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Proteasomes regulate hepatitis B virus replication by degradation of viral core-related proteins in a two-step manner

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Abstract The cellular proteasomes presumably inhibit the replication of hepatitis B virus (HBV) due to degradation of the viral core protein (HBcAg). Common proteasome inhibitors, however, either enhance or inhibit HBV replication. In this study, the exact degradation process of HBcAg and its influences on HBV replication were further studied using bioinformatic analysis, protease digestion assays of recombinant HBcAg, and proteasome inhibitor treatments of HBV-producing cell line HepG2.2.15. Besides HBcAg and hepatitis B e antigen precursor, common hepatitis B core-related antigens (HBcrAgs), the small and the large degradation intermediates of these HBcrAgs (HBcrDIs), were regularly found in cytosol of HepG2.2.15 cells. Further, the results of investigation reveal that the degradation process of cytosolic HBcrAgs in proteasomes consists of two steps: the limited proteolysis into HBcrDIs by the trypsin-like (TL) activity and the complete degradation of HBcrDIs by the chymotrypsin-like (chTL) activity. Concordantly, HBcrAgs and the large HBcrDI or HBcrDIs (including the small HBcrDI) were accumulated when the TL or chTL activity was inhibited, which generally correlated with enhancement and inhibition of HBV replication, respectively. The small HBcrDI inhibited HBV replication by assembling into the nucleocapsids and preventing the victim particles from being mature enough for envelopment. The two-step degradation manner may highlight some new anti-HBV strategies.

Keywords Hepatitis B virus · HBcrAgs · Viral replication · Protein degradation · Proteasomes

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Introduction

Hepatitis B virus (HBV) infection annually causes 1 million deaths worldwide [1, 2]. This misfortune will last for decades unless current antiviral options including recombinant interferon-α (IFN-α) and nucleotide/nucleoside analogs are improved or replaced by some radical drugs. The replication kinetics of HBV within hepatocytes has received much attention because it influences the innate immune responses that subsequently determine whether the HBV infection can be ultimately resolved [3–5], implying the importance to completely clarify the impact of intracellular factors on HBV replication.

HBV is an enveloped hepadnavirus. The viral core protein (HBcAg, 21 kDa) self-assembles and encapsidates pregenomic RNA (pgRNA) to form nucleocapsids in the cytosol. The envelopment is completed during penetration of the membrane of endoplasmic reticulum after reverse-transcription of the pgRNA [6]. HBcAg is a multifunctional protein involved in capsid assembly, pgRNA encapsidation, and nucleocapsid envelopment, which renders the downregulation of the intracellular HBcAg to lead to HBV replication inhibition [7, 8]. Cellular proteasomes

are responsible for the degradation of intracellular HBcAg [9]. Nuclear factor (NF)-κB and chaperone Hsp40 inhibit HBV replication via disassembling capsids and promoting the trafficking of HBcAg to proteasomes [7, 10]. These facts suggest that proteasomes play an inhibitory role in HBV replication by degradation of HBcAg, and that proteasome inhibition may lead to HBcAg accumulation and viral replication enhancement. Surprisingly, the common proteasome inhibitors MG132 and lactacystin accumulate some truncated HBcAg and do not significantly influence the replication of the wild-type viruses [9, 11], or another proteasome inhibitor, bortezomib, even inhibits HBV replication at a moderate dose in transgenic mice [12]. Since the major anti-HBV effect of recombinant IFN-α, one of the commonest antiviral therapy options in clinical practice [13], is to accelerate decay of viral nucleocapsids in a proteasome-dependent manner [14, 15], these above disharmonies let us become interested in understanding the exact influence of proteasomes on HBV replication.

HBcAg is encoded by the core gene within the C open reading frame of the viral genome. This frame also encodes a hepatitis B e antigen (HBeAg) precursor (pre-HBe, 22 kDa) that contains the entire amino acid sequence of HBcAg in C-terminus. The nascent peptide of pre-HBe is directed into the lumen of the endoplasmic reticulum by a signal peptide encoded within the precore region of the same frame. After the signal peptide is removed, pre-HBe is generated and transported to the trans-Golgi network for further processing by proprotein convertase furin, encoded by FES upstream region (Fur), to generate mature HBeAg (17–20 kDa) [16]. A portion of pre-HBe is retrotransported via endoplasmic reticulum-associated degradation pathway from the endoplasmic reticulum into cytosol where it is finally degraded in proteasomes [17]. Due to the contribution of HBeAg to chronic HBV infection, furin inhibitors reducing HBeAg secretion without affecting cellular albumin and prothrombin secretions are therapeutically promising for chronic hepatitis B [18]. Unfortunately, furin inhibitor I (decanoyl-RVKR-chloromethylketone), the most effective inhibitor of HBeAg secretion, has been found to accumulate full-length HBcAg and to enhance the replication of the HBeAg-defective variant (G1896A) [19].

Taken together with the induction of truncated HBcAg by common proteasome inhibitors [9], the above observation raises the possibility that HBcAg is partially proteolyzed into truncated HBcAg by furin or furin-like proteases prior to degradation in proteasomes. Our previous study, however, demonstrated that furin inhibitor I enhances HBV replication and accumulates full-length HBcAg and pre-HBe by inhibiting the trypsin-like (TL) activity of proteasomes, suggesting that cytosolic HBcAg and pre-HBe are directly degraded in proteasomes [20]. The different effects of common proteasome inhibitors and furin inhibitor I on HBcAg accumulation urge for further studies on the exact degradation process of HBcAg and pre-HBe in proteasomes. HBcAg, HBeAg, and pre-HBe are usually called Hepatitis B core-related antigens (HBcrAgs) because these antigens all originate from the C open reading frame of HBV and react with common commercial antibodies against HBcAg.

