Calcium ions affect the hepatitis B virus core assembly

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Abstract

Previous report showed that cytosolic Ca$^{2+}$ induced by hepatitis B virus X protein (HBx) promotes HBV replication. In this study, in vitro experiments showed that (i) HBV core assembly in vitro was promoted by Ca$^{2+}$ through the sucrose density gradient and the analytical ultracentrifuge analysis. Also, (ii) transmission electron microscope analysis demonstrated these assembled HBV core particles were the capsids. Ex vivo experiments showed that the treatment of BAPTA-AM and cyclosporine A (CsA) reduced HBV capsids in the transfected HepG2 cells. In addition to that, the treatment of Thapsigargin (TG) increased HBV capsids in the transfected HepG2 cells. Furthermore, we investigated the increased HBV core assembly by HBx. The results show that the increased cytosolic calcium ions by HBx promote the HBV core assembly.

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Introduction

Hepatitis B virus (HBV), a member of the hepadnaviridae family, is a liver-tissue-specific enveloped DNA virus (Nassal and Schaller, 1993; Seeger and Mason, 2000). The virus is also associated with the development of hepatocellular carcinoma and liver cirrhosis (Ganem and Varmus, 1987). Today, more than two billion people have been infected by HBV, and over 350 million people remain chronically infected (Vanlandschoot et al., 2003). HBV genome has four open reading frames (ORFs) (preC/C, preS1/preS2/S, P and X) (Nassal and Schaller, 1993). One ORF, preC/C (precore–core) region, encodes both the hepatitis B core antigen (HBcAg) and the hepatitis B e antigen (HBeAg). HBeAg is secreted into blood; however, it plays no role in viral assembly (Ganem and Prince, 2004). HBcAg consists of 183 or 185 amino acids, depending on the virus subtypes, ayw, adr, or adw (Tiollais et al., 1981). HBV core protein, which is assembled around HBV polymerase and pregenomic RNA (pgRNA) (Birnbaum and Nassal, 1990; Nassal, 1996), is required for HBV replication. Dimeric HBV core proteins assemble to form a capsid, an icosahedral core particle, through the trimers of dimers (Nassal et al., 1992; Zhou and Standring, 1992; Zlotnick et al., 1999a). In the HBV capsids, there are two size variants: a smaller capsid consisting of 90 dimeric core proteins with a triangulation number of $T = 3$ and a larger capsid consisting of 120 dimeric core proteins with $T = 4$ (Zlotnick et al., 1996). The overall diameters measured to the tips of the spikes are 32 nm for $T = 3$ and 36 nm for $T = 4$ (Crowther et al., 1994; Pante and Kann, 2002). HBV core proteins consist of two domains: the assembly domain (amino acids 1–149) and the protamine domain (amino acids 150–183). The protamine domain is the region for pgRNA binding (Nassal, 1992; Watts et al., 2002). The assembly domain, whose minimum length for the core assembly is 140 amino acids from amino-terminal group (Watts et al., 2002), spontaneously forms a spherical shell in adequate conditions in vitro and in vivo (Cohen and Richmond, 1982; Stahl et al., 1982). Generally, Core protein 149 (Cp149), a truncated form of 34 residues of the HBcAg in its carboxyl-terminal group, has been used for the study of core protein structure because of its facility of over-
expression in the *Escherichia coli* (*E. coli*) system and its structural similarity to HBeAg (Naito et al., 1997; Wynne et al., 1999). Also, it is known that the majority of assembled Cp149 in the *E. coli* and native capsids isolated from infected human livers are in the *T* = 4 form (Crowther et al., 1994; Kenney et al., 1995; Zlotnick et al., 1996). Cp149 can form the capsids with NaCl. This method was well established by Zlotnick et al. (1996). The Cp149 capsids produced by this method were mainly the *T* = 4 form, according to the analysis by analytical ultracentrifuge and cryoelectron microscope (Zlotnick et al., 1996).

