The Journal of Microbiology, December 2009, p. 746-752
DOI 10.1007/s12275-009-0272-x

Vol. 47, No. 6

Hepatitis B Virus Core Interacts with the Host Cell Nucleolar Protein, Nucleophosmin 1

Su Jin Lee, Hee Youn Shim, Antony Hsieh, Ji Young Min, and Gu hung Jung*

School of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea

(Received September 7, 2009 / Accepted October 7, 2009)

Hepatitis B virus (HBV) genome replication requires the packaging of viral factors (pregenomic RNA and polymerase) as well as host factors, including heat shock proteins and protein kinase C. Previous reports have suggested that there are several unidentified host factors that affect this encapsidation step. In this study, we identified a new host factor, nucleophosmin (B23) that interacts with the HBV core protein 149 (Cp149). We analyzed this factor using NHS-activated sepharose resin and MALDI-TOF MS. Using the BIAcore analysis system, we were also able to deduce that the B23.1 residues 259-294 were required for the interaction between Cp149 and B23.1 *in vitro*.

***Keywords*:** hepatitis B virus, core, capsid assembly, host factor, B23, nucleophosmin

The human hepatitis B virus (HBV) has infected more than 2 billion people and over 350 million people are chronically infected worldwide (Vanlandschoot *et al*., 2003; Wright, 2006; Seeger *et al*., 2007). HBV causes acute and chronic B type hepatitis in humans, and chronic HBV infection is associated with the development of hepatocellular carcinoma (HCC) and liver cirrhosis (Kang *et al.*, 2006). HBV is a member of the Hepadnaviridae family (Seeger *et al*., 2007), and has a partially double-stranded 3.2-kb DNA genome that is converted into covalently closed circular DNA in the nucleus (Seeger *et al*., 2007). HBV replicates through reverse transcription of the pregenomic RNA to make minus strand DNA, which encodes 4 viral proteins: the core protein (Cp), the surface protein (S), polymerase (P), and the X protein (Nassal and Schaller, 1996; Kang *et al.*, 2006; Seeger *et al.*, 2007). HBV Cp, depending on its subtype, is 183 or 185 amino acids long, consisting of an N-terminal assembly domain (amino acids 1-149; Cp149) and a C-terminal protamine domain (amino acids 150-183 or 185; Cp183) known to regulate viral replication (Zlotnick *et al.*, 1996; Kang *et al.*, 2006).

Nucleophosmin (B23) is a multi-functional phosphoprotein that exists in at least 2 isoforms in human cells; nucleophosmin 1 (residues 1-294; B23.1) and nucleophosmin 2 (residues 1-259; B23.2) (Okuwaki *et al.*, 2002). B23 plays a role in ribosome biogenesis, chaperon activity, nucleic acid binding activity, and ribonuclease activity (Wang *et al.*, 1994; Hingorani *et al*., 2000; Okuwaki *et al.*, 2002). B23 is overexpressed or mutated in human cancer cells, and is therefore a candidate prognostic marker in gastric, colon, ovarian, and prostate carcinomas (Hiscox, 2002; Grisendi *et al*., 2006). Interestingly, B23 is known to bind to several viral proteins, including the human immunodeficiency virus (HIV) Rev and Tat proteins, and the hepatitis delta protein (Huang *et al*., 2001; Seeger *et al*., 2007). B23 may be a target for viral manipulation to favor viral transcription and translation or cause perturbations in the cell cycle to promote virus replication (Hiscox *et al*., 2002; Grisendi *et al*., 2006). This is supported by evidence that B23.1 is up-regulated by binding with the hepatitis delta antigen to enhance hepatitis delta virus RNA replication (Huang *et al*., 2001). The C-terminal end of B23.1 directly interacts with HCV core proteins and the levels of B23.1 are enhanced in a cell line expressing the HCV core (Mai *et al*., 2006). In the present study, we suggest that a B23.1 deletion mutant (residues 7-294) is a new HBV host factor that interacts with HBV Cp149.

* To whom correspondence should be addressed.
(Tel) 82-2-880-7773; (Fax) 82-2-880-4424
(E-mail) drjung@snu.ac.kr

Materials and Methods

HBV Cp149 expression and purification

The HBV Cp149 gene was amplified by PCR from pHBV1.2x (subtype: *adr*, NCBI accession number: AB299858) as previously described (Choi *et al*., 2005), and was cloned directly into the pET28b vector (Novagen). This construct was transformed into BL21(DE3) + pLysS (Novagen) in *Escherichia coli* and purified as previously described (Zlotnick *et al.*, 1996; Choi *et al*., 2005). Purified Cp149 was mixed with 5× sample buffer (60 mM Tris-HCl; pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue) and boiled at 100°C for 10 min. Samples were resolved with 15% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and analyzed by immunoblotting as previously described (Choi *et al*., 2005). Purified Cp149 was confirmed as a dimer with a gel filtration column (Hiload 16/60 Superdex G-75 prep grade; Amersham Pharmacia Biotech) (Supplementary data Fig. S1).