Proteasomes, large protein complexes inside all eukaryotes, are multicatalytic, displaying TL, chymotrypsin-like (chTL) and caspase-like (casp-L) activities. In this study, we demonstrated that the degradation process of cytosolic HBcrAgs (HBcAg and pre-HBe) in proteasomes involved two independent steps, which allow accumulating either full-length HBcrAgs or truncated HBcrAgs (hepatitis B core-related degradation intermediates, HBcrDIs) and provide a novel proteasomal mechanism to either enhance or inhibit HBV replication. These findings are likely to be important in the future study of the pathogenesis of chronic hepatitis B.

Materials and methods

Cell culture and protease inhibitor treatments

HepG2.2.15 is a cell line that carries two copies of HBV genome in a consecutive manner and can expresses all native viral antigens and complete virion of HBV. HepG2.2.15 cells were grown in Dulbecco's modified Eagle's medium supplemented with 380 g/mL of geneticin. Cells were treated with furin inhibitor I (EMD Biosciences, La Jolla, CA, USA) for 48 h, or MG132 (A.G. Scientific, San Diego, CA, USA) and bortezomib (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 h in a growth arrest medium containing 0.5 % (vol/vol) fetal calf serum after confluent growth. The cells were harvested to evaluate HBV replication and HBcrAg expressions. To differentiate the transcriptional from the post-transcriptional accumulation of HBcrAgs, cells were cotreated with or without cycloheximide, a protein synthesis inhibitor (Sigma-Aldrich Corporation, St. Louis, MO, USA).

Detections of intracellular or nucleocapsid HBcrAgs

For the detections of intercellular HBcrAgs, total cytosolic and non-cytosolic cellular proteins were separately extracted as reported [17]. Capsid HBcrAgs were detected after capsids with and without envelopes were isolated [7]. The capsids and sorted cellular proteins were separated and transferred onto polyvinylidene fluoride membranes (Millipore Corporation, Billerica, MA, USA) using standard techniques. Immunoblot analysis was performed using polyclonal antibodies to HBcAg (DAKO, Carpinteria, CA, USA).

Detections of core-associated HBV DNA

The isolation of intracellular core particles was performed as reported [7]. Because the low expression of HBcrDI-1 and HBcrDI-2 in HepG2.2.15 cells, Western blot often requires excessive exposure to detect clear signal. The caspid of HBV was extracted based on the reference. In brief, after cells grew to a confluence of 10^6 cells/6-well, cells were cultured for further 48 h with 0.5 % low serum medium. Cells were then lysed, and the supernatants were collected after centrifugation. DNase and RNase were added and stored at 37 °C for 1 h. The polyethylene glycol-800 was added to the mixture and stored at 4 °C for 1 h. After centrifugation at 4 °C for 1 h, the supernatant was discarded, and 50 μL 10 mM Tris–HCl was added for the storage of caspid at −80 °C. The core-associated HBV DNA was detected by Southern blot analysis. Sampling was balanced based on the protein level in initial cell lysate. The core-associated HBV DNA was detected used Southern blot analysis. The isolated DNA was separated and transferred onto nylon membranes (Roche Applied Science, Indianapolis, IN, USA). After hybridized with digoxigenin-labeled DNA probes, all membranes were incubated with horseradish peroxidase-labeled anti-digoxigenin antibody (Roche Applied Science), and developed with an enhanced chemiluminescence reagent (Invitrogen Corporation, Shanghai, China).

Protease digestion assay

The chymotrypsin (Sigma-Aldrich Corporation) digestion of recombinant HBc, a fragment of 156 amino acids (aa 1-156 of HBcAg, the homologous region of HBcAg and pre-HBe) with a 6× histidine (His)-tag in the C-terminus (Millipore Corporation), was performed with 0.1 mol/L Tris–HCl (pH 7.8), 10 mmol/L $CaCl_2$, and 4 μg/mL recombinant HBc (30 °C for 30 min) [21]. The trypsin and chymotrypsin codigestion was performed within the chymotrypsin system, but trypsin was added 10 min before chymotrypsin to avoid the possible influence resulting from chymotrypsin proteolyzing trypsin. The possibility of trypsin proteolyzing chymotrypsin was dismissed because chymotrypsin itself is a trypsin-proteolyzed fragment. Trypsin predominantly cleaves proteins at the carboxyl side of the amino acids lysine and arginine. Chymotrypsin prefers large hydrophobic residues.

Proteasome activity assay

The TL and chTL activities of the proteasomes were measured using commercial cell-based kits (Proteasome-Glo™, Promega, Madison, WI, USA) as described previously [22]. Luminescence was recorded using a luminometer (Promega).