Another ORF, named X, encodes the hepatitis B virus X protein (HBx) consisting of 154 amino acids, which is required for viral infection and replication (Doria et al., 1995). This protein has been scientifically explored for a long time because of its relation with hepatocarcinogenesis (Bouchard et al., 2001; Zoulim et al., 1994). HBx is separated into two distinct populations in cytoplasm (in the mitochondria and in the cytosol) (Henkler et al., 2001; Murakami, 2001), and it releases Ca2+ to cytosol either from mitochondria or from endoplasmic reticulum (ER) or from both. The released Ca2+ triggers the action of pyk2 kinase, and the activated pyk2 kinase stimulates the Src-family kinases. As a result, HBV reverse transcription is up-regulated (Bouchard et al., 1997; Klein et al., 1999). Ca2+ triggers the action of pyk2 kinase, and the activated pyk2 kinase stimulates the Src-family kinases. As a result, HBV reverse transcription is up-regulated (Bouchard et al., 1997; Klein et al., 1999).

In our study, we investigated the effect of Ca2+ on HBV core assembly through the different Ca2+ concentrations in vitro and with BAPTA-AM, Cyclosporine A (CsA) and Thapsigargin (TG) ex vivo. Furthermore, we investigated the increased HBV core assembly by HBx. Our results suggest that increased cytosolic calcium ions by HBx promote the HBV core assembly.

**Results**

**Analysis of assembled Cp149 by Ca2+ in vitro through sucrose density gradient centrifugation**

To determine whether Ca2+ affects the assembly of HBV core protein in vitro, Cp149 was expressed in the *E. coli* expression system and confirmed by Western blotting, as mentioned in Materials and methods (Fig. 1C) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (data not shown). Over-expressed Cp149 in *E. coli* expression system was purified and stored at −70 °C as dimeric forms as previously described (Wingfield et al., 1995; Zlotnick et al., 1996); on the other hand, purified Cp149 was stored in 100 mM glycine buffer (pH 9.5) instead of 100 mM sodium bicarbonate buffer (pH 9.5) because CaCO3 precipitation was formed when CaCl2 solution was mixed with the 100 mM sodium bicarbonate storage buffer (pH 9.5), and this precipitation affected the sucrose gradient and analytical ultracentrifuge analysis. To investigate the effect of Ca2+ on HBV core assembly, a sucrose density gradient analysis and an analytical ultracentrifugation were performed with the purified Cp149 as described in Materials and methods. As shown in Fig. 2A, Cp149 assembly was increased more progressively with increasing CaCl2 concentration (the 5-mM and the 10-mM panels) compared to the absence of CaCl2 (the control panel). In these data, dimeric Cp149 was in the top of the sucrose gradient (fraction number: 1–2) whereas assembled Cp149 was in the middle of the sucrose gradient (fraction number: 5–7). Also, the analysis of the integrated density of individual bands by 1D Image Analysis Software (Eastman Kodak Company) showed that the more dimeric Cp149 was reduced, the more assembled Cp149 was increased in the same total protein amounts (Fig. 2B). Fig. 2C shows the native agarose gel electrophoresis of the assembled core particles obtained from the previously performed sucrose density gradient. In this experiment, Cp149 capsids were prepared with NaCl as previously used by Zlotnick et al. (1996) (Fig. 2C, lane 1). This result shows that the core particles assembled by Ca2+ (Fig. 2C, lanes 3, 4) were similar to the Cp149 capsids (Fig. 2C, lane 1) from their electrophoretical mobility in the native agarose gel.

*Analytical ultracentrifuge and transmission electron microscope (TEM) measurements of assembled Cp149 by Ca2+*

