Ceruloplasmin inhibits the production of extracellular hepatitis B virions by targeting its middle surface protein

Kaitao Zhao,1,2 Chunchen Wu,1,* Yongxuan Yao,1,2 Liang Cao,1,2 Zhenhua Zhang,3,4 Yifei Yuan,1,2 Yun Wang,1 Rongjuan Pei,1 Jizheng Chen,1 Xue Hu,1 Yuan Zhou,1 Mengji Lu1,5 and Xinwen Chen1,2

Abstract

Ceruloplasmin (CP) is mainly synthesized by hepatocytes and plays an essential role in iron metabolism. Previous reports have shown that CP levels correlate negatively with disease progression in patients with chronic hepatitis B. However, the function of CP in the hepatitis B virus (HBV) life cycle and the mechanism underlying the above correlation remain unclear. Here, we report that CP can selectively inhibit the production of extracellular HBV virions without altering intracellular viral replication. HBV expression can also downregulate the expression of CP. Knockdown of CP using small interfering RNA significantly increased the level of extracellular HBV virions in both Huh7 and HepG2.2.15 cells, while overexpression of CP decreased this level. Mechanistically, CP could specifically interact with the HBV middle surface protein (MHB). Using an HBV replication-competent clone unable to express MHBs, we demonstrated that the overexpression of CP did not affect the production of extracellular HBV virions in the absence of MHBs. Furthermore, introduction of an MHB expression construct could rescue the impairment in virion production caused by CP. Taken together, our results suggest that CP may be an important host factor that targets MHBs during the envelopment and/or release of virions.

INTRODUCTION

Hepatitis B virus (HBV) is a serious pathogen that causes a widespread viral infection with approximately 240 million chronic active carriers worldwide. HBV mainly infects hepatocytes and causes various liver diseases, such as chronic hepatitis, liver cirrhosis and even hepatocellular carcinoma [1, 2].

HBV belongs to the family Hepadnaviridae and is a small, enveloped virus that contains a partially double-stranded DNA genome measuring approximately 3.2 kb. The three envelope, or surface, proteins of the virus, called large surface protein (LHB), middle surface protein (MHB) and small surface protein (SHB) [3–5], are expressed from S ORFs. They share the same C-terminal region and only differ in length because of their N-terminal regions. LHBs, MHBs and SHBs are present in the envelopes of mature/infectious virions (42 nm large Dane particle) at a ratio of approximately 1 : 1 : 4 [6]. Although SHB is the predominant component of viral particles, LHB is indispensable for the formation and budding of virions. The absence of LHBs completely abolishes the generation of new virions [7]. In contrast, MHB is highly conserved within all Orthohepadnaviruses, although it is not essential for secretion of virions, subviral particle formation, or infectivity of HBV or hepatitis D virus. [5, 8–10]. However, the absence of MHBs reduces virion secretion and the virions from these MHB-minus constructs contain more mature relaxed circular (RC) forms of the HBV DNA [11]. Furthermore, the MHB-minus mutants frequently arise during the later stage of chronic HBV infection [8, 11–13]. Therefore, the function of the MHB still remains an enigma. Growing evidence suggests that HBV maturation and egress is mediated by multivesicular bodies (MVBs) from the late endosomal compartment [14, 15]. During this process, LHBs, MHBs and SHBs are recruited to MVB membranes and facilitate budding of the viral capsid into MVBs with the aid of the endosomal sorting complex required for transport (ESCRT) [16]. Given that all three envelope proteins are co-translationally integrated into the endoplasmic reticulum (ER) membrane, it is conceivable that transportation of envelope proteins from the...
pre-Golgi/ER membrane to the late endosome occurs during HBV maturation and egress. However, it remains unclear whether host factors affect the transportation of envelope proteins and thus interfere with the envelopment and/or release of HBV virions.