Results

HBcrDIs exist in cytosol of HepG2.2.15 cells

HBcrAgs all react with the commercial antibodies against HBcAg (anti-HBc). When Western blot analysis employed such antibodies, two regular HBcrAgs (HBcAg and pre-HBe) and two unidentified HBcrAgs of 19 and 17 kDa were demonstrated in cytosol of HepG2.2.15 cells (Fig. 1a). The origins of these unidentified HBcrAgs were unclear. However, the different band patterns between the cytosolic and non-cytosolic samples dismissed the possibility of contamination from HBeAg in non-cytosolic compartments (Fig. 1a). Furin inhibitor I has been found to accumulate cytosolic HBcrAgs by inhibiting the TL activity of proteasomes [20]. Here, furin inhibitor I was found to accumulate the unidentified HBcrAg of 19 kDa and to reduce the unidentified HBcrAg of 17 kDa (Fig. 1b), suggesting that the unidentified HBcrAgs were the HBcrDIs of cytosolic HBcrAgs in proteasomes. For convenience, those intermediates were referred to as HBcrDI-1 (19 kDa) and HBcrDI-2 (17 kDa).

Proteasomes may degrade cytosolic HBcrAgs in a two-step manner

Cytosolic HBcrAgs mainly consist of HBcAg and pre-HBe (Fig. 1). Like HBcAg, pre-HBe is assembly-competent [23]. Thus, it exists as a dimer or a capsid in cytosol, which means that cytosolic HBcrAgs like recombinant HBcAg in vitro only let the C-terminus (39 amino acid residues) be protease-sensitive [21]. Based on analysis using the method of bioinformatics (http://web.expasy.org/peptide_cutter/), there are multiple putative cleaving sites of trypsin, but there is a lack of putative cleaving sites of chymotrypsin in the protease-sensitive C-terminus of these antigens (Fig. 2a). Since the chTL activity is responsible for the major degradation of proteasomes [24], trypsin only limitedly digested recombinant HBcAg in vitro [21], and HBcrAg degradations were blocked by inhibiting the TL activity alone [20], suggesting that cytosolic HBcrAgs are degraded in proteasomes in two steps: the limited proteolysis into HBcrDIs by the TL activity and the complete degradation of HBcrDIs by the chTL activity. This degradation manner requires that the chTL activity cannot directly degrade HBcrAgs. Indeed, chymotrypsin did not proteolyze native recombinant HBcAg in concentrations up to 10 mg/L (30 times higher than recommended) as illustrated in (Fig. 2b, top). HBcAg without C-terminus is unstable [23], suggesting that HBcrDIs are sensitive to the chTL activity. Although chymotrypsin did not digest the native trypsin-proteolyzed fragment (Fig. 2c, top), the chTL activity of proteasomes could still be effective

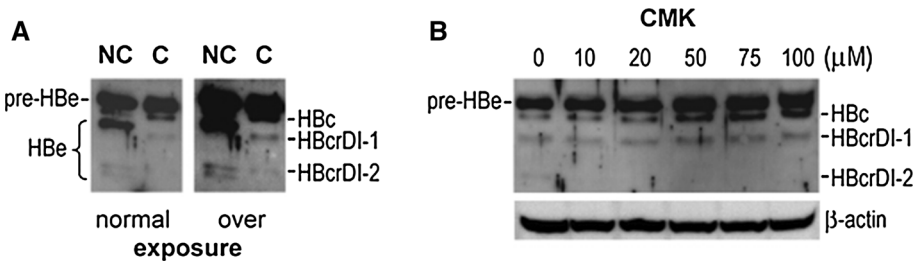

Fig. 1 HBcrDIs in HepG2.2.15 cells. HBcrAgs (HBcAg and pre-HBe), HBeAg (HBe), and HBcrDIs (HBcrDI-1 and HBcrDI-2) were detected using Western blot analysis. **a** HBcrDI-1 and HBcrDI-2 were found in cytosol (C), irrelevant to HBcrAgs in non-cytosol (NC). **b** Furin inhibitor I (CMK) accumulated HBcrAgs and HBcrDI-1, and reduced HBcrDI-2 in cytosol of HepG2.2.15 cells

