard error of 3.5 ng/ μ g (Fig. 2B). This level was significantly higher than that in Huh7 cell lysates, where the mean PP2Ac expression level was 16.1 ng/ μ g total protein (standard error, 2.2 ng/ μ g). Inducing HBV replication by cultur-

genuine viral polyadenylation signal is contained in the 3' terminal redundancy of the inserted HBV genome. The positions of the HBV genes are indicated by boxes. MCS, multiple cloning sites. (B) HBs antigen is expressed in the perinuclear cytoplasm in Huh7.93 cells. H7TA61 (left panels) and Huh7.93 (right panels) were stained for HBsAg expression. The cell nuclei were stained with Hoechst dye (middle panels). The merge of the two pictures (lower panels) shows the cytoplasmic location of the HBsAg. (C) Huh7.93 cells constitutively express HBsAg. Cells were grown in culture medium supplemented with doxycycline. The culture supernatant was sampled at the indicated time points, and HBsAg was quantified on an automated Elecsys 2010 system. The amount of HBsAg is expressed as a signal/cutoff ratio. The cutoff ratio was determined with an HBsAg standard provided by the manufacturer. (D) Derepression of Huh7.93 cells induces the expression of HBV core protein. The Western blot shows the samples with 30, 100, and 300 ng of recombinant truncated HBV core protein (amino acids 1 to 149), which appears as a monomer of 16/17 kDa and a dimer of 32 to 34 kDa, and lysates of Huh7.93 cells grown for 6 days without (-) (lane 1) or with Dox (+) (lane 2). The asterisk indicates the position of the 21-kDa full-length core protein in the cells.

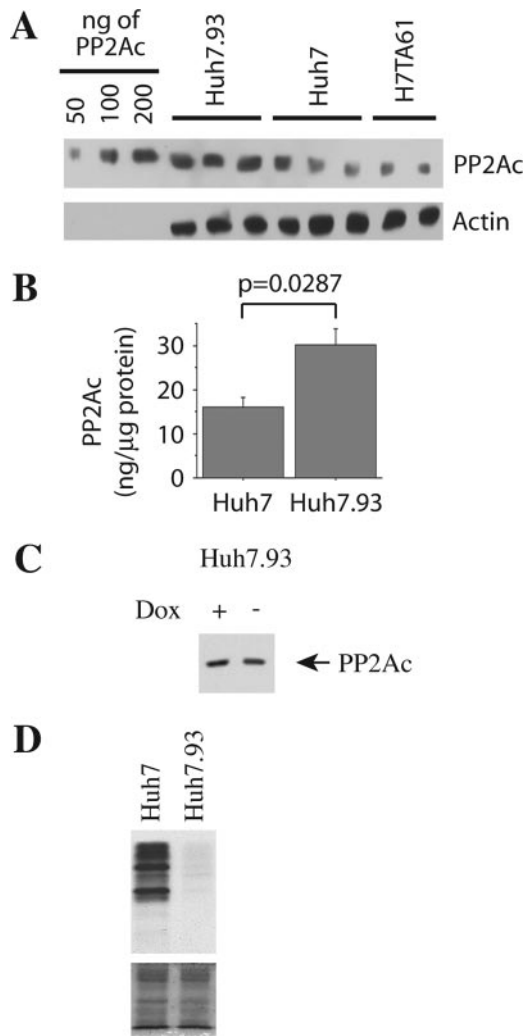


FIG. 2. (A) Expression of PP2Ac in human hepatoma cells expressing HBV proteins. The Western blot shows PP2Ac standard samples with 50, 100, and 200 ng of purified PP2Ac in lanes 1 to 3 (used to generate a calibration curve) and three samples each of Huh7 and Huh7.93 cell lysates as well as two samples of H7TA-61 cells (5 μ g total protein in each lane). The lower panel is a loading control showing the housekeeping protein actin. (B) Graph of quantified PP2Ac expression levels in Huh7 versus Huh7.93 cells. Shown is the mean of three independent samples with error bars representing the standard errors of the means. The difference in the expression of PP2Ac between control cells and HBV protein-expressing cells is statistically significant ($P = 0.0287$, Mann-Whitney U test). (C) Huh7.93 cells were grown in medium with (+) and without (-) doxycycline as indicated. PP2Ac expression was measured by Western blot analysis. No further increase of PP2Ac expression was observed in derepressed cells. (D) PRMT1 activity is inhibited in Huh7.93 cells. Twenty micrograms of whole-cell lysate from each cell line was incubated in the presence of 3 μ l of 14 C-AdoMet for 2 h at 37°C. The reactions were then stopped by adding 5 μ l of sample loading buffer, the reaction mixtures were boiled for 5 min, and the proteins were 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.

ing the cells without Dox did not further increase the expression level of PP2Ac (Fig. 2C). PP2Ac expression was also significantly increased in Huh7.93 cells compared to that in H7TA-61 cells (Fig. 2A). Hence the increased PP2Ac

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 stimulation of cells with IFN- α in Huh7 and Huh7.93 cells. The inhibition of the Jak-STAT pathway in Huh7.93 cells on the level of STAT1 methylation resulted in a significantly lower induction of IP10 (Fig. 4C).

