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Hepatitis B Virus-Induced Calreticulin Protein Is Involved in IFN Resistance

Xin Yue,*† Hui Wang,* Fanpeng Zhao,* Shi Liu,* Jianguo Wu,* Wendan Ren,* and Ying Zhu*

IFN-α is a widely used treatment for hepatitis B virus (HBV) infection, and IFN resistance caused by viral and/or host factors is currently a challenging clinical problem. A better understanding of the molecular mechanisms underlying IFN immunotherapy in the treatment of viral infection would be very beneficial clinically and is of immense clinical importance. Calreticulin (CRT) is an endoplasmic reticulum luminal calcium-binding chaperone that is involved in the regulation of calcium homeostasis, the folding of newly synthesized proteins, and many other cellular functions. However, little is known about the role of CRT in HBV infection. In this study, we observed high levels of CRT expression in the sera and PBMCs of patients with HBV relative to those of healthy individuals. HBV upregulated the expression of CRT at the transcriptional level. Further investigation showed that HBV-induced CRT enhanced HBV replication by antagonizing the IFN pathway. CRT suppressed the production of endogenous IFN-α by reducing the nuclear translocation of IFN regulatory factor-7 but not IFN regulatory factor-3. Furthermore, CRT also suppressed the antiviral activity of IFN-α by inhibiting the phosphorylation of STAT1 and decreasing the expression of two IFN-α downstream effectors, protein kinase R and 2′,5′-oligoadenylate synthetase. Our results offer new insights into the pathogenesis of HBV infection and may provide potential targets for anti-HBV therapy. The Journal of Immunology, 2012, 189: 279–286.

Hepatitis B virus (HBV) can cause both transient and chronic infections of the liver and is the leading cause of cirrhosis and hepatocellular carcinoma (1). HBV infection affects ~350 million people worldwide and likely causes more than 1 million deaths from liver cancer each year (2). In China, ~120 million people are chronically infected with HBV (3). To date, the immunomodulator IFN-α is one of the few antiviral agents licensed for the treatment of chronic HBV infection.

Calreticulin (CRT) is a 46-kDa endoplasmic reticulum (ER) luminal protein whose main functions are to bind Ca2+ and participate in the folding of newly synthesized proteins (4, 5). Calreticulin contains an N-terminal amino acid signal sequence that targets it to the ER and a C-terminal KDEL sequence that leads to its retention in the ER. Within the lumen of the ER, CRT forms a folding cycle with calnexin and ERp57 to control protein folding (6). As a modulator of Ca2+ homeostasis, CRT increases the capacity for Ca2+ storage in the ER by binding large quantities of Ca2+ and thus influences a variety of cellular functions (7).

Besides the lumen of the ER, CRT is also localized to the intracellular, cell surface, and extracellular compartments, suggesting that it may have multiple functions. Cell surface CRT has been shown to be required for Ag presentation (8), complement activation (9), cell adhesion (10, 11), the immunogenicity of cancer cells (12), the clearance of cells (13), and wound healing (14, 15), whereas cytoplasmic CRT has been suggested to serve as a signal transducer by both coupling calcium release and calcium influx (16) and acting as an inhibitor of steroid-sensitive gene transcription through interacting with hormone receptors (17, 18). CRT has also been suggested to be related to nuclear translocation (19, 20) and to serve as a component of the nuclear matrix in hepatocellular carcinomas (21, 22). Recent studies have shown altered CRT expression in tumors and identified it as a marker for several human cancers (23, 24).

There are few reports about the relationship between CRT and HBV infection in the literature. In this study, we identified a previously unrecognized function of CRT during HBV replication. HBV upregulated the expression of CRT, and the increased expression of CRT antagonized the IFN pathway, leading to increased viral protein synthesis and HBV DNA replication. CRT inhibited the production of endogenous IFN-α and suppressed the expression of the IFN-α downstream effectors protein kinase R (PKR) and 2′,5′-oligoadenylate synthetase (OAS). Our results reveal the importance of host factors in IFN resistance during HBV expression and replication.

Materials and Methods

Clinical samples

Peripheral blood samples were obtained from 110 patients with chronic hepatitis B (89 males and 21 females with a mean age of 46.5 ± 12.7 y).
Patients were matched for sex and age with 105 healthy individuals (85 males and 20 females with a mean age of 41.6 ± 14.5 y) with no history of liver disease that were randomly selected as controls from the local blood donation center. The study was conducted according to the principles of the Declaration of Helsinki and approved by the Institutional Review Board of the College of Life Sciences, Wuhan University, in accordance with the guidelines for the protection of human subjects. Written informed consent was obtained from each participant.