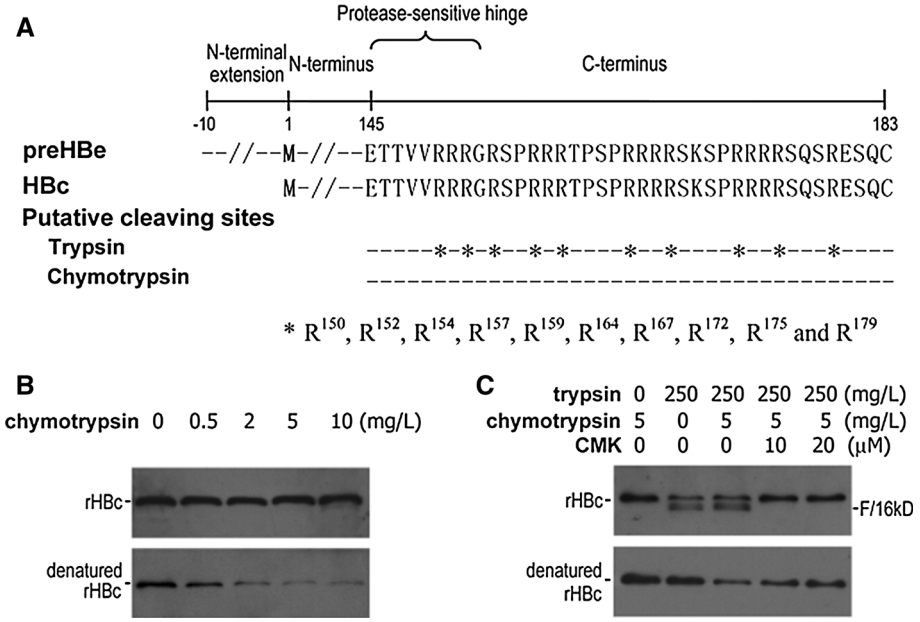

Fig. 2 Chymotrypsin did not digest native recombinant HBc. Putative cleaving sites of trypsin and chymotrypsin in the C-terminus (39 amino acid residues) of HBcrAgs (HBcAg and pre-HBe) were predicted using the method of bioinformatics (http://web.expasy.org/peptide_cutter/). Native or denatured (at 100 °C for 10 min) recombinant HBc with the 6× His-tag in the C-terminus (rHBc) was digested by trypsin and/or chymotrypsin, and the products were demonstrated using Western blot analysis. **a** Putative cleaving sites of trypsin and chymotrypsin in the C-terminus of HBcrAgs. **b** Chymotrypsin only digested the denatured rHBc. **c** Chymotrypsin did not digest the trypsin-fragment (F/16kD), but codigested the denatured rHBc with trypsin more efficiently

because HBcrDIs could be destabilized by some cellular mechanisms, such as NF-κB and Hsp40 [7, 10]. This notion is further supported because chymotrypsin digested the denatured recombinant HBc (Fig. 2b, bottom), and trypsin and chymotrypsin codigestion of the denatured recombinant HBcAg was more efficient (Fig. 2c, bottom).

Inhibition of the chTL activity or the TL or/and chTL activities of proteasomes was responsible for accumulation of HBcrDIs or HBcrAgs, respectively

Until now, all commercial proteasome inhibitors preferably inhibited the chTL activity, especially at lower concentrations [22, 24]. Therefore, the two-step degradation manner would be confirmed in cell-based testing if proteasome inhibitors accumulate HBcrDIs at low concentrations and HBcrAgs at high concentrations. Since proteasome inhibitors lead to cell apoptosis [25, 26], the concentrations of MG132 and bortezomib, ≤50 μmol/L and ≤200 nmol/L, respectively, were selected based on a cut-off value of 90 % of cell viability. MG132 inhibited the chTL activity and slightly accumulated HBcrDI-1 at lower concentrations (≤15 μmol/L) and inhibited the chTL and TL activities and accumulated cytosolic HBcrAgs (pre-HBe was less remarkable than HBcAg perhaps because its base level was too high) at higher concentrations (25 and 50 μmol/L) (Fig. 3a, c). Unexpectedly, MG132 also increased the level of cytosolic HBcAg at lower concentrations (5 and 10 μmol/L). It was noticeable that only 15 μmol/L MG132

did not increase the level of HBcAg (Fig. 3c). Bortezomib preferably inhibited the chTL activity at all interesting concentrations (Fig. 3b). It significantly accumulated HBcrDI-1 at lower concentrations (≤50 nmol/L) (Fig. 3d, bottom) and slightly increased HBcAg at 10 nmol/L (Fig. 3d, top), but all of those antigens were decreased at higher concentration (200 nmol/L) (Fig. 3d, top). Furthermore, the observed HBcAg accumulation of MG132 at high concentrations was post-transcriptional (proteasomal degradation-related) as expected, but that at low concentrations was transcriptional (proteasomal degradation-unrelated) (Fig. 3e). Therefore, these above findings, together with the HBcrAg accumulation effect of furin inhibitor I (Fig. 1b), make the two-step degradation manner of HBcrAgs in proteasomes believable since HBcrDIs or HBcrAgs had tendency to be accumulated when the chTL activity only or the TL or/and chTL activities of proteasomes were inhibited, respectively. In addition, the lack of