In order to analyze the core particles assembled by Ca2+, analytical ultracentrifugation was performed as described in Materials and methods. As shown in Fig. 3, the apparent sedimentation coefficient distribution ([g*(S)] was relatively more increased as the Ca2+ concentration was increased from 5 to 10 mM compared to the control performed without CaCl2 in the sedimentation velocity between 40S and 45S. However, the corrected sedimentation coefficients (*S*20, w) of these assemblies of Cp149 were 45S. To identify whether these particles were capsids or aggregates, having the same density and electrophoretical mobility in the native gel as the Cp149 capsids, electron microscopy was used (Fig. 4). Assembled Cp149 was negatively stained and was observed by TEM as in Materials and methods. As shown in Fig. 4B, dimeric Cp149 formed the capsids with Ca2+ as the control (Fig. 4A). In the control experiment, Cp149 capsids were prepared by NaCl as previously done by Zlotnick et al. (1996). From these data, the approximate diameter of the Cp149 capsids as a control (Fig. 4A) was 36 nm (average diameter, 35.57 nm), and the internal cavity was 27 nm (average diameter, 27.26 nm). The approximate diameter of the assembled core particles by Ca2+ (Fig. 4B) was 37 nm (average diameter, 37.38 nm), and the internal cavity was 27 nm (average diameter, 26.82 nm). Also, we could observe small particles (approximate diameter, 32 nm), thought to be of the *T* = 3 form considering their shape and size (Newman et al., 2003) (Fig. 4B, white arrow). From the electron microscopy data, the assembled core particles by Ca2+ (Fig. 4B) is supposed to be identical as the Cp149 capsids (Fig. 4A) with respect to their shapes. This shows that the
assembled core particles by Ca\(^{2+}\) were not a result of aggregation; they possess the same density in the sucrose gradient and mobility in the native agarose gel. Considering the analytical ultracentrifuge study that showed that the corrected sedimentation coefficients (S\(_{20, w}\)) of these assemblies of Cp149 were 45S, and electron microscope studies that showed their shapes and diameters, we supposed that these assembled core particles by Ca\(^{2+}\) are mainly capsids of the T = 4 form. In the next experiment, this association with the HBV core protein and Ca\(^{2+}\) was further investigated ex vivo.

**The reduced HBV capsids assembly by BAPTA-AM and CsA ex vivo**

To determine whether Ca\(^{2+}\) affects the HBV capsids assembly, BAPTA-AM (10 \(\mu\)M) and CsA (1 \(\mu\)M) were treated to the pHBV1.2x (Park et al., 2003) and the pCMV/\(\beta\)-gal transfected HepG2 cells, respectively. BAPTA-AM was used as a cell-permeable cytosolic calcium chelator (Bouchard et al., 2001). CsA was also used to decrease cytosolic calcium (Ahlers et al., 1999; Clapham, 1995). In our experiments, the \(\beta\)-galactosidase assay (\(\beta\)-gal assay) was used to normalize the total expressed HBV core proteins. As shown in Fig. 5A, cytosolic HBV capsids were reduced by 1.9-fold through BAPTA-AM (lane 3) and by 2.3-fold through CsA (lane 4), respectively, in comparison to the control (no chemical treatment) (lane 2). Also, the total expressed HBV core proteins in this experiment were almost identical (Fig. 5A top panel). These results show that the cytosolic HBV capsids could be reduced by BAPTA-AM and CsA.

**The increased HBV core assembly by HBx and TG ex vivo**

In order to focus on the HBV core assembly promoted by Ca\(^{2+}\) and HBx, BAPTA-AM (10 \(\mu\)M), CsA (1 \(\mu\)M), and TG...
We investigated if the HBV capsids level could be recovered in the HBx-deficient HBV mutant by HBx. For this experiment, pHBV1.2x(-X), which is HBx-deficient HBV mutant, was constructed (Fig. 1B) and transfected to the HepG2 cells with pCMV/β-gal. One well of six-well plates was co-transfected with pCMV-HA/HBx to investigate the recovery of core assembly by HBx in the HBx-deficient HBV mutant. The total expressed HBV core proteins were normalized by β-gal assay. As a result, the level of assembled HBV capsids of HBx-deficient HBV (Fig. 5D, lane 3) was reduced by 4.5-fold more compared to the HBV1.2x transfected HepG2 cells (lane 2). However, this low level of assembled HBV capsids was re-leveled when the pCMV-HA/HBx was co-transfected (lane 4). As shown in Fig. 5D, the assembled HBV capsids were increased 3.5-fold by HBx compared to the HBV1.2x(-X) transfected HepG2 cells (lane 3). These results suggest that the recovery of HBx brought about the level elevation of assembled HBV capsids in the HBx-deficient condition.