We purified the Cp149 capsid as previously described (Birnbaum and Nassal, 1990; Konig *et al*., 1998), except for

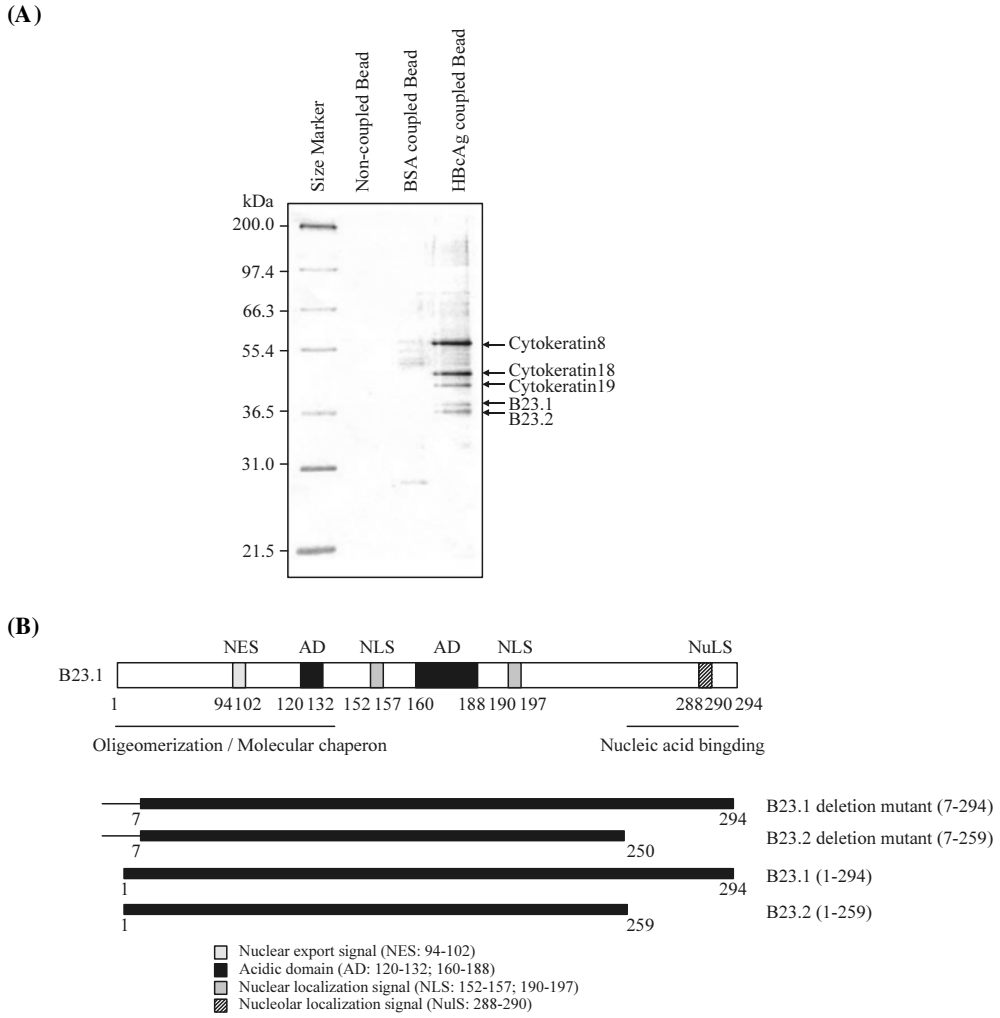

Fig. 1. (A) Identification of a new host factor, B23, which binds to HBV Core protein 149 (Cp149). HepG2 total lysate was added to purified HBV Cp149-coupled NHS-activated resin. Coomassie brilliant blue staining showing size marker, non-coupled resin and BSA-coupled resin (as acontrol and Cp149 coupled resin. (B) The domain region of B23 and the B23 deletion mutants. The positions of the B23 deletion mutants are indicated. These mutants (residues 7-294, 7-259) were cloned by insertion of a fragment from an *Nde*I site to an *Xho*I site into the His-tagged pET21a vector.

the sucrose density gradient analysis buffer in which 150 mM HEPES (pH 7.5) was used.

Expression and purification of B23 with residues 7-294, residues 7-259, and residues 1-161

Human B23 (a gift from Kenji Fukasawa, Department of Cell Biology, Neurobiology, and Anatomy, University of Cincinnati College of Medicine) was cloned into the expression vector pET-21a+ (Novagen). Each of the B23 deletion mutants for residues 7-294, 7-259, and 1-161 was constructed in a similar manner. These constructs added an 8-residue tag containing hexa-histidine to the C-terminus of the recombinant B23 core, and were over-expressed in *E. coli* BL21 (DE3). Protein expression was induced by 1 mM isopropyl β-D-thiogalactopyranoside (IPTG; Duchefa Biochemie) and the cells were incubated for an additional 4 h at 37°C. The proteins were extracted by sonication of the cells in lysis buffer [20 mM Tris-HCl; pH 7.5, 500 mM NaCl, and 5% (v/v) glycerol] containing 5 mM imidazole. The crude lysate was centrifuged at 15,000×g for 60 min. The supernatant was applied to an affinity chromatography column of nickel-nitrilotriacetic acid-agarose (Ni-NTA; QIAGEN). The protein was eluted with lysis buffer containing 500 mM imidazole. The samples were gel filtered on a HiLoad 16/60 Superdex-200 prep-grade column (Amersham Pharmacia Biotech), using an elution buffer of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 2 mM β-mercaptoethanol.

Cell culture

The human liver cell line HepG2 was cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (Gibco). Cells were seeded in 100-mm dishes and were harvested and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.2% Nonidet P-40.