In addition to infectious virions, envelope proteins can assemble into two kinds of nucleocapsid-free subviral particles (SVPs), spherical SVPs and filamentous SVPs, which are secreted in 1000- to 100 000-fold excess relative to infectious virions [17]. Spherical SVPs are mainly composed of MHB secreted in 1000- to 100 000-fold excess relative to infectious (SVPs), spherical SVPs and filamentous SVPs, which contain an integrated HBV (subotype ayw) genome and stably express HBV, were also decreased in comparison with those measured in HepG2 cells (Fig. 1c, d). These results indicate that HBV expression resulted in the downregulation of CP.

**CP inhibits HBV virion production**

To reveal the function of CP in the HBV life cycle, Huh7 cells were co-transfected with pSM2 and pHA-CP plasmids or an empty vector. Levels of HBV virions, HBV naked nucleocapsids, hepatitis B surface antigen (HBsAg) and the HBV protein HBeAg secreted into cell culture supernatants were assayed at the indicated time points. HBV virions were immunoprecipitated with mixed monoclonal anti-HBs antibodies and subjected to HBV DNA extraction followed by quantitative real-time PCR (RT-PCR) analysis. Overexpression of CP had no toxicity to cells (Fig. S1, available in the online Supplementary Material) but considerably reduced the supernatant HBV virion level by approximately 80, 70 and 80% at 48, 72 and 96 h post transfection (p.t.), respectively (Fig. 2b). In contrast, HBsAg levels were slightly increased by approximately 30 and 15% at 48 and 96 h p.t. (Fig. 2c), while HBeAg levels were not affected (Fig. 2d). The function of CP in the HBV life cycle was further examined in HBV-replicating HepG2.2.15 cells. Consistently, the levels of HBV virions in the culture supernatants were reduced by 48% (Fig. 2g) after CP was overexpressed, whereas the levels of HBsAg and HBeAg were not affected (Fig. 2h, i). These results imply that the overexpression of CP may mainly impair the production of extracellular HBV virions.

In addition to HBV virions, another type of viral particle, namely, naked nucleocapsid, can be produced in HBV-replicating cells and extracellularly secreted [26]. Therefore, the influence of CP on the level of extracellular naked nucleocapsids was also assessed. To accomplish this, naked nucleocapsids were immunoprecipitated with an anti-core antibody and subjected to HBV DNA extraction followed by quantitative RT-PCR analysis. Compared to the mock vector-transfected control, overexpression of CP had no significant impact on the production of extracellular naked nucleocapsids by Huh7 cells at 72 h p.t. (Fig. 2e) or by HepG2.2.15 cells (Fig. 2f). Furthermore, to investigate whether depletion of CP could have a consistent effect on the HBV life cycle, the CP-specific shCP expression vector pShCP or the nonspecific shNC expression vector pShNC was used to knockdown the level of CP in Huh7 cells transiently transfected with pSM2 or in HepG2.2.15 cells. The results showed that the levels of
extracellular virions increased by approximately 230 and 115% (Fig. 3b, g), while the levels of extracellular HBsAg slightly decreased by approximately 15 and 16% (Fig. 3c, h) in Huh7 cells and HepG2.2.15 cells, respectively. HBV naked nucleocapsid and HBeAg levels were not affected (Fig. 3d, e, i, j). These data further indicate that CP is possibly involved in the HBV life cycle mainly by regulating the production of extracellular HBV virions.

**CP has no impact on HBV replication**

The downregulation of extracellular HBV virion production by CP may result from impaired HBV replication. To address this issue, the influence of CP on viral replication was further investigated. Southern blot assay showed that quantities of HBV replication intermediates did not change in the context of overexpression (Fig. 4a) or knockdown (Fig. 4c) of CP. Quantitative RT-PCR analysis further confirmed that the level of CP had no significant effect on HBV DNA copy numbers (Fig. 4b, d), indicating that CP may not affect HBV genomic replication.