**Plasmids and reagents**

Coding regions of CRT were generated by PCR amplification. The PCR products were digested with EcoRI/XhoI and cloned directly into the pCMV-Tag2B expression vector to generate pCMV-CRT. pHBV-1.3 (ayw) was generated from the HBV genome (genotype D, subtype ayw, GenBank accession no. U95551; http://www.ncbi.nlm.nih.gov/Genbank/), digested with EcoRI/Sall, and inserted into pBlueScript II. pHBV-1.3 (adw) was generated from the HBV genome (genotype B, subtype adw, GenBank accession no. JN406371; http://www.ncbi.nlm.nih.gov/Genbank/), digested with EcoRI/Sall, and inserted into pBlueScript II.

Specific small interfering RNA (siRNA) against calreticulin (siCRT) was synthesized based on a previously reported target sequence (25). Abs against CRT, PKK, OAS2, STAT1, p-STAT1, IFN regulatory factor (IRF)-3, IRF-7, p65, p50, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs against CRT, PKK, OAS2, STAT1, p-STAT1, IFN regulatory factor (IRF)-3, IRF-7, p65, p50, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs against CRT, PKK, OAS2, STAT1, p-STAT1, IFN regulatory factor (IRF)-3, IRF-7, p65, p50, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs against CRT, PKK, OAS2, STAT1, p-STAT1, IFN regulatory factor (IRF)-3, IRF-7, p65, p50, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell culture**

The human hepatoma cell lines HepG2 and HuH7 were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate at 37°C in 5% CO2. The HepG2.2.15 cell line, which was derived from HepG2 cells and stably expresses HBV (subtype ayw), has been described in a previous study (26). The HuH7.37 cell line, which was derived from HuH7 cells and also stably expresses HBV (subtype adw), was constructed in this study. The stable cell lines were maintained in DMEM containing 400 µg/ml G418.

**Quantitative RT-PCR analysis**

Quantitative RT-PCR analysis was performed to determine relative mRNA levels. Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA). Cellular RNA samples were reverse transcribed with random primers. Real-time PCR was performed in a Light Cycler 480 (Roche, Indianapolis, IN). GAPDH was amplified as an internal control, and the following primers were used: CRT forward 5′-TGGCTGTGGGCAAGG-3′; CRT reverse 5′-AGGCCCTACGAAGAAT-3′; GAPDH forward 5′-AAAGCCCTACGAAGAATAGG-3′; and GAPDH reverse 5′-TGGAGGATTGGTGTGCG-3′.

**Transfection and luciferase reporter gene assays**

Cells were plated at a density of 4 × 10⁴ cells per 24-well or 6-well plate, depending on the experiment, and were grown to 80% confluence prior to transfection. Cells were transfected with Lipofectamine 2000 (Invitrogen) for 24 h, serum starved for an additional 24 h, and then harvested. A Renilla luciferase reporter vector pRL-TK was used as an internal control. The dual-luciferase reporter assay system (Promega, Madison, WI) was used to measure the luciferase activity of each sample 48 h after transfection, and Renilla luciferase activities were determined as internal controls for transfection efficiency. Assays were performed in triplicate, and the results were expressed as mean percentage ± SE relative to the vector or mock control samples, which were set at 100%.

**Western blot analysis**

Whole-cell lysates were prepared by lysing cells in PBS (pH 7.4) that contained 0.01% Triton X-100, 0.01% EDTA, and 10% protease inhibitor mixture (Roche). To separate and collect the cytosolic and nuclear protein fractions, cells were washed with ice-cold PBS and collected by centrifugation, and the resulting pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.5 mM DTT, 10% protease mixture inhibitor) for 15 min on ice and then vortexed for 10 s. Nuclei were pelleted by centrifugation at 13,000 rpm for 1 min, and the pellets and cytosolic protein-containing supernatants were collected. The protein concentration of each sample was determined with the Bradford assay kit (Bio-Rad, Hercules, CA). A 100-µg aliquot of each sample was subjected to 12% SDS-PAGE and then transferred to a nitrocellulose membrane. Blots were blocked with nonfat dry milk prior to incubation with Abs as indicated in the figures. Blots were developed with the SuperSignal Chemiluminescent reagent (Pierce, Rockford, IL).