HBV Cp149-coupled affinity chromatography and MALDI-TOF analysis

We made Cp149-coupled affinity resin by using NHS-activated sepharose 4 Fast Flow (Amersham Pharmacia Biotech). Cp149, purified from *E. coli* as described above, was loaded on the NHS-activated resin and immobilized according to the manufacturer's protocol. HepG2 total cell lysate was loaded batchwise on the affinity resin and incubated at 4°C for 1 h. After a brief centrifugation at 10,000×g and removal of the supernatant, the beads were washed 4 times with HepG2 lysis buffer containing 50 mM NaCl. Samples were analyzed by 12% SDS-PAGE and Coomassie brilliant blue staining. Mass spectrometry on selected protein bands was performed as previously described (Choi *et al*., 2005).

Co-immunoprecipitation of HBV Cp149 and B23

To determine which B23 isoform interacted with Cp149, 0.2 μM of purified Cp149 was added to protein A sepharose (Sigma) conjugated to HBV Cp rabbit polyclonal antibody (polyAb; DakoCytomation) for 2 h at 4°C. After incubation, 0.1 μM of purified B23.1 (residues 7-294) or B23.2 (residues 7-259) was added to this mixture and incubated for 2 h at 4°C. Immunoprecipitants were analyzed by 15% SDS-PAGE, followed by immunoblot analysis with B23 mouse monoclonal Ab (monoAb; Sigma) and HBV Cp polyAb.

Purified Cp149 was added to HepG2 total cell lysate and incubated at 4°C for 2 h for the co-immunoprecipitation experiment. The purified Cp149 and B23.1 were co-immunoprecipitated with 30 μl of 50% protein A/G-sepharose (Sigma), conjugated to 2 μg of HBV Cp polyAb or B23 monoAb, respectively, at 4°C for 2 h. Immunoprecipitated lysate was analyzed by 15% SDS-PAGE, followed by immunoblot analysis with HBV Cp polyAb and B23 monoAb.

Surface plasmon resonance experiments

Surface plasmon resonance measurements were performed with the BIAcore 3000 apparatus at 25°C. For all experiments, purified Cp149 was first immobilized onto CM5 sensor chips (BIAcore AB) via amine coupling, using the manufacturer's protocol. In a typical experiment, 6 different dilutions (100, 50, 10, 5, 1, and 0.05 μM) of B23.1 (residues 7-294), B23.2 (residues 7-259), and B23.1 (residues 1-161), purified from *E. coli* cultures, were injected at 20 μl/min for 3 min. TN buffer (20 mM Tris-HCl; pH 7.5 and 100 mM NaCl) was then injected at 20 μl/min for 1.5 min to remove nonspecifically bound supernatant components from the surface of the sensor chip. The flow cells were monitored in real time and shown as binding curves of response units for changes in the surface plasmon resonance from experimental flow cells. The sensorgram was analyzed using the BIA evaluation software, version 3.1 (BIAcore AB).

In vitro HBV Cp149 assembly, sucrose density gradient analysis, and dot-blot assay

For *in vitro* Cp149 assembly, the assembly reaction was performed and confirmed as previously described (Choi *et al*., 2005). A protein mixture of purified Cp149 capsid with B23.1 (residues 7-294) and purified Cp149 dimer with B23.1 (residues 7-294) was prepared and sedimented under Cp capsid assembly conditions by sucrose density gradient analysis as previously described (Choi *et al*., 2005), except for the gradient ranges: 1 ml of 50%, 1 ml of 40%, 1 ml of 35%, 1 ml of 30%, and 0.8 ml of 20% sucrose in 150 mM HEPES (pH 7.5) and 0.2 ml of reaction mixture. After sedimentation, the fractions of each tube were detected by HBV Cp polyAb and B23 monoAb by immunoblot analysis and dot-blot assay, which were performed according to the instructions in the Bio-Dot Microfiltration Apparatus manual (Bio-Rad Laboratories).

Binding assay of B23.1 with HBV Cp149 dimer or Cp149 capsid

For the binding assay of B23.1 with the Cp149 dimer or Cp149 capsid, B23.1 (residues 7-294), Cp149 dimer, and Cp149 capsid were purified separately from *E. coli* as described above. One microgram of His-tagged B23.1 (residues 7-294) was bound to 200 μl of 50% Ni-NTA slurry (QIAGEN) at 4°C for 1 h. The B23.1 (residues 7-294) bound to the Ni-NTA mixture was loaded into a column (Bio-Rad Laboratories) with the bottom outlet capped. The bottom cap was removed and the column was drained, 1 μg of Cp149 dimer or Cp149 capsid was then added to the column. Ni-NTA resin-coupled proteins were washed with TN buffer 4 times and the proteins were eluted with TN buffer containing 500 mM imidazole. Eluted proteins were analyzed by 15% SDS-PAGE followed by immunoblot analysis with B23 monoAb and HBV Cp polyAb.

Results

Identification of new a host factor, B23 that bound to HBV Cp149

HBV Cp149 was used as the model HBV Cp in this study as it is the minimum truncated protein that is still able to spontaneously form Cp149 capsid under suitable conditions *in vitro* and *in vivo* (Wingfield *et al*., 1995). It has also been used for the study of Cp structure as it can be over-expressed in *E. coli* (Wynne *et al*., 1999; Kang *et al*., 2008). Cp149 was expressed and purified in *E. coli*, which was confirmed by immunoblot analysis. To identify new binding partners for this protein, purified Cp149 was loaded onto NHS-activated affinity resin and exposed to HepG2 cell lysate. We identified 5 new host factors that could bind to Cp149 (Fig. 1A) in repeated experiments and analyzed them using MALDI-TOF MS (data not shown). Putative Cp149 binding partners were identified as cytokeratin 8, 18, and 19, and two isoforms of B23 designated as B23.1 (38 kDa) and B23.2 (36 kDa), which are polypeptides of 294 and 259 amino acids, respectively (Fig. 1A).