**CP has no impact on the transcription and expression of structural proteins from HBV**

Given that CP had no impact on HBV replication, we reasoned that the downregulation of extracellular HBV virion production by CP may be related to the envelopment and/or release of virion. To test this hypothesis, we first analysed the influence of CP levels on the transcription and expression of structural proteins from HBV, including the LHBs, MHBs, SHBs and core proteins. Northern blot assay showed that overexpression of CP did not affect the levels of HBV transcripts in Huh7 cells (Fig. 5a, b), which was further confirmed in HepG2.2.15 cells (Fig. 5c, d). Western blot assay showed that quantities of LHB, MHB, SHB and core proteins did not change in the context of overexpression of CP (Fig. 5e, f). Moreover, quantities of capsids were also unaffected (Fig. 5g). These results indicate that CP has no impact on the transcription and expression of structural proteins from HBV.

**CP interacts with MHBs to impair the production of extracellular virions but facilitate MHBs secretion**

Considering that CP had no impact on the transcription and expression of LHBs, MHBs and SHBs, it is possible that CP regulates the production of extracellular virions through interactions between CP and the viral structural proteins. To test this hypothesis, an immunofluorescence assay was performed to detect whether CP colocalizes with viral structural proteins. As shown in Fig. 6(a), MHBs colocalized with CP in the cytoplasm when CP was co-expressed with LHBs, MHBs, SHBs or core (Fig. 6a). We also calculated colocalization coefficients as described previously [27]. Statistical analysis showed that the
The colocalization coefficient of CP and MHBs was significantly higher than the other three groups (Fig. 6b). Besides, a co-immunoprecipitation (co-IP) assay further demonstrated that MHBs could be pulled down by CP (Fig. 6c), implying that an interaction forms between MHBs and CP. In contrast, there was no specific interaction between LHBs, SHBs or core and CP (Figs 6c and S2). To validate whether MHBs could interact with CP directly, a bimolecular fluorescence complementation (BiFC) assay was performed as described previously [28]. For the BiFC assay, mCherry was split into the amino-terminal half, MN-159, and the carboxyterminal half, MC-160. Only when MN-159 and MC-160 are brought together by interactions between proteins fused to each fragment, can whole mCherry be formed and red fluorescence observed (Fig. 6d). Therefore, BiFC assay can be used to visualize direct protein–protein interactions in living cells. Considering that albumin protein can bind to (BiFC) assay was performed as described previously [28]. For the BiFC assay, mCherry was split into the amino-terminal half, MN-159, and the carboxyterminal half, MC-160. Only when MN-159 and MC-160 are brought together by interactions between proteins fused to each fragment, can whole mCherry be formed and red fluorescence observed (Fig. 6d). Therefore, BiFC assay can be used to visualize direct protein–protein interactions in living cells. Considering that albumin protein can bind to
MHBs [29, 30], the plasmid MC-160-ALB, which overexpresses albumin, was constructed and used here for a positive control. As shown in Fig. 6(e), compared to the positive control, the red mCherry fluorescence was also observed in cytoplasm when MN-159-CP and MC-160-M were co-expressed (Fig. 6e). In contrast, no specific fluorescence was observed when MN-159-CP and MC-160-L were co-expressed (Fig. 6e). These results indicated that CP could interact with MHBs directly and specifically.

To determine whether CP impaired the production of extracellular HBV virions via interaction with MHBs, an HBV replication-competent clone unable to express MHBs or an MHB expression construct was co-transfected with the CP-overexpressing plasmid as indicated (Fig. 7a). Overexpression of CP did not affect the production of extracellular HBV virions in the absence of MHBs (Fig. 7a, lanes 3 and 4), although the overall secretion of virions was decreased compared to the wild-type (Fig. 7a, lanes 1 and 3). However, supplementation of MHBs could rescue the impairment caused by CP on extracellular virion production (Fig. 7a, lanes 5 and 6).