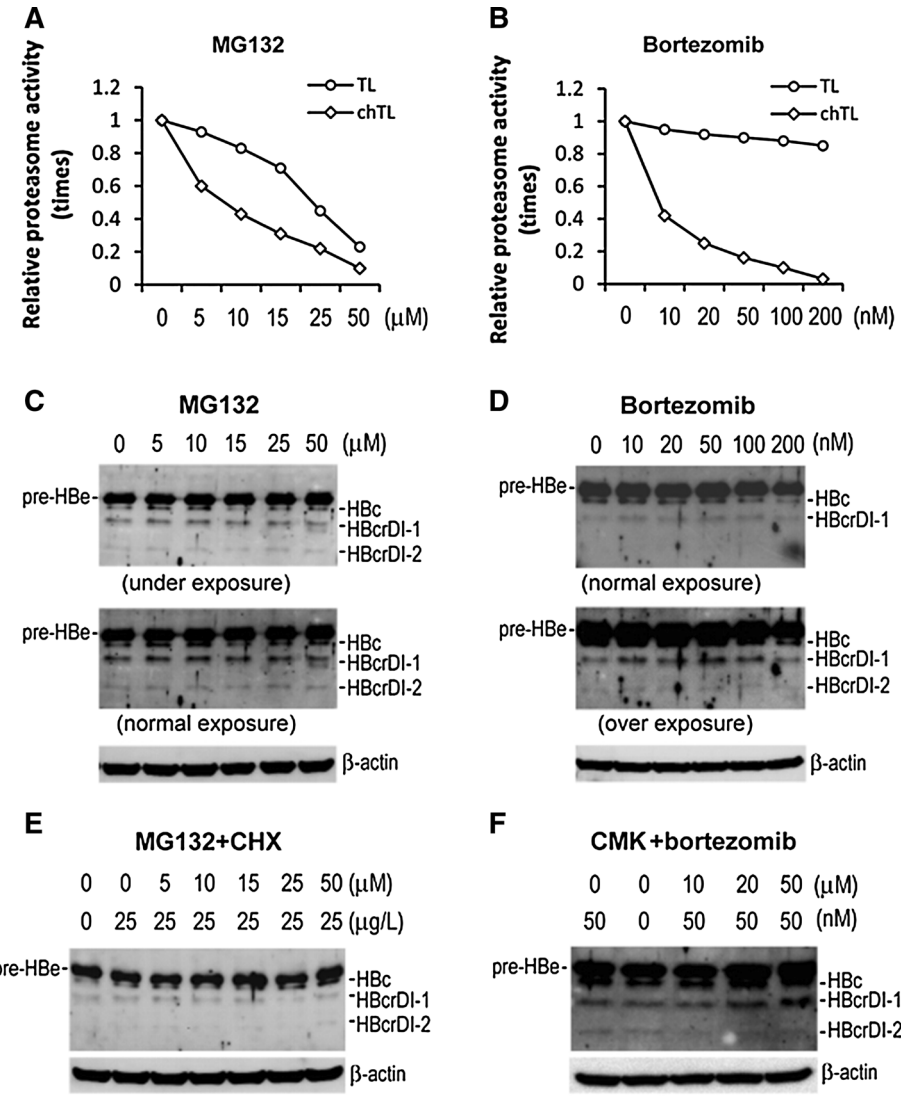

Fig. 3 Proteasome inhibition and the degradation of cytosolic HBcrAgs in HepG2.2.15 cells. Cytosolic HBcrAgs (HBc and pre-HBe) and their HBcrDIs (HBcrDI-1 and HBcrDI-2) were determined using Western blot analysis. Proteasome activities were demonstrated using a commercial system, and results were expressed as times of the protease activities of the treatment groups over those of the control group. **a** MG132 preferably inhibited the chTL activity at lower concentrations and the chTL and TL activities at high concentrations. **b** Bortezomib had similar effects as MG132 except that of no inhibition of the TL activity at high concentrations. **c** MG132 slightly accumulated HBcrDI-1 at low and moderate concentrations (15 μmol/L), and significantly accumulated HBc (*bottom*) and slightly accumulated pre-HBe (*top*) at low (5 and 10 μmol/L) and high (25 and 50 μmol/L) concentrations. **d** Bortezomib significantly accumulated HBcrDI-1 at low concentrations (50 nmol/L) and slightly accumulated HBcrAgs at concentration of 10 nmol/L. Both HBcrAgs and HBcrDI-1 decreased at high concentration (200 nmol/L). **e** HBcrAg accumulations disappeared at low concentrations of MG132, but the accumulations at high concentrations still existed when new protein biosynthesis was abrogated by cycloheximide (CHX). **f** Furin inhibitor I (CMK) significantly minimized the effect of bortezomib on HBcrDI-2

HBcrAg accumulations at high concentrations supports the two-step degradation manner from the reverse side because bortezomib rarely affected the TL activity (Fig. 3b). Compared with HBcrDI-1, HBcrDI-2 did not regularly change as the doses of these inhibitors (Fig. 3c, d). However, furin inhibitor I significantly minimized the effect of bortezomib on HBcrDI-2 (Fig. 3f), suggesting that the degradation process of HBcrAgs in proteasomes indeed needs the prerequisite step dependent on the TL activity.

HBcrAg degradation bidirectionally influenced HBV replication

To address the effects of the degradation process of cytosolic HBcrAgs on HBV replication, the relationship between proteasome inhibitors and HBV replication was studied. MG132 affected HBV replication bidirectionally, with inhibition at a moderate concentration (15 μmol/L) and enhancement at low (10 μmol/L) and high (25 μmol/L) concentrations (Fig. 4a, top). Likewise, bortezomib inhibited HBV replication at 20 and 50 nmol/L and enhanced HBV replication at the concentrations of 10 and 200 nmol/L (Fig. 4a, bottom). In general, HBV replication enhancement correlated with the accumulations of HBcrAgs (especially, HBcAg) except for bortezomib at higher concentration, and HBV replication inhibition correlated with the accumulation of HBcrDIs and the lack of increase in HBcAg (Figs. 1b, and 3c, d).

HBcrDIs interfered with HBV maturation

The protein composition of the nucleocapsids in the cytosol mainly consisted of HBcAg and HBcrDI-2 (Fig. 4b, top). When the nucleocapsids in cytosol and non-cytosol were compared, bortezomib with accumulation of HBcrDI-2 at low concentrations primarily increased the level of nucleocapsids in cytosol (Fig. 4b, top), and without accumulation of HBcrDI-2 at high concentrations significantly increased the level of nucleocapsids in non-cytosol (Fig. 4b, bottom). HBcrDI-2 in nucleocapsids appeared to prevent the victim particles from being mature enough to penetrate the endoplasmic reticulum membrane for envelopment.