Co-immunoprecipitation and binding assay of B23 with HBV Cp149

To determine the specific B23 isoform that interacted with Cp149, we performed co-immunoprecipitation with *E. coli* purified proteins: Cp149 against B23.1 (residues 7-294) and Cp149 against B23.2 (residues 7-259). To improve the expression level of B23, the first 6 highly hydrophobic amino acids were deleted (Fig. 1B) (Hingorani *et al*., 2000). We found that only B23.1 (residues 7-294) could interact with Cp149 (Fig. 2A). To confirm this interaction we conducted

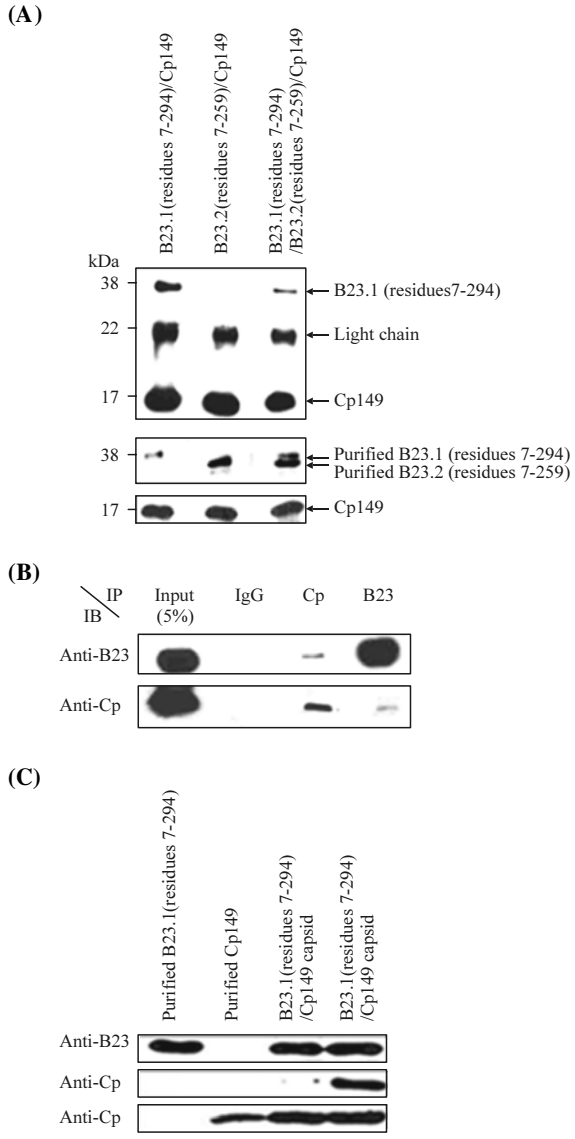

Fig. 2. Co-immunoprecipitation of B23 with HBV Cp149 and binding assay of Ni-NTA-coupled B23.1 (residues 7-294). (A) Co-immunoprecipitation was performed to determine which B23 isoforms bind to Cp149 *in vitro*. All proteins were purified from *E. coli.* B23.1 (residues 7-294) and Cp149, B23.2 (residues 7-259) and Cp149, the 2 bottom panels are provided to indicate the size difference between B23.1 (residues 7-294) and B23.2 (residues 7-259). (B) Co-immunoprecipitation of cellular B23.1 and *E. coli* purified Cp149. The input fraction of total cell lysate was mixed with Cp149 and used as a control (lane 1). A mixture of rabbit and mouse IgG was used as a negative control (lane 2), HBV Cp polyAb (lane 3), and B23 monoAb (lane 4). (C) Binding assay of B23.1 (residues 7-294) with the HBV Cp149 dimer and capsid. Both B23.1 (residues 7-294) and Cp149 were purified from *E. coli* and eluted through Ni-NTA resin as a marker (lanes 1 and 2), a mixture of the Cp capsid and B23.1 (residues 7-294) (lane 3), and a mixture of the Cp149 dimer and B23.1 (residues 7-294) (lane 4). The third panel demonstrates that the same quantity of the Cp149 dimer and capsid (lower panel) were loaded.

co-immunoprecipitation of purified Cp149 against cellular B23.1, which demonstrated that cellular B23.1 interacted