The interaction between CP and MHBs may affect the secretion of MHBs. To address this issue, LHB, MHB and SHB levels were measured in both culture supernatants and cell lysates when CP was overexpressed (Fig. 7b, c). The results showed that the amount of MHBs secreted into culture supernatant increased after CP was overexpressed. In contrast, the overexpression of CP did not change the amounts of LHBs and SHBs in either the supernatants or cell lysates. Furthermore, CP was knocked down using short hairpin RNA (shRNA), and LHBs, MHBs and SHBs were again detected in both supernatants and cell lysates. Consistently, the knockdown of CP reduced the amount of MHBs secreted into the supernatant (Fig. 7d, e).

Collectively, these results indicate that CP interacts with MHBs to impair the production of extracellular virions but facilitate MHB secretion.
DISCUSSION

It has been reported that CP is downregulated in patients with CHB and that the levels of CP expression in these patients negatively correlate with their disease progression [23, 24]. Here, for the first time to our knowledge, CP has been shown to be involved in the HBV life cycle. Specifically, CP interacts with MHBs to impair the production of extracellular virions and thus represents an important factor for HBV replication.

CP, the major copper-carrying protein in the blood, plays an essential role in iron metabolism [22] and is mainly synthesized by hepatocytes. In the present study, both IHC staining and in vitro assay indicated that HBV expression downregulates the expression of CP in CHB patient liver tissues and HBV-replicating cell lines, an observation that is concordant with previously reported clinical data. It has been reported that serum CP levels are decreased in patients with HBV-related liver disease and that CP levels negatively correlate with inflammation and fibrosis stages in patients with CHB [23]. In addition, serum CP levels were also decreased in patients with severe hepatitis, Wilson’s disease and alcoholic liver steatosis [31–33]. Even though CP is not involved in HBV genomic replication, knockdown of CP enhanced HBV virion secretion. Thus, decreased CP levels in patients with CHB are beneficial to HBV virion production, which in turn may promote disease progression. Further experiments are needed to identify whether HBV expression decreases the expression of CP through an IFN-gamma-activated inhibitor of translation-mediated translational silencing mechanism [34].

So far, the exact role of MHBs in the HBV life cycle is not well understood. Previously, mutant HBV genomes unable to produce MHBs were identified in the serum of a chronic HBV carrier. These genomes were replication competent and could result in efficient secretion of virions [35]. Thus, MHB expression is not essential for HBV replication, virion morphogenesis or virion secretion. However, a recent report and our current data show that MHBs may play a regulatory role in HBV envelopment and/or release because ablating MHB expression reduced the level of extracellular virion, while trans-complementation of MHBs rescued the production of extracellular virions to a certain degree (Fig. 7a) [11]. Moreover, over-expression of CP impaired the production of extracellular virions when MHB was provided in trans. These data demonstrate that the impact of CP on the production of extracellular virions is mainly mediated by MHB. Previous studies have shown that MHB (aa 3–17) binds modified human serum albumin in vivo, which may aid attachment of HBV to liver and interfere with the induction of anti-preS2 specific immune response [29, 30]. These reports and our current data indicate that MHBs may play a particular role in HBV infection by interacting with host factors. Although N-glycosylation in the pre-S2 domain of MHBs is necessary for the secretion of virions [36], both glycosylated bands of MHBs could be detected whenever CP was overexpressed or knocked down (Fig. 7b, d). Thus, CP-mediated regulation of HBV virion production does not involve this N-glycosylation.

In the present study, CP could specifically interact with MHBs but not with LHBs and SHBs, although LHBs, MHBs and SHBs share the majority of their amino acid sequences. Previous reports have shown that different host factors are required during the folding of LHBs, MHBs and SHBs [15], suggesting that they may have different topological
structures. Our observation of the specific interaction between CP and MHBs further supports this hypothesis. Currently, it remains unclear how MHB is involved in envelopment and/or release of HBV virions. Based on our results, it is hypothesized that interactions between CP and MHBs may interfere with the participation of MHBs in envelopment and/or release of HBV virions.