**Analysis of HBV DNA replicative intermediates by real-time PCR**

HBV DNA replicative intermediates, which are found in HBV core particles inside the transfected cells, were analyzed by quantitative real-time PCR. Cells were lysed and centrifuged, and then magnesium chloride was added to the supernatant. DNA that was not protected by HBV core protein was digested with DNase I. After the inactivation of DNase I, cell lysates were treated with proteinase K, and the DNA was extracted with phenol/ chloroform. Core-associated HBV DNA was recovered by ethanol precipitation and quantified by real-time PCR per the manufacturer’s protocol (PG Biotech, Shenzhen, China). The primers used in RT-PCR were as follows: P1, 5′-ATCTTGCTGCTCACTCT-3′; and P2, 5′-ACC-GTGGGAAAAGCTTCGAAAAGTTTTG-3′. The probe was used was 5′-TGGCTGTTTCTGATTTGATAAG-3′, and the PCR was carried out and analyzed with an LC480 (Roche).

**Quantification of HBV e-Ag and HBV surface Ag**

Cells were transfected as indicated and were cultured for an additional 24 h in DMEM without FBS or antibiotics. The conditioned media were collected, and a standard ELISA kit was used to quantify HBV e-Ag (HBsAg) and HBV surface Ag (HBsAg) (Shanghai KeHua Biotech, Shanghai, China).

**Statistical analysis**

All of the experiments were reproducible and carried out in duplicate or quadruplicate. Each set of experiments was repeated at least three times with similar results. The results are presented as the means ± SE. Student’s t test for paired samples was used to determine statistical significance. Differences were considered statistically significant at a p value ≤ 0.05.

**Results**

**CRT expression is elevated in HBV patients**

High levels of CRT were found by ELISA in the sera of patients with chronic HBV infection. As shown in Fig. 1A and Table I, the serum levels of CRT were significantly higher in patients with chronic hepatitis B infection than in healthy control individuals (median ± SEM: 72.31 ± 18.90 versus 37.53 ± 8.01 ng/ml, p < 0.05), suggesting that HBV infection resulted in the upregulation of CRT.

On the basis of the high level of circulating CRT found in the chronic HBV patients, we performed quantitative RT-PCR (qRT-PCR) analysis to quantify the CRT mRNA level in the PBMCs of hepatitis B patients. The results demonstrated that CRT mRNA was significantly elevated in the PBMCs of patients compared with those of healthy individuals (Fig. 1B). The expression of CRT was also increased in freshly isolated PBMCs after treatment with the culture supernatants of HepG2.2.15 cells, which contained HBV (8.5 × 10⁶ copies/ml), and this elevation in CRT was reversed by human hepatitis B Ig, an HBV neutralizing Ab, at both the mRNA and protein levels (Fig. 1C). To determine the cellular source of CRT elevation in response to the stimulation of HBV, PBMCs were separated based on adherence to the culture plate: Nonadherent lymphocytes were removed, leaving adherent monocytes, and each cell type was then cultured separately. The induction of HBV resulted in no significant difference of CRT production between monocytes and lymphocytes (Fig. 1D).

**CRT is induced by HBV at both the transcriptional and posttranscriptional levels**

To test if the expression of HBV influences the expression of CRT, the CRT mRNA level in HepG2 human hepatoma cells was compared with that in HepG2.2.15 cells, which contain an integrated HBV (subtype ayw) genome and stably express HBV. qRT-PCR showed that the CRT mRNA expression in HepG2.2.15 cells was
elevated in comparison with that of the HepG2 cells (Fig. 2A). To ensure that this was not a cell-specific event, another pair of human hepatoma cells, Huh7 and Huh7.37 (Huh7.37 cells contain an integrated HBV [subtype adw] and stably express HBV), were also tested in regard to CRT expression. As with the HepG2 cells, the cell line that expresses HBV, Huh7.37, had a higher level of CRT mRNA expression as measured by qRT-PCR (Fig. 2B). pHBV-1.3, a 1.3-fold overlength fragment of the HBV genome (subtype ayw or adw), which retains the ability to produce mature HBV virions, was then transfected into HepG2 cells or Huh7 cells, and the level of CRT protein expression was detected by Western blot analysis. The results showed that HBV stimulated the expression of CRT in a dose-dependent manner (Fig. 2C).

To find out at what level HBV influences the expression of CRT, pCRT-Luc or pCRT-UTR-Luc and pHBV-1.3 were cotransfected into HepG2 cells. Luciferase activity was measured in each sample, and we observed that HBV activated both CRT promoter-Luc and CRT 3′ UTR-Luc activities (Fig. 2D, 2E), indicating that CRT was induced by HBV at both the transcriptional and posttranscriptional levels.