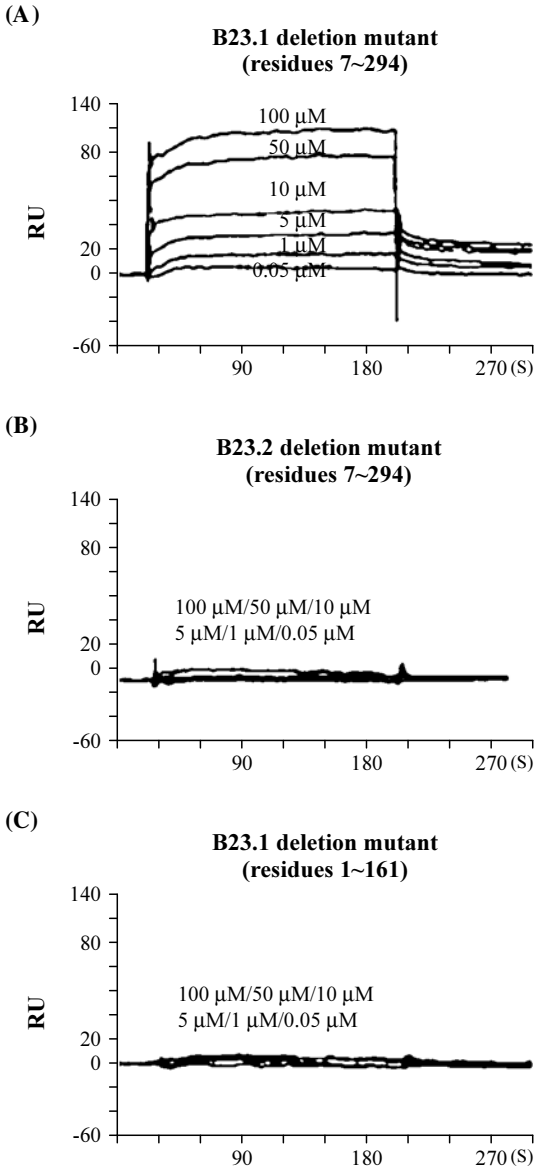

Fig. 3. Residues 259-294 of B23.1 were required for its interaction with HBV Cp149. Sensorgrams depict the binding of B23.1 (residues 7-294), B23.2 (residues 7-259), and B23.1 (residues 1-161) with Cp149 purified from *E. coli*. (A) Cp149 was immobilized on a CM5 chip and then injected with 6 different concentrations (0.05, 1, 5, 10, 50, and 100 μM) of B23.1 (residues 7-294). (B) Cp149 was immobilized on a CM5 chip and injected with 6 different concentrations (0.05, 1, 5, 10, 50, and 100 μM) of B23.2 (residues 7-259). (C) Cp149 was immobilized on a CM5 chip and 6 different concentrations (0.05, 1, 5, 10, 50, and 100 μM) of B23.1 (residues 1-161) were injected.

with Cp149 (Fig. 2B). As shown in Fig. 2B, we detected less Cp149 following immunoprecipitation with B23.1, compared to the amount of B23.1 detected following Cp149 immunoprecipitation. This result might suggest that only a minor proportion of B23 interacted with Cp149, and the rest exists as a free form.

To further investigate our assessment, we conducted a

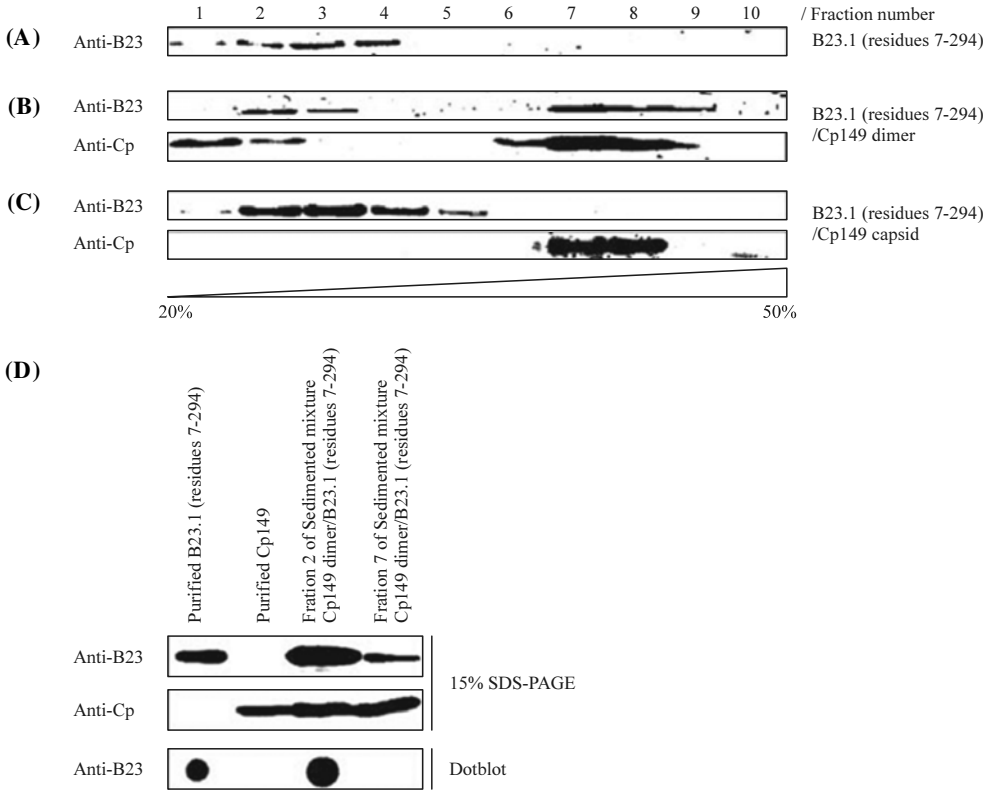

Fig. 4. B23.1 (residues 7-294) interacted with the Cp149 dimer and not with the surface of the Cp149 capsid *in vitro*. To analyze whether B23.1 (residues 7-294) interacted with the Cp149 dimer, and not with the surface of the Cp149 capsid, 20~50% sucrose density gradient analysis was performed. (A) B23.1 (residues 7-294) as a control. (B) Cp149 (lower panel: dimer, lanes 1-3 and capsid, lanes 6-9), B23.1 (residues 7-294) (upper panel: lanes 1-3 and lanes 7-9). (C) Cp149 (lower panel: capsid, lanes 7-8), B23.1 (residues 7-294) (upper panel: lanes 1-5). (D) The 2 samples from fractions 2 and 7 of Fig. 4B were analyzed by 15% SDS-PAGE (denaturing conditions) and dot blotting (non-denaturing conditions), and then probed with the B23 monoAb and HBV Cp polyAb. Both Cp149 (lane 1) and B23.1 (residues 7-294) (lane 2) were purified from *E. coli* and used as markers, sample from fraction 2 of Fig. 4B (lane 3), and sample from fraction 7 of Fig. 4B (lane 4).