Discussion

HBV contains a pregenomic RNA (pgRNA) and the enzyme reverse transcriptase (RT), both of which are located in the HBV capsid. The core particles assembled with three components, HBV core protein, pgRNA and RT, provide the machinery for replication (Nassal and Schaller, 1993). Thus, the assembling of HBV capsids is an important step within its life cycle. Furthermore, cytosolic Ca\textsuperscript{2+} is an important factor for intracellular signaling (Carafoli et al., 2001; Clapham, 1995). Based on the facts above, we focused on the assembly of HBV capsids by HBx in vitro. We found that the level of assembled HBV capsids could be recovered by HBx in the HBx-deficient HBV mutant by HBx. For this experiment, pHBV1.2x(-X), which is HBx-deficient HBV mutant, was constructed (Fig. 1B) and transfected to the HepG2 cells with pCMV/β-gal. One well of six-well plates was co-transfected with pCMV-HA/HBx to investigate the recovery of core assembly by HBx in the HBx-deficient HBV mutant. The total expressed HBV core proteins were normalized by β-gal assay. As a result, the level of assembled HBV capsids of HBx-deficient HBV (Fig. 5D, lane 3) was reduced by 4.5-fold more compared to the HBV1.2x transfected HepG2 cells (lane 2). However, this low level of assembled HBV capsids was re-leveled when the pCMV-HA/HBx was co-transfected (lane 4). As shown in Fig. 5D, the assembled HBV capsids were increased 3.5-fold by HBx compared to the HBV1.2x(-X) transfected HepG2 cells (lane 3). These results suggest that the recovery of HBx brought about the level elevation of assembled HBV capsids in the HBx-deficient condition.

Fig. 2. Sucrose density gradient analysis of assembled Cp149 by CaCl\textsubscript{2} in vitro. Sucrose density gradient (10–50%) for the Cp149 assembly by Ca\textsuperscript{2+} was performed with concentrations of 5 and 10 mM CaCl\textsubscript{2} (final concentration). (A) After sucrose density gradient, 10 fractions (500 μl each) were collected from top to bottom, and the same amounts were loaded and separated by 15% SDS-PAGE and visualized with SYPRO orange. (B) The integrated density of individual bands was analyzed by 1D Image Analysis Software. (C) Native agarose gel was performed about the fraction number 5 (the asterisk mark in A). Lane 1 is the assembled Cp149 by NaCl by the previous method of Zlotnick et al. (1996) and is used as a control.

Fig. 3. The analysis of Cp149 assembly with Ca\textsuperscript{2+} by analytical ultracentrifugation. Sedimentation velocity of the Cp149 assembly was analyzed by analytical ultracentrifugation. This experiments was performed with 5 mM (-----) and 10 mM (- - - -) CaCl\textsubscript{2}, compared with the control (---), which was performed without CaCl\textsubscript{2}. The ordinate, g*S\textsubscript{20,w}, is the apparent sedimentation coefficient distribution. The abcissa is Svedbergs (S) and has the units 10\textsuperscript{-13} s. The corrected sedimentation coefficients (S\textsubscript{20, w}) of assembled Cp149 were 45S.
capsids promoted by Ca\(^{2+}\) and demonstrated this through in vitro and ex vivo experiments.