In addition to being a component in virions, MHBs can assemble into SVPs together with SHBs and/or LHBs. It has been shown that SVP production is also sustained by MHBs [37]. SVPs may assemble in the ER–Golgi intermediate compartment and egress from cells through the constitutive secretion pathway. CP is also secreted extracellularly through the constitutive secretion pathway [22]. Therefore, it is reasonable to hypothesize that CP affects the secretion of SVPs due to their common secretion pathway. Consistent with this speculation, CP overexpression increased HBsAg levels in culture supernatants, while CP knockdown decreased HBsAg levels in culture supernatants when CP plasmid and HBV replication-competent clones were co-transfected. Considering that the overexpression or the knockdown of CP only affected the secretion of MHBs and not that of SHBs or LHBs (Fig. 7b, d), the increase in SVP secretion in the presence of CP potentially resulted from interactions formed between CP and MHBs.

Overall, we identified a novel cellular factor, CP, which regulates the secretion of HBV virions by targeting the transportation of MHBs. These findings have important implications for studies of HBV replication, pathogenesis and therapy.
Fig. 6. CP interacts with MHBs. (a) Huh7 cells were co-transfected with pFLAG-CP and pHA-L, pHA-M, pHA-S or pHA-core. The cells were immunostained with an anti-CP antibody and an anti-HA antibody 72 h p.t. The nuclei were stained with Hoechst 33258. Higher magnification images of the selected area are also shown. The intensity profile describes the distribution of the L/M/S/core- and CP-specific fluorescence along the indicated line. (b) Statistical analysis of the colocalization coefficient of CP and L/M/S/core. The colocalization coefficient is represented as percentage of signals of CP that were positive for L/M/S/core. Quantifications were performed using Velocity software. A two-tailed t-test was used to determine differences in multiple comparisons. *P<0.05; ***P<0.001. (c) 293T cells were transfected with the indicated plasmids. The cells were lysed and subjected to a co-IP assay at 48 h p.t. An anti-CP antibody was used for the co-IP, and mouse-derived IgG served as a control. The precipitates were analysed by Western blotting using anti-CP.
and anti-HA antibodies. Core proteins in the precipitates and whole-cell lysate (WCL) were detected using anti-core. (d) Principle of the BiFC system and schematic view of the fusion protein constructs. (e) Vero cells were co-transfected with MN-159-M and MC-160-CP, MN-159-M and MC-160-ALB (as positive control), MN-159-L and MC-160-ALB (as negative control), MN-159-LTag and MC-160-p53 (as positive control), MN-159-mLTag and MC-160-mp53 (as negative control), respectively. Cells then were transferred to 4°C for 1–2 h before imaging at 24 h p.t. The nuclei were stained with Hoechst 33258.

**METHODS**

**Cells**

Huh7, HepG2, HepG2.2.15, 293T and Vero cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% FBS (Life Technologies) and 100 U ml⁻¹ penicillin/streptomycin (Life Technologies). All cells were cultured under humidified conditions with 5% CO₂ at 37°C as described previously [38, 39].