Discussion

Proteasomes represent the major pathway of non-lysosomal proteolysis of intracellular proteins. Their involvement in the degradation of cytosolic HBcrAgs has been confirmed by many published researches [7, 9, 15, 17]. Our previous study based on furin inhibitor I ascertained that cytosolic HBcrAgs were directly degraded in proteasomes [20]. In the current study, cytosolic HBcrAgs were found to be degraded via a two-step degradation manner in proteasomes. This degradation manner allowed HBcAg or/and pre-HBe to generate HBV replication-inhibitory degradation intermediate when the chTL activity was more profoundly inhibited than the TL activity of proteasomes. In contrast, it would lead to HBcrAgs accumulation and HBV replication enhancement if the TL activity was profoundly suppressed. Since the anti-HBV effect of IFN-α depends on proteasome activities to degrade viral nucleocapsids [14, 15], upregulation of the TL activity combined with selective inhibition of the chTL activity by inhibitors developed based on HBV replication-inhibitory effect may be strategy to improve the antiviral response of recombinant IFN-α in the future. Moreover, the two-step degradation manner also

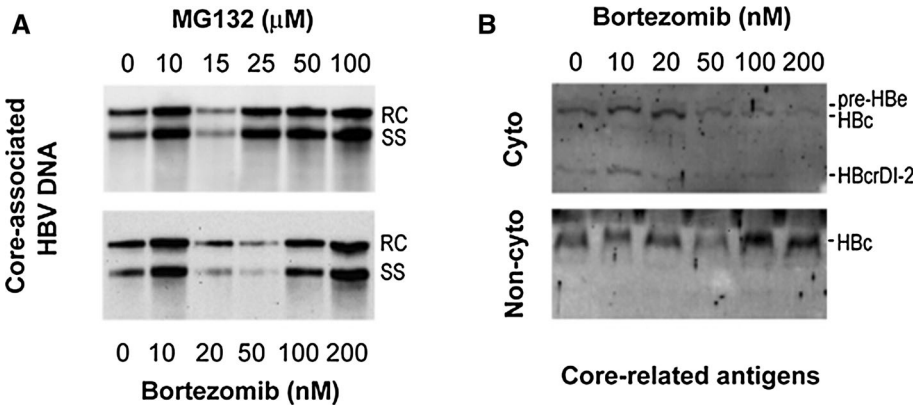

Fig. 4 HBcrDIs and HBV replication in HepG2.2.15 cells. The intracellular core-associated HBV DNA was demonstrated using Southern blot assay. Relaxed circular (RC) HBV DNA was the HBV genome, and single-stranded (SS) HBV DNA was the intermediates of HBV replication. Capsid-related HBcrAgs (HBc and pre-HBe) and their HBcrDIs were detected using Western blot analysis. **a** Both MG132 and bortezomib enhanced HBV replication at low and high concentrations, but inhibited replication at moderate concentrations. **b** Capsids mainly consisted of HBc and HBcrDI2. Bortezomib at low concentrations preferably increased capsids in cytosol (cyto), but at high concentrations increased capsids in non-cytosol (non-cyto)

provides a novel cellular mechanism to either inhibit or enhance HBV replication through accumulation of HBcrDIs or HBcrAgs by inhibiting the chTL activity or the TL activity, respectively. Since the activities of proteasomes in liver decline with patient age, with the chTL activity declining faster than the TL activity [27, 28], the two-step degradation manner may correlate with the formation of inactive hepatitis B surface antigen carrier state and reactivation of the HBV infection (HBeAg-negative chronic hepatitis B) by accumulating HBcrDIs at first and HBcrAgs at last, respectively.

HBcrAgs exist as either capsids or nucleocapsids in cells. Hsp40 protein (hTid1) and the activation of NF-κB accelerate HBcAg degradation by the destabilization of capsids [7, 10], suggesting that HBcrAgs degrade in proteasomes in dimers. It may be the assembly-competence of N-terminus of HBcrAgs that only renders the C-terminus to be sensitive to some proteases [21]. Proteasomes have three major protease activities: the TL, chTL, and casp-L activities. HBcAg in literature and HBcrAgs in this study were accumulated by inhibiting the TL activity alone, there was no putative cleaving sites of chymotrypsin in protease-sensitive C-terminus, and recombinant HBcAg was not proteolyzed by chymotrypsin in vitro, suggesting that only the TL activity of proteasomes can initiate the degradation process. However, recombinant HBcAg was limitedly proteolyzed by trypsin. Thus, the above findings further imply that the degradation process of HBcrAgs consists of two steps: the limited proteolysis into HBcrDIs by the TL activity and the complete degradation of HBcrDIs by the chTL activity. Indeed, it is the chTL activity that degrades most cellular proteins [24], and HBcrDIs unlike HBcrAgs may be sensitive to the chTL activity because the dimers without C-terminus are instable [23]. Until now, commercial proteasome inhibitors all preferentially inhibit the chTL activity of proteasomes. Thus, the preference of these inhibitors and the two-step manner explain why common proteasome inhibitors at some concentrations accumulate truncated HBcAg or remain the intracellular HBcAg level unaffected [9, 10].