binding assay using B23.1 (residues 7-294) coupled to Ni-NTA resin, with the Cp149 dimer and Cp149 capsid to determine which Cp149 form interacted with B23.1 (residue 7-294) (Fig. 2C). Immunoblot analysis using HBV Cp polyAb and B23 monoAb showed that B23.1 (residues 7-294) bound to the Cp149 dimer, but not to the Cp149 capsid (Fig. 2C, middle panel).

Residues 259-294 of B23.1 were required for interaction with HBV Cp149

As shown in Fig. 1A, we found that Cp149 bound to both B23.1 and B23.2. However, Fig. 2A indicated that Cp149 only interacted with B23.1 (residues 7-294) and not with B23.2 (residues 7-259). Thus, we analyzed those specific amino acid sites of B23 that interacted with Cp149. Sensorgrams (RU; resonance unit) of the interaction between Cp149 and the B23 deletion proteins (residues 7-294, 7-259, and 1-161) are shown in Fig. 3. Detailed accounts of the kinetics of the interactions of between Cp149 and B23.1 (residues 7-294) are listed in Table 1. The association rate constant (K_a) is the rate of formation of new complexes and K_d is the rate of complex dissociation. The χ^2 value is a standard statistical measure of the closeness of fit and values of χ^2 below 10 are frequently acceptable. The values of K_a and K_d fit well to a 1:1 Langmuir binding model. The equilibrium dissociation constant (K_D) is defined as K_d/K_a, which represents the affinity between molecules (Ceres and Zlotnick, 2002).

Table 1. Binding parameters obtained from kinetic analysis

Molecule A	Molecule B	$(K_a)^a$	$(K_d)^b$	$(K_D)^c$	Chi2(X^2)
Cp149	B23.1 (residues7-294)	9.00×10^{2}	2.27×10^{-3}	2.51×10^{-6}	3.29

Sensorgrams of the detailed accounts of the kinetics of the interactions and affinities Cp149 dimer and B23.1 (residues 7-294) are shown in Fig. 3A.
[a] Association rate of constant (K_a) for A + B → AB
[b] Dissociation rate of constant (K_d) for AB → A + B
[c] Equilibrium dissociation of constant (K_D) = K_d/K_a

With gradually increasing concentrations of B23.1 (residues 7-294), we observed that the binding response of Cp149 and B23.1 (residues 7-294) was increased to 120 RU (Fig. 3A). However, the same concentrations of B23.2 (residues 7-259) and B23.1 (residues 1-161) did not change the binding response of Cp149 with the respective B23.1 (residues 7-259) and B23.1 (residues 1-161) (Fig. 3B and C).

As previously reported, the kinetic value (K_D) of the interaction between Cp149 and Cp149 was 1.3×10^{-5} (Kang *et al*., 2006).

B23.1 (residues 7-294) interacted with the HBV Cp149 dimer during Cp149 capsid assembly

Fig. 1, 2, and 3 indicated that the Cp149 dimer interacted with B23.1. The HBV core protein and Cp149 are known to spontaneously form capsid *in vitro* and *in vivo*. To determine if B23.1 (residues 7-294) interacted with the Cp149 dimer during capsid formation we performed sucrose density gradient analysis (20∼50%). Under Cp capsid assembly conditions, 2 individual reactions, Cp149 capsid and B23.1 (residue 7-294), and Cp149 dimer and B23.1 (residue 7-294), were sedimented. These were then analyzed with immunoblot analysis using HBV Cp polyAb and B23 monoAb (Fig. 4A, B, and C). In our previous study, the Cp149 dimer (34 kDa), which was sedimented by sucrose density gradient analysis, existed in fractions 1-4 and 6-8 (data not shown, order from top to bottom). Fig. 4A indicates that B23.1 (residues 7-294, 38 kDa) existed in fractions 1 to 4 after sucrose density gradient analysis. In Fig. 4B, B23.1 (residues 7-294) and the Cp149 dimer reacted under Cp capsid assembly conditions, and were sedimented together. Interestingly, we found that B23.1 (residues 7-294) was detected in fractions 1-3 and 7-9 (Fig. 4B). Furthermore, the Cp149 dimer and Cp149 capsid from the same experiment were both detected in fractions 1-3 and 6-9 (Fig. 4B). In Fig. 4C, B23.1 (residues 7-294) and the Cp149 capsid were mixed together and sedimented. B23.1 (residues 7-294) was only detected in fractions 1-5 (Fig. 4C) while the Cp149 capsid was detected in fractions 7-8 (Fig. 4C) further suggesting that the interaction of B23 with Cp149 might be involved with capsid assembly. On the basis these results, each of the samples from fractions 2 and 7 in Fig. 4B was analyzed by 15% SDS-PAGE and dot-blot assay (Fig. 4D). Under denaturing SDS-PAGE conditions, both Cp149 and B23.1 (residues 7-294) were detected in both fractions; whereas, by dot-blot assay (Fig 4D), which has non-denaturing conditions and allows the Cp149 capsid to remain intact, B23.1 (residues 7-294) was detected only in fraction 2 (Fig. 4D, lane 3) of Fig. 4B and not in fraction 7 (Fig. 4D, lane 4) of Fig. 4B. Based on the interaction between B23.1 (residues 7-294) and the Cp149 dimer in sucrose density gradient analysis, dot-blot assay suggested that B23.1 (residues 7-294) might be packaged into the Cp149 capsid during Cp149 assembly *in vitro*.