**Construction of plasmids**

The coding sequence of CP (GenBank accession number NM_000096.3) was PCR-amplified with the following primer pair: sense, 5¢-CCGCTCGAGATGAAGATTTTGATAC-3¢ (XhoI); antisense, 5¢-AAACTGCAGTCAGCCAGATTTGGTG-3¢ (HinIII). The PCR fragments were inserted into pXJ40-HA and pXJ40-FLAG plasmids via the restriction sites XhoI/HindIII to generate pHA-CP and pFLAG-CP, respectively. The HA tag is a major sequential epitope of influenza haemagglutinin with the amino acid sequence MYPYDVP-DYA. The FLAG tag is an epitope of flagelliform silk protein with the amino acid sequence of DYKDDDDK. The HBV replication-competent clone pSM2, harbouring a head-to-tail tandem dimer of HBV genome (HBV genotype D, GenBank accession number V01460) and driven by its own HBV promoter to initiate the transcription of pre-genomic RNA (pgRNA), was used as described previously [40]. The HBV replication-competent clone N16, the M-minus mutant (XhoI); antisense, 5¢-AAACTGCAGTCAGCCAGATTTGGTG-3¢ (HindIII). The PCR fragments were inserted into pXJ40-HA and pXJ40-FLAG plasmids via the restriction sites XhoI/HindIII to generate pHA-CP and pFLAG-CP, respectively. The HA tag is a major sequential epitope of influenza haemagglutinin with the amino acid sequence MYPYDVP-DYA. The FLAG tag is an epitope of flagelliform silk protein with the amino acid sequence of DYKDDDDK. The HBV replication-competent clone pSM2, harbouring a head-to-tail tandem dimer of HBV genome (HBV genotype D, GenBank accession number V01460) and driven by its own HBV promoter to initiate the transcription of pre-genomic RNA (pgRNA), was used as described previously [40]. The HBV replication-competent clone N16, the M-minus mutant

![Fig. 7.](image-url) CP interacts with MHBs to impair the production of extracellular HBV virions. (a) Huh7 cells were transfected with the indicated plasmids and supernatants were collected 72 h p.t. N16, HBV replication-competent clone. 1.5×10⁶N16LS⁺M⁺S⁺, M-minus mutant HBV replication-competent clone. 0.7×10⁶N16LS⁻M⁺S⁺, M protein expression plasmid. HBV virions in supernatants were detected as described in Fig. 2. M and CP proteins in cell lysate were detected by Western blotting using anti-HBs and anti-CP antibodies. A two-tailed t-test was used to determine differences in multiple comparisons. *P<0.05; **P<0.01. (b, d) Huh7 cells were transfected with the indicated plasmids. Culture supernatants and cell lysates were collected and subjected to Western blot assay using anti-CP and anti-HA antibodies. The ratios of band intensity in the supernatants and cell lysates were calculated. The mean values from three independent experiments are shown.
1.5×N16L−M∗S∗and the M protein expression construct 0.7×N16L−M∗S∗ were kindly provided by Professor Dr Shuping Tong (Brown University, USA) [11]. An shCP oligo (AGC TTAAAAACCGAGAAAGTAAACAAAGATCCTTCTGAA ATCTTTGTTTTACTTTCTCGGGGG, a short hairpin RNA targeting the mRNA of CP to knockdown the expression of CP protein) and an shNC oligo (AGCTTTAACAAGC TAAAGTTAAGTCCTCCTCTTGAAGAGCG AGGGCAGCTTAACTTAGGGG, a control short hairpin RNA that has no targeting RNA) were inserted into a pSUPER.retro.neo vector (OligoEngine) via the restriction sites BglII/HindIII to generate pShCP and pShNC, respectively. To construct surface-protein expression plasmids, pUC19-HBV1.3-B was used as a template to amplify the ORFs of LHB, MHB and SHB. Then, the ORF of LHB was cloned into pcDNA3.1-HA to generate the LHB expression plasmid pHa-L, in which the ATG codon that initiated the translation of M and S was mutated to GCG (Ala). The ORF of MHB was cloned into pXJ40-HA to generate the MHB expression plasmid pHA-M, in which the ATG codon that initiated the translation of S was mutated to GCG (Ala). The ORF of SHB was cloned into pXJ40-HA to generate pHa-S. The ORF of HBV core was amplified from pHY106 and cloned into pXJ40-HA to generate pHa-core. The plasmids MN-159-LTag, MC-160-p53, MN-159-mLTag and MC-160-mp53 were kindly provided by Professor Dr Xian-En Zhang (Wuhan Institute of Virology, PR China). MN-159-M, MN-159-L, MC-160-ALB and MC-160-CP were constructed as described previously [28], using the restriction sites HindIII/Sall and HindIII/PstI, respectively.