Sucrose density gradient analysis and native agarose gel analysis have been widely used for detecting the core particles (Konig et al., 1998; Koschel et al., 1999). In this study, we showed that the core particles assembled by Ca\(^{2+}\) have the same electrophoretical mobility in the native gel as the Cp149 capsids (Fig. 2). These results with analytical ultracentrifugation data revealed information about the assembled core particles promoted by Ca\(^{2+}\). First, Ca\(^{2+}\) facilitates the Cp149 assembly, and this was aspect is quite novel because previous reports were mainly focused on the Cp149 assembly by Na\(^{+}\) (Ceres and Zlotnick, 2002; Wingfield et al., 1995; Zlotnick et al., 1999a). Second, these assembled core particles promoted by Ca\(^{2+}\) showed the same electrophoretical mobility as the Cp149 capsids in the native agarose gel. Even though native agarose gel was not as specific as the sucrose density gradient centrifugation in the detection of capsid formation, it has been widely used because of its facility to compare each capsid (Koschel et al., 1999). In our results, the core particles assembled by Ca\(^{2+}\) showed the same electrophoretical mobility as the Cp149 capsids in the native agarose gel. If their shape or surface charge were different due to lack of the accurate capsid formation, each capsid would show different mobility in the native agarose gel (Konig et al., 1998; Preikschat et al., 2000). Also, from the analytical ultracentrifugation analysis and the transmission electron microscope data as we mentioned above, these assembled core particles were capsids in their shapes and could be \(T = 4\) form. Based on the illustrated reasons, we conclude that Ca\(^{2+}\) promotes the Cp149 capsid formation in in vitro conditions.

The relationship with Ca\(^{2+}\) was studied ex vivo by using BAPTA-AM and CsA, and TG. These chemicals were used in previous reports (Bouchard et al., 2001, 2003), and their working mechanism is relatively well established (Ahlers et al., 1999; Bouchard et al., 2001; Clapham, 1995; Thastrup et al., 1990). In ex vivo experiments, first of all, we confirmed the reduction of HBV capsids from the treatment of BAPTA-AM and CsA in the pHBV1.2x-transfected HepG2 cells (Fig. 5A). When the pHBV1.2x was transfected to HepG2 cells, they produce hole virus proteins. This data confirmed the possibility of the reduction of HBV capsids by decreasing Ca\(^{2+}\) in the total HBV core protein-normalized condition. Second, we introduced pCMV/FLAG-Core to HepG2 cells, and we could compare the level of assembled core particles in the total FLAG-tagged core protein-normalized condition (Figs. 5B,C). From these results, we found that the assembly of HBV core particles is affected by Ca\(^{2+}\).

HBx has been known as a nuclear transcription factor and it causes the release of Ca\(^{2+}\) into cytosol, which eventually acts on the mitochondria or the ER or both (Ganem, 2001). As previously reported, released Ca\(^{2+}\) up-regulated the HBV reverse transcription, and this was confirmed by HBx-deficient HBV mutant (Ganem, 2001; Klein et al., 1999). In this mutant, the HBV replication was reduced more than the wild type when the pgRNA level remained the same. However, this reduced replication in HBx-deficient HBV mutant could be recovered by HBx or by several agents such as valinomycin, TG, and glibenclamide, which increase the cytosolic calcium level (Bouchard et al., 2003; Ganem, 2001; Klein et al., 1999). Concerning the relationship between HBx and increased HBV replication, previous reports suggested that the HBx protein altered the cellular environment to enhance the polymerase activity, rather than acting on the polymerase itself (Bouchard et al., 2003). Due to these reasons, we suggested that cytosolic Ca\(^{2+}\) increased by HBx affected the enhanced HBV replication not only from the sequential downstream calcium signaling but also from the promoted HBV core assembly.