The C-terminus of HBcrAgs has four arginine-rich domains (ARDs), ARD1 (^{150}RRRGR^{154}), ARD2 (^{157}RRR^{159}), ARD3 (^{164}RRRR^{167}), and ARD4 (^{172}RRRR^{175}), according to HBV of genotype B (GenBank accession no. D00630.1). These ARDs may be sensitive to the TL activity of proteasomes; however, only two HBcrDIs, HBcrDI-1 and HBcrDI-2, were regularly found in HepG2.2.15 cells. HBcrDI-1 increased and HBcrDI-2 decreased when the TL activity was selectively inhibited by furin inhibitor I, suggesting that HBcrDI-1 was an intermediate product and HBcrDI-2 was the end product of the TL activity of proteasomes. Thus, these HBcrDIs were derived from the same HBcrAg, possibly pre-HBe due to its higher level in cytosol. Based on the molecular weight, the cleaving sites were at R^{154} and R^{164} of pre-HBe. Theoretically, the HBcrDIs from HBcAg also exist. Since HBcrDI-1 is relatively sensitive to the changes of the activities of proteasomes, the preferable cleavage site of the TL activity may be R^{164}. In this regard, the band of HBcrDI-2 could also have included HBcAg-related HBcrDI-2 because its molecular weight is similar to that of pre-HBe-related HBcrDI-2 (R^{154}) if HBcAg is cleaved at R^{164}. Since pre-HBe-related HBcrDI-2 decreases and HBcAg-related HBcrDI-2 increases if the TL activity is gradually inhibited by inhibitors, the change of HBcrDI-2 with the activities of proteasomes being not as regular as HBcrDI-1 supports the heterologous nature of HBcrDI-2. Indeed, the lack of pre-HBe and HBcrDI-1 in capsids indicated that the HBcrDI-2 in capsids comes from HBcAg. In addition, R^{164} (ARD3) as the preferable cleavage site of the TL activity is inconsistent with the hot spot of trypsin-cleaving site (R^{150} in ADR1) in the protease-sensitive hinge of recombinant HBcAg [21]. The possible explanation for this is that R^{164} may topographically be more accessible because it is farther away from the assembly-competent domain. Trypsin, a small protease, may freely access those sites (R^{150} and R^{164}). In contrast, proteasomes, which are large protein complexes, may only be able to access R^{164}.

The influences of proteasome activities on HBV replication have not completely been clarified. In this study, HBV replication was bidirectionally influenced by proteasome inhibitors. Both MG132 and bortezomib enhanced HBV replication at low concentrations, inhibited at moderate concentrations, and enhanced replication again at high concentrations. These results are in concordance with the notion that bortezomib at moderate doses inhibited HBV replication in transgenic mice [12]. Although proteasome inhibitors enhancing or inhibiting HBV replication generally correlated with the accumulations of HBcrAgs or HBcrDIs, HBcrAg degradation manner was not the only mechanism involved. Enhanced HBV replication at low concentrations correlated with the transcriptional accumulation of HBcrAgs, possibly due to the transactivation of hepatitis Bx (HBx) protein because proteasomes are also responsible for HBx protein degradation and proteasome inhibitors accumulate HBx protein more sensitively than do HBcAg [10]. Perhaps because the transactivation of HBx protein itself depends on proteasome activities [29], the transcriptional activation disappeared at higher concentrations of these inhibitors. Bortezomib, at high concentrations, enhanced HBV replication without accumulating HBcrAgs. This could potentially be attributable to improved capsid integrity. The activation of NF-κB leads to a disruption in capsid integrity, and bortezomib reportedly inhibits the activation of NF-κB [7, 30]. Thus, the possible mechanisms by which proteasome inhibitors inhibit HBV

replication at moderate concentrations correlate with the accumulation of HBcrDIs, the decline in transactivation of the HBx protein and the lack of NF-κB activation blockage. Among these mechanisms, only HBcrDIs are really HBV replication–inhibitory.

Pre-HBe in the cytosol is assembly-competent. It cross-assembles with HBcAg to inhibit HBV replication by forming pgRNA encapsidation-incompetent capsids [23]. In this study, however, pre-HBe accumulation correlating with HBV replication enhancement and the lack of pre-HBe in capsids suggest that the effect of cytosolic pre-HBe is limited in HepG2.2.15 cells. Similarly, the lack of HBcrDI-1 in capsids suggests that pre-HBe-related HBcrDIs have limited significance to HBV replication. These data suggest that pre-HBe and its HBcrDIs back from proteasomes have been modified, perhaps in the extensional region of N-terminus. Therefore, the influences of the degradation manner of HBcrAgs on HBV replication are mediated by HBcAg and its related HBcrDIs. It is well known that cellular HBcAg accumulation leads to HBV replication enhancement [7, 8, 19]. HBcAg-related HBcrDIs without the C-terminus is assembly-competent, but pgRNA encapsidation–incompetent [23]. This implies that HBcAg-related HBcrDIs inhibit HBV replication by interfering with the pgRNA encapsidation. However, HBcAg-related HBcrDIs were found to affect the maturation of the capsids that have already packed pgRNA. This finding seems to be in concordance with that amino acid residues 165–175 of HBcAg (ARD3 and ARD4) play key roles in HBV replication and the deletion of C-terminal 19 amino acid residues leads to abnormal encapsidation, packaging spliced viral genomes, but lack full-size DNA [31, 32]. The possible explanation is that HBcAg-related HBcrDIs usually exist in hybrid capsids with normal HBcAg since the capsids assembled by HBcAg without C-terminus are instable, but the hybrid capsids with normal HBcAg are stable enough [23]. The hybrid capsids with limited HBcAg-related HBcrDIs may be pgRNA encapsidation-competent, but reverse transcription- or nucleocapsid envelopment-incompetent.