Table I. Demographic and baseline characteristics and serum CRT levels of the patients and healthy individuals enrolled in this study

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy Individuals (n = 105)</th>
<th>Patients (n = 110)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>41.6 ± 14.5</td>
<td>46.5 ± 12.7</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>85/20</td>
<td>89/21</td>
</tr>
<tr>
<td>Race or ethnic group, % (Asian)</td>
<td>105 (100)</td>
<td>110 (100)</td>
</tr>
<tr>
<td>HBsAg, +/−</td>
<td>0/105</td>
<td>110/0</td>
</tr>
<tr>
<td>HBV genotype, a/b/c</td>
<td>NA</td>
<td>19/91</td>
</tr>
<tr>
<td>WBC, cells/μl</td>
<td>3479 ± 815</td>
<td>7948 ± 7230</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>&lt;30</td>
<td>151.7 ± 169.4</td>
</tr>
<tr>
<td>HBV DNA, copies/ml</td>
<td>&lt;500</td>
<td>9.6E + 07 ± 4.9E + 08</td>
</tr>
<tr>
<td>CRT, ng/ml</td>
<td>37.53 ± 8.01</td>
<td>72.31 ± 18.90</td>
</tr>
</tbody>
</table>

ALT, Alanine aminotransferase.

**CRT enhances the replication of HBV**

To determine the role of CRT during HBV expression, we first examined the effect of CRT on HBV replication. HepG2 cells were cotransfected with pHBV-1.3 at the indicated concentration gradient of pCMV-CRT plasmid or CRT siRNA. We found that overexpression of CRT stimulated the expression of HBe and HBs, whereas the knocking down of CRT suppressed the expression of HBeAg and HBsAg (Fig. 3A, 3B). We then examined the effect of CRT on the production of HBV DNA replicative intermediates. HepG2 cells were cotransfected with pHBV-1.3 and either pCMV-CRT plasmid or CRT-specific siRNA. Core-associated viral DNA, the replicative intermediates extracted from the transfected cells, was assayed by qRT-PCR 48 h posttransfection. The overexpression of CRT increased HBV DNA replication, whereas the knockdown of CRT by RNA interference inhibited HBV DNA replication (Fig. 3C). The efficiency of CRT-specific siRNA in knocking down the CRT mRNA level is shown in Fig. 3D.

**CRT restricts the antiviral activity of IFN-α**

IFN-α can inhibit the protein synthesis and DNA replication of HBV and is one of the antiviral agents used for the treatment of chronic HBV infection. As our results have demonstrated that CRT promotes the replication of HBV, we asked if CRT could affect the antiviral activity of IFN-α. pCMV-CRT was transfected along with pHBV-1.3 into HepG2 cells, which were subsequently treated with exogenous IFN-α (600 U/ml) 24 h posttransfection. Forty-eight hours after transfection, HBeAg and HBsAg levels were examined by ELISA. IFN-α strongly inhibited HBV expression, but this inhibition could be partially reversed by the expression of CRT (Fig. 4A).

To confirm further the cross talk between CRT and IFN-α, the vesicular stomatitis virus (VSV) and Vero cell infection system was chosen. VSV is a pathogen that is extremely sensitive to the actions of type I IFN, and Vero cells do not produce type I IFN due to genomic deletions. In contrast to the results observed in the
HepG2 cells, increased CRT expression did not highly increase viral replication (Fig. 4B, bars 1 and 4). However, exogenous IFN-α (600 U/ml) strongly inhibited viral replication (Fig. 4B, bars 1 and 2), and this inhibition could be reversed by the expression of CRT (Fig. 4B, bars 2 and 3). The increase of VSV replication by CRT with (Fig. 4B, bars 2 and 3) or without (Fig. 4B, bars 1 and 4) IFN-α is markedly different (p value being 0.0007 versus 0.0087).

CRT suppresses the expression of IFN-α downstream effectors ISG15, OAS, Mx proteins, and PKR vary in their responsiveness to type I IFNs. These effector proteins interfere with distinct steps in viral replication or trigger the degradation of viral RNA and/or protein. To study the effects of CRT on these effectors, HepG2 cells were transfected with either the pCMV-CRT vector or siCRT and then treated with IFN-α. qRT-PCR and Western blotting were used to measure the IFN-α–induced levels of PRK and OAS2.
at the mRNA and protein levels, respectively. As expected, CRT inhibited the expression of IFN-α-induced PKR and OAS2 (Fig. 5A, 5B), whereas CRT-specific siRNA promoted the expression of PKR and OAS2 (Fig. 5B, right panel).