Cell transfection, Western blotting and ELISA

Cells were seeded at approximately 30% confluence. Huh7 cells and HepG2 cells were transfected with Lipofectamine 2000 (Invitrogen) and Lipofectamine 3000 (Invitrogen), respectively, according to the manufacturer’s instructions. A Western blot assay was performed as described previously [41, 42]. The following antibodies were used: anti-CP (Santa Cruz), anti-HBs (Abcam), anti-preS1 (Santa Cruz), anti-HBc (Dako), anti-HA (Cell Signaling Technology) and anti-beta-actin (Santa Cruz). Relative band intensities for viral proteins were quantified using NIH ImageJ software. HBsAg and HBeAg in cell culture supernatants were detected by ELISA as described previously [41, 43, 44].

Immunofluorescence assay

Indirect immunofluorescence [45] staining of transfected cells was performed as described previously [39, 41, 43]. Anti-HA (Cell Signaling Technology) and anti-CP (Santa Cruz) were used as primary antibodies, while Alexa Fluor 488-conjugated antibodies and Alexa Fluor 568-conjugated antibodies (Life Technologies) were used as secondary antibodies. The nuclei were stained with Hoechst 33258 (Life Technologies).

BiFC assay

BiFC assay was performed as described previously [28]. Split mCherry is a new red bimolecular fluorescence complementation system for visualizing protein–protein interactions in living cells. The mCherry, a mutant monomeric red fluorescent protein, is split between aa 159 and 160 to be divided into two fragments, MN-159, for the amino-terminal half of mCherry, and MC-160, for the carboxy-terminal half of mCherry, in the context of plasmids. Only when MN-159 and MC-160 are brought together by interactions between proteins fused to each fragment, can whole mCherry be formed and red fluorescence could be observed. Therefore, BiFC assay can be used to visualize direct protein–protein interactions in living cells. Considering the strong interaction between SV40 large T antigen (LTag) and human p53 protein [46], LTag and p53 were fused with the amino-terminal half and the carboxy-terminal half of mCherry, respectively, to form the plasmids MN-159-LTag and MC-160-p53. The co-transfection of these two plasmids can produce red fluorescence and thus served as a positive control. In contrast, the co-transfection of MN-159-mLTag and MC-160-mp53 expressing mutant LTag (mLTag) and mutant p53 (mp53) could not produce red fluorescence and thus served as a negative control [46]. To study the interaction between CP and MHBs, the plasmid MN-159-CP was constructed by linking entire CP with the N-terminal 159 amino acids of mCherry at the carboxy terminal of CP. The plasmid MC-160-M was constructed by linking entire MHB with the C-terminal 160 amino acids of mCherry at the carboxy terminal of MHB. Then, Vero cells were co-transfected with the indicated plasmids and were incubated overnight at 37 °C. After that, the Vero cells were transferred to 4°C for 1–2 h before imaging. The nuclei were stained with Hoechst 33258.

Clinical samples and IHC

Liver tissue samples were obtained from ten patients with CHB virus infection (eight males and two females, with a mean age of 39.3 years and no history of antiviral drug therapy; the mean load of HBV DNA in serum is 6.6 log10 copies ml–1; alanine aminotransferase (ALT) levels: four cases were high (>40 U l–1), five cases were normal (≤40 U l–1), one case was unclear) and seven healthy individuals (two males and five females, with a mean age of 45 years; ALT levels of all cases were normal) with no history of viral hepatitis. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the First Affiliated Hospital, Anhui Medical University, in accordance with the guidelines for the protection of human subjects. Informed consent was obtained from each patient prior to inclusion in the study. Human liver tissue samples were subjected to IHC staining as described previously [42] and the intrahepatic CP was detected using anti-CP antibody (Abcam). The images were acquired and analysed by using a Pannoramic MIDI Digital Slide Scanner (3DHISTECH).