Discussion

HBV genome replication is essential to the HBV life cycle, hence, there have been many investigations on the replication cycle of HBV, such as Cp capsid assembly, RNP (pregenomic RNA and polymerase) complex encapsidation, polymerase activity, and host factors (heat shock proteins and protein kinase C) that are involved in HBV genome replication and packaging into the Cp capsid (Kann and Gerlich, 1994; Hu *et al*., 1997, 2004; Nassal, 1999; Park and Jung, 2001; Kang *et al*., 2008). Some reports have suggested that unknown host factors are involved in the encapsidation process (Hu *et al*., 1997; Nassal, 1999; Cao and Travis, 2004).

In this study, we identified (i) a new host factor, B23.1, that interacted with HBV Cp149 (Fig. 1A), (ii) residues 259-294 of this B23.1 (residues 7-294) were required for the interaction with HBV Cp149 (Fig. 3), and (iii) B23.1 (residues 7-294) interacted with the Cp149 dimer when Cp149 capsid assembly was induced *in vitro* (Fig. 4).

From Fig. 2 and 3, we determined that only B23.1 (residues 7-294) bound to Cp149, whereas from Fig. 1A, it was found that both B23.1 and B23.2 were able to bind to Cp149. These two disparate results can be explained as B23.1 and B23.2 form a hetero-oligomer complex (Okuwaki *et al*., 2002; Grisendi *et al*., 2006), and thus the B23.2 that was bound to B23.1 was detected in Fig. 1A. As shown in Fig. 2A and B, B23.1 (residues 7-294) was detected by co-immunoprecipitation using HBV Cp polyAb and immunoblot analysis using B23 monoAb. A binding assay was performed to detect interactions between B23.1 (residues 7-294) and the Cp149 capsid or dimer *in vitro*, and the results suggested that B23.1 (residues 7-294) might interact with the Cp149 dimer.

We conducted sucrose density gradient analysis of 3 different reaction mixtures containing either B23.1 (residues 7-294) alone, B23.1 (residues 7-294) and Cp dimer, and B23.1 (residues 7-294) and Cp capsid, under confirmed Cp capsid assembly conditions (Choi *et al*., 2005; Kang *et al*., 2008) (Fig. 4). We observed that B23.1 (residues 7-294) sedimented with HBV Cp149 dimer, while B23.1 (residues 7-294) alone or B23.1 (residues 7-294) with Cp149 capsid had no remarkable migration (Fig. 4A, B, and C). This result indicated that B23.1 (residues 7-294) and the Cp dimer could interact during the normal physiological formation of capsid. Interestingly, a dot-blot assay of sedimented samples from fractions 2 and 7 from the B23.1 (residues 7-294) and Cp149 dimer reaction (Fig. 4B) revealed that B23.1 (residues 7-294) might interact with Cp149 from within the Cp capsid (Fig. 4D; lanes 3 and 4).

It should be noted that this *in vitro* analysis required $10^7\sim10^{10}$ times higher concentrations of Cp capsid than the *in vivo* concentrations required to detect B23.1 (residues 7-294) by immunoblot analysis. *In vivo*, the concentration of Cp capsid in HBV-infected patient serum is 10^7 ml^{-1} to 10^{10} ml^{-1} (Kang *et al*., 2006), which is not sufficient to detect whether B23.1 might be related to Cp capsid assembly.

The K_D for the interaction between Cp149 with Cp149 was previously calculated to be 1.3×10^{-5} (Kang *et al*., 2006). In this study, we found that the kinetic value of interaction for B23.1 (residues 7-294) with Cp149, which was calculated from Fig. 3A, was 2.51×10^{-6} (Table 1). This inferred that the interaction strength of B23.1 (residues 7-294) with Cp149 was stronger than the interaction between Cp149 and Cp149. Therefore, we might consider that initially B23.1 (residues 7-294) interacted with the Cp149 dimer, before Cp149 be-

gan the process of capsid formation.

In conclusion, we identified a new host factor that may provide insights into the mechanisms of HBV replication. In previous reports, B23 stimulated the nuclear import of target proteins, as an *in vitro* molecular chaperone (Szebeni and Olson, 1999; Lim and Wang, 2006). Based on these reports and our findings, we suggest that B23 is involved in the HBV life cycle in a chaperone like manner.

Acknowledgements

We thank Kenji Fukasawa for providing human B23. This work was supported by the Korea Research Foundation grant funded by the Korean Government (KRF-2006-12-00322) and the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean government (No.009-0083302). Hee Youn Shim was supported by a research fellowship (BK21) from the Korean Ministry of Education and Human Resources Development and a Lotte fellowship. Su Jin Lee and Ji Young Min were supported by research fellowships (BK21) from the Korean Ministry of Education and Human Resources Development.

References

Birnbaum, F. and M. Nassal. 1990. Hepatitis B virus nucleocapsid assembly: primary structure requirements in the core protein. *J. Virol.* 64, 3319-3330.

Cao, F. and J.E. Tavis. 2004. Detection and characterization of cytoplasmic hepatitis B virus reverse transcriptase. *J. Gen. Virol.* 85, 3353-3360.