Upregulation 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/ μ g total protein in controls and 17.5 ng/ μ g 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

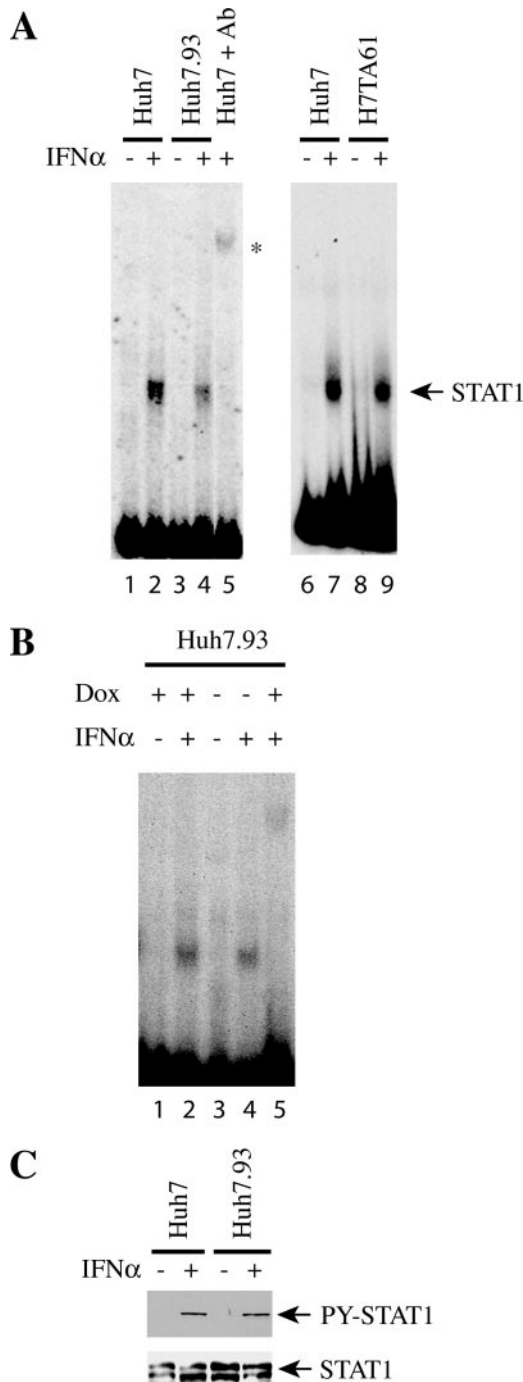


FIG. 3. (A) IFN- α -induced binding of activated STAT1 is impaired in Huh7.93 cells compared to that in the parental cell lines Huh7 and H7TA-61. Shown are EMSA results using the SIE-m67 oligonucleotide probe. Cells were left untreated (-) (lanes 1, 3, 6, and 8) or they were treated (+) for 20 min with 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 reblotted for STAT 1 as a loading control (lower part). +, with; -, without.