Generally, cytosolic calcium concentration is from 0.1 \(\mu\)M (resting state) to 1–3 \(\mu\)M (excited state) (Brini, 2003). However, the CaCl\(_2\) concentration for in vitro experiments was higher than general physiological relevant. In respect to this reason, we supposed that there is other unknown machinery to facilitate the HBV core assembly in vivo. Furthermore, it is possible that the difference in the amounts of assembled core proteins depends on their stability. However, we thought that this possibility would be low...
Fig. 5. The effects of BAPTA-AM, CsA, TG, and HBx on the HBV core assembly. HepG2 cell was transfected by each plasmid DNA. The chemicals were treated daily for 72 h. Assembled core particles in the cytoplasm, separated by sucrose step gradient, were resolved in 1% agarose gel. Results were normalized to the untreated pHBV1.2x and pCMV/FLAG-Core transfected control, which were set at 100% in all experiments, respectively. (A) pHBV1.2x and pCMV/β-gal transfected HepG2 cells. BAPTA-AM (10 μM) and CsA (1 μM) were daily treated, respectively, and the total core protein level was normalized by β-gal assay. Top panel indicates the total HBV core proteins separated by 12.5% SDS-PAGE after immunoprecipitation with a rabbit polyclonal anti-HBc Ab according to the normalization by β-gal assay. (1) Light chain of a rabbit polyclonal anti-HBc Ab, (2) HBV core proteins. (B) pCMV/FLAG-Core and pCMV-HA/HBx-transfected HepG2 cells. BAPTA-AM (10 μM) and CsA (1 μM) were daily treated, respectively, and the total core protein level was normalized by a mouse monoclonal anti-FLAG Ab (top panel). The middle and bottom panel show the corresponding Western blot analysis of HBx in 15% SDS-PAGE and the assembled core particles in 1% native agarose gel, respectively. (C) pCMV/FLAG-Core transfected and TG-treated HepG2 cells. TG (5 nM) was treated daily and the total core protein level was normalized by a mouse monoclonal anti-FLAG Ab (top panel). (D) pHBV1.2x(-X) with pCMV-HA/HBx-transfected HepG2 cells. However, one well of six-well plates (lane 4) was co-transfected with pCMV-HA/HBx to investigate the recovery of core assembly by the HBx in the HBx-deficient HBV mutant. The total core protein level was normalized by β-gal assay. *P < 0.05 by Student’s t test.
because HBV capsid particles are so robust that they can be dissociated at pH 2 or 14, at temperatures higher than 75 °C, or in 0.1% SDS (Newman et al., 2003).

In this report, we found that calcium ions affect the HBV core assembly in in vitro and in ex vivo conditions. Moreover, we discovered that the increased Ca\(^{2+}\) by HBx increased the HBV core assembly. Recently, it has been reported that HBV replication was activated by HBx-mediated calcium stimulation (Bouchard et al., 2001). Therefore, our data indicate that this activation of HBV replication is related to the increased HBV core assembly by calcium ions. These results may be helpful for further study of HBV replication mechanism and the role of HBx.

**Materials and methods**

**Plasmids**

For reducing the variation by different promoters, all plasmids for ex vivo experiments were cloned to pCMV-HA vector (Clontech, Palo Alto, CA) to have the same CMV promoter. The pHBV1.2x construct, subtype adr, was previously described (Park et al., 2003). This construct supports HBV replication by providing all required HBV transcripts. The pCMV/FLAG-Core was constructed by inserting the FLAG-tag into pCMV/Core (Park et al., 2003) at the N-terminus by PCR. The pCMV-HA/HBx was constructed by insertion of an HBx gene fragment generated by PCR into the EcoRI site in pCMV-HA (Fig. 1A). The Core149 gene was amplified by PCR using the forward primer, 5′-ACCATGGACATTGACCCGTATAAAG-3′ including NcoI and the reverse primer, 5′-ACTCGAGTAAACAACAGTAGTTCCGG-3′ including XhoI using pHBV1.2x as a template. The Core149 gene described above was ligated to pGEMT-easy vector (Promega, Madison, WI) after PCR amplification and was transformed to DH5α E. coli host. Amplified core149 gene was cut at the enzyme sites of NcoI and XhoI, and then ligated to pET28b E. coli expression vector (Novagen, Madison, WI). This ligated plasmid, pET28b/Core149 was transformed to BL21(DE3) + pLysS expression host (Novagen). The pHBV1.2x(-X) plasmid (Novagen, Madison, WI) was ligated to pET28b/Core149 was transformed to BL21(DE3) + pLysS expression host (Novagen). The pHBV1.2x(-X) plasmid (Novagen, Madison, WI) was ligated to be used for in vivo experiments under the control of CMV promoter.