Southern blot and Northern blot assays

Huh7 or HepG2.2.15 cells were seeded in six-well plates and harvested at 72 h after transient transfection. Encapsidated HBV replication intermediates were extracted and subjected...
to Southern blot analysis as described previously [47]. Total cellular RNA was prepared using TRIzol reagent (Invitrogen). Total RNA (20 µg) was used for the Northern blot assay according to the manufacturer’s protocols (Ambion; Life Technologies). Hybridization signals were quantified with NIH ImageJ software.

**HBV DNA extraction and detection**

Supernatants from transfected cells were collected 72 h p.t. and cleared by centrifugation at 4°C and 400 g for 10 min. To detect HBV DNA in mature viral particles, 1 ml of supernatant was pre-incubated with mixed monoclonal anti-HB antibodies, including A11, BJ11 and S1 antibodies (2 µg of each antibody, kindly provided by Professor Bin Yan), overnight at 4°C. Then the supernatant was mixed with protein G-conjugated agarose beads (Millipore) for another 4 h. Bead–particle complexes were washed three times with IP buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM NaCl, 1% Triton X-100, and 150 mM NaCl) and resuspended with 200 µl PBS, followed by digestion with DNase I overnight at 37°C. Then, virion-associated DNA was extracted from the samples using a QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer’s instructions. Quantification of HBV DNA was performed as described previously [41]. The following real-time PCR primers were used: forward 5′-ACCATGCCAGTCCAAGAA-3′ and reverse 5′-ACCACAGGAAAAATCCAGGC-3′. To detect HBV DNA in naked nucleocapsids, 2 µl anti-HBc (Dako) was added to 1 ml of supernatant to precipitate naked nucleocapsids, followed by HBV DNA extraction and detection as described above.

**HBV capsid extraction and native agarose gel electrophoresis**

Huh7 cells were transfected with pSM2 in six-well plates and then harvested at 72 h with 200 µl specific lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 50 mM NaCl, 8% sucrose, and 0.25% NP-40) and incubated on ice for 10 min followed by centrifugation at 16 000 g and 4°C for 10 min. The supernatants were transferred into a new 1.5 ml EP (Eppendorf tube), and 6 µM MgCl2, 200 µg µl-1 DNAse I and 1.5 mg µl-1 RNase was added and incubated for 20 min at 37°C followed by centrifugation at 16 000 g and 4°C for 10 min. The supernatants were harvested and submitted to 1.6% native agarose gel electrophoresis for 2.5 h (45 V) at 4°C. A nitrocellulose membrane was used for capsid transfer via adsorption overnight at room temperature. The subsequent steps were the same as those used for the Western blot assay above, and the anti-core (Dako) antibody was used for capsid detection.

**Co-IP assay**

Transfected 293 T cells were lysed with IP buffer 48 h p.t. and subjected to a co-IP assay as described previously [39]. The following antibodies were used: anti-CP (Santa Cruz) and mouse IgG (Proteintech) as a control.

**Statistical analysis**

Statistical analysis was carried out using GraphPad Prism 5.0 software (GraphPad Software). The statistical significance of differences in multiple comparisons was determined using Student’s t-test. P<0.05 was considered statistically significant. The results are presented as the means±SD.

**Funding information**

This work was supported by grants from the National Basic Research Priorities Program of China (2012CB519001, 2013CB911100), the National Natural Science Foundation of China (8146113019, 31200269) and the Youth Innovation Promotion Association of the Chinese Academy of Sciences (201603).

**Acknowledgements**

We are grateful to Professor Shuping Tong for the plasmids N16, 1.5×N16L’M’S’ and 0.7×N16L’M’S’. We also thank Mr Ding Gao, Ms Anna Du and Ms Juan Min (The Core Facility and Technical Support, Wuhan Institute of Virology) for their excellent technical support.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

The study protocol of clinical samples used in this paper conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the First Affiliated Hospital, Anhui Medical University, in accordance with the guidelines for the protection of human subjects.

**References**