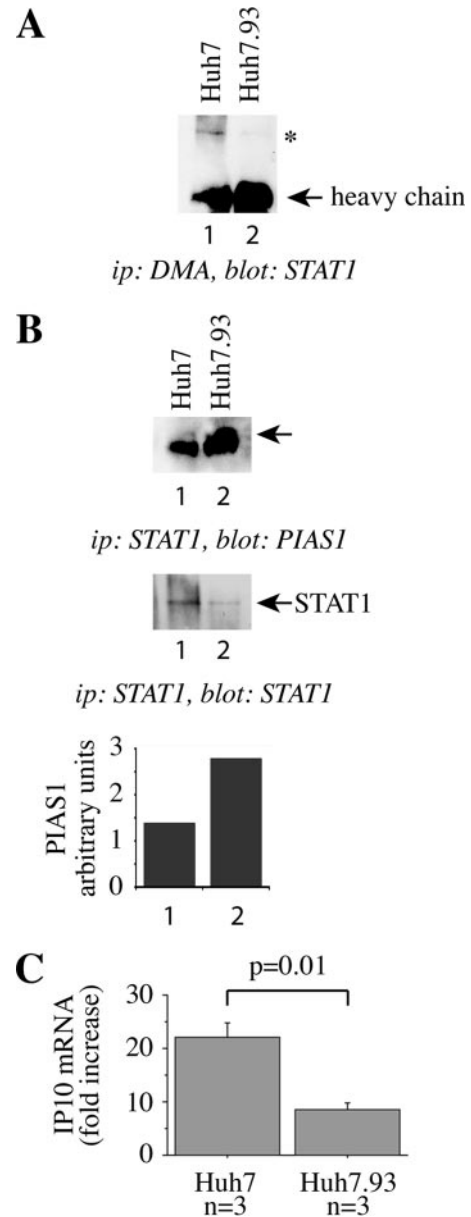


FIG. 4. (A) STAT1 methylation (asterisk), as detected by an immunoprecipitation (*ip*) with antibodies to monomethyl- and dimethylarginine and Western blotting with STAT1 antibodies, is impaired in cells expressing viral proteins (lane 2) compared to that in Huh7 cells (lane 1). (B) Binding of PIAS1 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 induction of IP10 mRNA was calculated as severalfold increase of the mRNA amounts in IFN- α -treated samples versus that in untreated samples. Shown are the mean values and the standard errors of the means (error bars). The *P* value was obtained using the analysis of variance test. The transcriptional induction of IP10 is inhibited in Huh7.93 cells (right bar) compared to that in the parental cell line Huh7 (left bar).

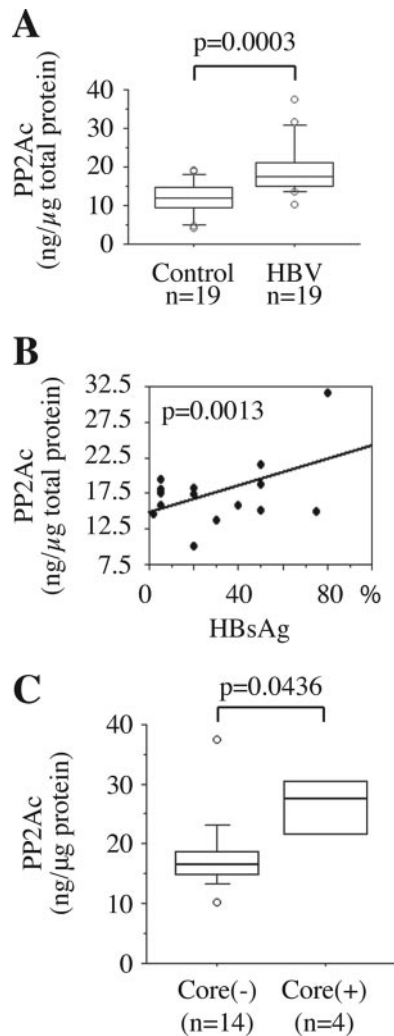


FIG. 5. (A) Expression of PP2Ac in human liver biopsies of patients with CHB. PP2Ac expression levels were analyzed in liver biopsies from patients with CHB and from controls by using semiquantitative Western blot analysis. On each Western blot gel, 50, 100, and 200 ng of purified PP2Ac was loaded to generate a calibration curve. The Western blot signal intensities of these standards and of the samples were measured using NIH Image software, and the amount of PP2Ac in each sample was calculated according to the calibration curve. Shown is a box plot diagram of quantified PP2Ac expression levels in the liver biopsies of 19 patients with chronic hepatitis B and those of 19 control patients without parenchymal liver disease. The difference in the expression of PP2Ac between the two groups is statistically significant ($P = 0.0003$, Mann-Whitney U test). (B) Correlation between PP2Ac expression and HBsAg expression. Liver biopsies from patients with CHB were stained for HBsAg expression. The percentage of hepatocytes with positive staining was assessed. The expression of PP2Ac correlated positively with HBsAg expression ($r = 0.565$, $P = 0.021$, Fisher's Z test). (C) Correlation between PP2Ac expression and HBcAg expression. HBcAg expression was positive in 4 samples and negative in 14 samples. PP2Ac expression was higher in the group with HBcAg expression. Shown is a box plot diagram.

expression was found in only 4 of the 18 biopsies. The PP2Ac expression level tended to be higher in these HBcAg-positive samples, but the small sample size limits the significance of this result (Fig. 5C). There was no correlation between PP2Ac

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- α 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 modulates IFN- α -induced transcription of interferon target genes (25). Methylated STAT1 has a lower affinity to PIAS1, an inhibitor of DNA binding of activated STAT dimers. Because PRMT1 activity is inhibited in HBV antigen-expressing cells, STAT1 is present in mainly its unmethylated form (Fig. 4A) and has a higher affinity for PIAS1, as shown in coimmunoprecipitation experiments (Fig. 4B). The increased binding of STAT1 by PIAS1 results in a reduced affinity of STAT1 to its response elements in IFN- α 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- α 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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