**Cp149 expression and purification**

Cp149, a truncated form of 34 residues from its carboxy-terminal group of the HBcAg, was expressed after induction with isopropyl-β-D-1-thiogalactoside (IPTG) (Duchefa biochemie, Haarkem, Netherlands) and purified as previously described (Zlotnick et al., 1996). However, we substituted the 100 mM sodium bicarbonate buffer (pH 9.5) for 100 mM glycine buffer (pH 9.5) in the final purification step. Stock solutions of Cp149 dimers [2 mg/ml in 100 mM glycine (pH 9.5) containing 10% glycerol] were stored at −70 °C. The yield of purified HBV dimer proteins was 7.9 mg per g weight (wt) of starting cells. Purified Cp149 was identified by Western blot with a rabbit polyclonal anti-HBc antibody (Ab) (DAKO, Glostrup, Denmark) and MALDI-TOF MS. MALDI-TOF MS analysis was performed at the National Center for Inter-University Research Facilities (NCIRF, SNU, Korea) by a Voyager-DE STR Biospectrometry Workstation (Applied Biosystems Inc., Foster City, CA).

**In vitro Cp149 assembly and sucrose density gradient analysis**

Purified Cp149 was set at a concentration of 90 μM. For Cp149 assembly, the reaction buffer [150 mM HEPES (pH 7.5) including 15 mM NaCl] was mixed with Cp149 at a ratio of 2:1 and the concentrations of 5 mM and 10 mM CaCl\(_2\) (final concentration) were added to the reaction, respectively. In vitro assembly reaction was performed at 37 °C for 1 h. After the assembly reaction, sucrose density gradient [1 ml of 50% (wt/vol), 1 ml of 40%, 1 ml of 30%, 1 ml of 20%, 0.85 ml of 10% sucrose in 150 mM HEPES (pH 7.5) and 150 μl of reaction sample] was performed by spinning for 1.5 h at 46,000 rpm at a temperature of 20 °C using a P55ST2 rotor of CP-100x (Hitachi koki Co., Ltd, Tokyo, Japan). Ten fractions of each of the 500 μl were collected from top to bottom. Each fraction 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 was boiled at 100 °C for 5 min. Identical amounts of each fraction were loaded and separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with SYPRO orange (Bio-Rad, Hercules, CA), then scanned on an FLA-2000 (Fuji Photo Film Co., Ltd, Tokyo, Japan) and bands were determined quantitatively by use of 1D Image Analysis Software (Windows Version 3.0.2, Eastman Kodak company, Rochester, NY). The integrated density of individual bands was plotted as a function of the gradient fraction number.

**Analytical ultracentrifugation**

Analytical ultracentrifugation was performed at the National Instrumentation Center for Environmental Management (NICEM, SNU, Korea) by XL-A analytical ultracentrifuge (Beckman Coulter Inc., Fullerton, CA) with An-60 Ti rotor and 12-mm standard double sector centerpiece cells as previously mentioned (Zlotnick et al., 1996, 1999b). Cp149 dimer proteins (2 mg/ml) in 100 mM Glycine (pH 9.5) were immediately mixed by the reaction buffer [150 mM HEPES (pH 7.5) including 150 mM NaCl] at a ratio of 2:1 and concentrations of 5 mM...
and 10 mM CaCl$_2$ (final concentration) were added to the reaction, respectively. Sedimentation velocity was measured at 20,000 rpm for 2 h at 20 °C with data collection every 10 min. Data was analyzed using the SEDFIT program (http://www.analyticalultracentrifugation.com) using the method of Schuck (2000).

**Electron microscopy**

In vitro Cp149 assembly by 10 mM CaCl$_2$ was performed as mentioned above. However, Cp149 was also assembled with the reaction buffer [150 mM HEPES (pH 7.5) including 300 mM NaCl] at a ratio of 2:1 as a control (Fig. 4A) (Zlotnick et al., 1996). For negative staining, 10 μl of a solution containing the assembled core particles was applied to a carbon-coated grid and incubated for 1 min. The grid was washed with water and stained with 2% uranyl acetate for 1 min. Transmission electron micrographs were taken on a JEM 1010 (JEOL Ltd, Tokyo, Japan) operating at 80 kV at the NICEM.