Conclusions

The degradation process of HBcrAgs in proteasomes consists of two independent steps: the limited proteolysis into HBcrDIs by the TL activity and the complete degradation of HBcrDIs by the chTL activity. By providing a novel cellular mechanism to either inhibit or enhance HBV replication, the two-step degradation manner may be involved in the mechanism of inactivation and reactivation of HBV during the natural history of infection. More studies on the biological functions of HBcrDIs and the relationships between proteasome activities and the antiviral responses of recombinant IFN-α or different disease states of HBV infection are warranted in the future.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

References

1. D. Ganem, A.M. Prince, N. Engl, J. Med. **350**, 1118–1129 (2004)
2. Y.F. Liaw, Liver Int. **29**(Suppl 1), 100–107 (2009)
3. M. Ait-Goughoulte, J. Lucifora, F. Zoulim, D. Durantel, Viruses **2**, 1394–1410 (2010)
4. G.J. Webster, S. Reignat, M.K. Maini, S.A. Whalley, G.S. Ogg, A. King, D. Brown, P.L. Amlot, R. Williams, D. Vergani, G.M. Dusheiko, A. Bertoletti, Hepatology **32**, 1117–1124 (2000)
5. A. Bertoletti, A.J. Gehring, J. Gen. Virol. **87**, 1439–1449 (2006)
6. J. Beck, M. Nassal, World J. Gastroenterol. **13**, 48–64 (2007)
7. M. Biermer, R. Puro, R.J. Schneider, J. Virol. **77**, 4033–4042 (2003)
8. Y.C. Chou, M.L. Chen, C.P. Hu, Y.L. Chen, C.L. Chong, Y.L. Tsai, T.L. Liu, K.S. Jeng, C. Chang, Hepatology **46**, 672–681 (2007)
9. S. Braun, A. Zajakina, J. Aleksejeva, A. Sharipo, R. Bruvere, V. Ose, P. Pumpens, H. Garoff, H. Meisel, T. Kozlovska, J. Med. Virol. **79**, 1312–1321 (2007)
10. S.Y. Sohn, S.B. Kim, J. Kim, B.Y. Ahn, J. Gen. Virol. **87**, 1883–1891 (2006)
11. Z. Zhang, E. Sun, J.H. Ou, T.J. Liang, J. Virol. **84**, 9326–9331 (2010)
12. P. Bandi, M.L. Garcia, C.J. Booth, F.V. Chisari, M.D. Robek, Agents Chemother. **54**, 749–756 (2010)
13. Y. Zheng, L. Zhao, T. Wu, S. Guo, Y. Chen, T. Zhou, Virol. J **6**, 99 (2009)
14. C. Xu, H. Guo, X.B. Pan, R. Mao, W. Yu, X. Xu, L. Wei, J. Chang, T.M. Block, J.T. Guo, J. Virol. **84**, 9332–9340 (2010)
15. M.D. Robek, S.F. Wieland, F.V. Chisari, J. Virol. **76**, 3570–3574 (2002)
16. K. Ito, K.H. Kim, A.S. Lok, S. Tong, J. Virol. **83**, 3507–3517 (2009)
17. M. Duriez, J.M. Rossignol, D. Sitterlin, J. Biol. Chem. **283**, 32352–32360 (2008)
18. Y.J. Pang, X.J. Tan, D.M. Li, Z.H. Zheng, R.X. Lei, X.M. Peng, Liver Int. **33**, 1230–1238 (2013)

19. M. Sugiyama, Y. Tanaka, F. Kurbanov, N. Nakayama, S. Mochida, M. Mizokami, Virology **365**, 285–291 (2007)
20. H.Y. Yang, N.Q. Zheng, D.M. Li, L. Gu, X.M. Peng, Virol. J **11**, 165 (2014)
21. M. Seifer, D.N. Standring, J. Virol. **68**, 5548–5555 (1994)
22. R.A. Moravec, M.A. O'Brien, W.J. Daily, M.A. Scurria, L. Bernad, T.L. Riss, Anal. Biochem. **387**, 294–302 (2009)
23. P.P. Scaglioni, M. Melegari, J.R. Wands, J. Virol. **71**, 345–353 (1997)
24. T. Jung, B. Catalgol, T. Grune, Mol. Aspects Med. **30**, 191–296 (2009)
25. I.M.S. Seda, J.L. Mott, Y. Akazawa, F.J. Barreyro, S.F. Bronk, S.H. Kaufmann, G.J. Gores, Hepatol. Res **40**, 701–710 (2010)
26. B. Pandit, A.L. Gartel, Am. J. Pathol. **178**, 355–360 (2011)
27. K.A. Rodriguez, M. Gaczynska, P.A. Osmulski, Mech. Ageing Dev. **131**, 144–155 (2010)
28. T. Hayashi, S. Goto, Mech. Ageing Dev. **102**, 55–66 (1998)
29. Z. Hu, Z. Zhang, E. Doo, O. Coux, A.L. Goldberg, T.J. Liang, J. Virol. **73**, 7231–7240 (1999)
30. J.B. Sunwoo, Z. Chen, G. Dong, N. Yeh, C.C. Bancroft, E. Sausville, J. Adams, P. Elliott, C. Van Waes, Clin. Cancer Res **7**, 1419–1428 (2001)
31. J. Jung, H.Y. Kim, T. Kim, B.H. Shin, G.S. Park, S. Park, Y.J. Chwae, H.J. Shin, K. Kim, PLoS One **7**, e41087 (2012)
32. J. Köck, M. Nassal, K. Deres, H.E. Blum, F. von Weizsäcker, J. Virol. **78**, 13812–13818 (2004)