Ceres, P. and A. Zlotnick. 2002. Weak protein-protein interactions are sufficient to drive assembly of hepatitis B virus capsids. *Biochemistry* 41, 11525-11531.

Choi, Y., S.G. Park, J.H. Yoo, and G. Jung. 2005. Calcium ions affect the hepatitis B virus core assembly. *Virology* 332, 454-463.

Grisendi, S., C. Mecucci, B. Falini, and P.P. Pandolfi. 2006. Nucleophosmin and cancer. *Nat. Rev. Cancer* 6, 493-505.

Hingorani, K., A. Szebeni, and M.O. Olson. 2000. Mapping the functional domains of nucleolar protein B23. *J. Biol. Chem.* 275, 24451-24457.

Hiscox, J.A. 2002. The nucleolus--a gateway to viral infection? *Arch. Virol.* 147, 1077-1089.

Hu, J., D. Flores, D. Toft, X. Wang, and D. Nguyen. 2004. Requirement of heat shock protein 90 for human hepatitis B virus reverse transcriptase function. *J. Virol.* 78, 13122-13131.

Hu, J., D.O. Toft, and C. Seeger. 1997. Hepadnavirus assembly and reverse transcription require a multi-component chaperone complex which is incorporated into nucleocapsids. *EMBO. J.* 16, 59-68.

Huang, W.H., B.Y. Yung, W.J. Syu, and Y.H. Lee. 2001. The nucleolar phosphoprotein B23 interacts with hepatitis delta antigens and modulates the hepatitis delta virus RNA replication. *J. Biol. Chem.* 276, 25166-25175.

Kang, H.Y., S. Lee, S.G. Park, J. Yu, Y. Kim, and G. Jung. 2006. Phosphorylation of hepatitis B virus Cp at Ser87 facilitates core assembly. *Biochem. J.* 398, 311-317.

Kang, H., J. Yu, and G. Jung. 2008. Phosphorylation of hepatitis B virus core C-terminally truncated protein (Cp149) by PKC increases capsid assembly and stability. *Biochem. J.* 416, 47-54.

Kann, M. and W.H. Gerlich. 1994. Effect of core protein phosphorylation by protein kinase C on encapsidation of RNA within core particles of hepatitis B virus. *J. Virol.* 68, 7993-8000.

Konig, S., G. Beterams, and M. Nassal. 1998. Mapping of homologous interaction sites in the hepatitis B virus core protein. *J. Virol.* 72, 4997-5005.

Lim, M.J. and X.W. Wang. 2006. Nucleophosmin and human cancer. *Cancer Detect. Prev.* 30, 481-490.

Mai, R.T., T.S. Yeh, C.F. Kao, S.K. Sun, H.H. Huang, and Y.H. Wu Lee. 2006. Hepatitis C virus core protein recruits nucleolar phosphoprotein B23 and coactivator p300 to relieve the repression effect of transcriptional factor YY1 on B23 gene expression. *Oncogene* 25, 448-462.

Nassal, M. 1999. Hepatitis B virus replication: novel roles for virus-host interactions. *Intervirology* 42, 100-116.

Nassal, M. and H. Schaller. 1996. Hepatitis B virus replication-an update. *J. Viral. Hepat.* 3, 217-226.

Okuwaki, M., M. Tsujimoto, and K. Nagata. 2002. The RNA binding activity of a ribosome biogenesis factor, nucleophosmin/B23, is modulated by phosphorylation with a cell cycle-dependent kinase and by association with its subtype. *Mol. Biol. Cell.* 13, 2016-2030.

Park, S.G. and G. Jung. 2001. Human hepatitis B virus polymerase interacts with the molecular chaperonin Hsp60. *J. Virol.* 75, 6962-6968.

Seeger, C., F. Zoulim, and W. Mason. 2007. Hepadnaviruses, p. 2977-2993. *In* D.M. Knipe and P.M. Howley (eds.), Fields Virology. Fifth ed, Lippincott Williams & Wilkins, Philadelphia, USA.

Szebeni, A. and M.O. Olson. 1999. Nucleolar protein B23 has molecular chaperone activities. *Protein Sci.* 8, 905-912.

Vanlandschoot, P., T. Cao, and G. Leroux-Roels. 2003. The nucleocapsid of the hepatitis B virus: a remarkable immunogenic structure. *Antiviral Res.* 60, 67-74.

Wang, D., A. Baumann, A. Szebeni, and M.O. Olson. 1994. The nucleic acid binding activity of nucleolar protein B23.1 resides in its carboxyl-terminal end. *J. Biol. Chem.* 269, 30994-30998.

Wingfield, P.T., S.J. Stahl, R.W. Williams, and A.C. Steven. 1995. Hepatitis core antigen produced in *Escherichia coli*: subunit composition, conformational analysis, and *in vitro* capsid assembly. *Biochemistry* 34, 4919-4932.

Wright, T.L. 2006. Introduction to chronic hepatitis B infection. *Am. J. Gastroenterol.* 101 (Suppl 1), S1-S6.

Wynne, S.A., R.A. Crowther, and A.G. Leslie. 1999. The crystal structure of the human hepatitis B virus capsid. *Mol. Cell.* 3, 771-780.

Zlotnick, A., N. Cheng, J.F. Conway, F.P. Booy, A.C. Steven, S.J. Stahl, and P.T. Wingfield. 1996. Dimorphism of hepatitis B virus capsids is strongly influenced by the C-terminus of the capsid protein. *Biochemistry* 35, 7412-7421.