As the phosphorylation and dimerization of the STAT proteins are essential for the expression of IFN-stimulated antiviral genes, we investigated the effect of CRT on IFN-induced STAT expression and phosphorylation. As shown in Fig. 5C, expression of CRT had no influence on the IFN-α-induced expression of total STAT1 and STAT2. However, CRT inhibited the phosphorylation of IFN-α-activated STAT1 but not STAT2. These results indicate that CRT can restrict the expression of IFN-stimulated antiviral genes through the inhibition of STAT1 phosphorylation.

CRT suppresses the expression of endogenous IFN-α

The production of IFNs is very important in the protective immunity against most viruses. Thus, we next investigated the influence of CRT on the endogenous expression of IFN-α. The overexpression of CRT strongly inhibited the Sendai virus (SeV)-induced activation of the IFN-β promoter in reporter assays (Fig. 6A). Further,
In this study, we first identified that HBV upregulates the expression of CRT. We then demonstrated a previously unknown mechanism in which CRT enhances HBV replication by suppressing endog-
ylation of EIF2α. There are four different forms of OAS (OAS1, OAS2, OAS3, and OASL), and there seems to be differential expression and induction of all four of these forms in humans (32). When induced by dsRNA, the OAS proteins are able to synthesize oligoadenylates that activate the latent form of RNase L and then trigger the degradation of viral RNA. OAS proteins may have additional antiviral functions independent of RNase L activity (33, 34). In this report, we showed that CRT was able to suppress the expression of IFN-α–induced PKR and OAS2 through the inhibition of STAT1 phosphorylation, which is an essential step in the expression of IFN-stimulated antiviral genes. In IFN-deficient Vero cells, the ability of CRT to reverse the IFN-induced inhibition of VSV further confirmed the inhibition of IFN-mediated antiviral activity by CRT.

It has been well demonstrated that the induction of type I IFNs is required for the activation of the IRF-3/7 and NF-kB pathways (35). IRF-3, a constitutively expressed phosphoprotein, is activated by virus- and dsRNA-induced phosphorylation. This phosphorylation leads to the homodimerization and heterodimerization and nuclear localization of IRF-3, as well as its association with the coactivator CBP/p300 (36, 37). IRF-3 is a multifunctional protein with transcriptional activity that, similar to IRF-3, depends on phosphorylation and nuclear translocation (38, 39) and is well known as an important regulator of virus-induced IFN-α expression (40). In most cells, the expression of IRF-7 is very low constitutively and is virus and IFN inducible. The requirement for NF-kB in IFN-β regulation has been recognized for decades (41, 42). The NF-kB pathway is extensively involved in a large number of cellular processes, including immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. Activation of NF-kB signaling requires the phosphorylation of the I-kB inhibitor. In this study, we showed that CRT was able to suppress the production of endogenous IFN-α and that this suppression was mediated by the reduction of IRF-3 in the nucleus. However, whether this reduction is due to CRT leading to the reduction of IRF-7 translocation from the cytosol to the nucleus or the result of the CRT-facilitated translocation of IRF-7 from the nucleus to the cytosol during HBV replication is still not clear.

To figure out the exact role of CRT during HBV infection, it is important to correlate infection of HBV, level of CRT, and suppression of IFN-α. However, a major barrier to the study of HBV infection is the lack of a reliable and sensitive in vitro infection system. We made some attempts in HBV transfected liver cells. IFN-α was upregulated by the expression of HBV at an early stage. When CRT expression increased to a certain level, the expression of IFN-α then dropped down (Supplemental Fig. 1).

In conclusion, we identified a previously unrecognized function of CRT during HBV expression. A hypothetical model detailing this function of CRT is proposed in Fig. 7. HBV was able to upregulate the expression of CRT at both the transcriptional and posttranscriptional levels. The HBV-induced CRT then enhanced the replication of HBV through inhibiting the IFN pathway by suppressing the production of endogenous IFN-α by antagonizing the nuclear translocation of IRF-7 and inhibiting IFN-α downstream antiviral effectors. Our research offers new insights into the pathogenesis of HBV infection and may provide potential targets for anti-HBV therapy.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.


Supplemental Fig. 1. The relationship of HBV expression, induced levels of CRT and production of IFN-α.

HepG2 cells were transfected with pHBV-1.3 and then collected at indicated time points. Expressions of CRT and IFN-α were detected by qRT-PCR, respectively.