**Cell culture, transfection, and chemical treatment**

HepG2 cells were cultured on a six-well multidish (Nunc, Rochester, NY) in a minimal essential medium (MEM) (Sigma) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 1× antibiotic–antimycotic (Invitrogen) and was maintained at 37 °C in 5% CO$_2$. Cells were transfected with FuGENE 6 (Roche, Mannheim, Germany) transfection agents as instructed by the manufacturer. After 6 h, the cells were washed once by 1× PBS and treated with chemicals 10 μM BAPTA-AM (Sigma), 1 μM Cyclosporine A (CsA) (Sigma), and 5 mM Thapsigargin (TG) (Sigma) in MEM, respectively. Chemical concentrations were determined by triplicated MTT assay. Cells were incubated in light-protective condition with daily chemical treatment for 72 h. Experiments were triplicated, separately.

**β-galactosidase assay (β-gal assay)**

Ten microliters of cell lysate was mixed with 1 μl of 100× MgCl$_2$ (which included 4.5 M 2-mercaptoethanol), 22 μl of 1× o-Nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma), and 67 μl of 100 mM sodium phosphate (pH 7.3). The reaction was performed at 37 °C for 1 h and then stopped with 100 μl of 1 M Na$_2$CO$_3$. The reactant density was measured by a spectrometer (Gene spec III) (Naka Instruments, Ibaraki, Japan) at 420 nm.

**Detection of HBV core proteins in the cytoplasm by SDS-PAGE**

The HBV core proteins in the cytoplasm of pHBV1.2x transfected HepG2 cells cultured on a 100-mm dish (Nunc) were immunoprecipitated with a rabbit polyclonal anti-HBc Ab (1:4,000) (DAKO) as previously described (Schlicht, 1991). Immunoprecipitated HBV core proteins were absorbed to Protein A-Sepharose CL4B (Sigma). After being washed twice with 1× PBS, precipitated proteins were solubilized in the sample buffer and separated on a 12.5% SDS polyacrylamide gel. Denatured proteins with SDS-PAGE were transferred onto PVDF (PerkinElmer Life Sciences, Inc., Boston, MA) and Western blotted with a rabbit polyclonal anti-HBc Ab (1:4,000) (DAKO).

**Detection of assembled HBV core particle in the cytoplasm by agarose gel electrophoresis**

The assembled HBV core particles in the cytoplasm were separated by sucrose step gradient as previously described (Park et al., 2003), but the sucrose step gradient buffer was in 100 mM HEPES (pH 7.5). Also, the pellet was suspended in 50 μl of 100 mM HEPES (pH 7.5) by sonication (three strokes, 1 s each). Electrophoresis was carried out at 40 V and then the resolved proteins in the gel were transferred overnight onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH) by capillary transfer using 10× SSC containing 1.5 M NaCl and 1.5 M sodium citrate (pH 7.0). The cytosolic core particles on the membrane were detected by Western blot analysis with a rabbit polyclonal anti-HBc Ab (1:4,000) (DAKO).

**Western blotting**

Denatured proteins with SDS-PAGE were transferred onto PVDF (PerkinElmer Life Sciences) membrane by semi-dry transfer cell (Bio-Rad) for 1 h at 24 V. Assembled HBV core particles in the cytoplasm (in nondenatured condition) were transferred onto nitrocellulose membrane (Schleicher & Schuell). PVDF membrane and nitrocellulose membrane were blocked by 10% fat-free milk in PBS-T, which included 0.1% Tween 20 for 1 h, and then each blot was incubated for 2 h at 4 °C with a mouse monoclonal anti-FLAG Ab (5 μg/ml) (Sigma), or a rabbit polyclonal anti-HBc Ab (1:4,000) (DAKO), or a mouse monoclonal anti-HA Ab (1:10,000) (Sigma). After three washes with PBS-T for 1.5 h, horseradish-conjugated anti-mouse or anti-rabbit Ab (1:40,000) (Sigma) were incubated for 2 h at 4 °C. After three washes for 30 min with PBS-T, the HRP signal was detected with an enhanced chemiluminescence (ECL) (PerkinElmer Life Sciences) on the X-ray film (Fuji Photo Film Co., Ltd). The detected antibodies on the X-ray film were quantified through 1D Image Analysis Software (Eastman Kodak Company).

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Appendix A. Supplementary data


References


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