이학석사학위논문

Heat shock protein 90 facilitates formation of the HBV capsid via interacting with the HBV core protein dimers

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열 충격 단백질 90 에 의한 HBV 캡시드 형성 촉진

2012 년 8 월

서울대학교 대학원
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지도교수 정 구 홍

이 논문을 이학석사 학위논문으로 제출함

2012 년 8 월

서울대학교 대학원

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논문제목: **Heat shock protein 90 facilitates formation of the HBV capsid via interacting with the HBV core protein dimers**

학위구분 : 석사
학 과 : 생명과학부
학 번 : 2007-22844
연 락 처 :
저 작 자 : 심 희 연 (인)

제 출 일 : 2012 년 8 월 1 일

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Abstract

Heat shock protein 90 facilitates formation of the HBV capsid via interacting with the HBV core protein dimers

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The mechanism by which host factors contribute to hepatitis B virus (HBV) capsid formation during the viral life cycle remains unclear. This study analyzed the interaction between heat shock protein 90 (Hsp90), a host factor, and the HBV core protein. Hsp90 was found to bind to HBV core protein dimers, which was then encapsidated into the HBV capsid. Furthermore, activated Hsp90 may facilitate the formation of the human HBV capsid by catalyzing core assembly and reducing the degree of capsid dissociation at various temperatures, both *in vitro* and *in vivo*, and when subjected to detergent treatments *in vitro*. In addition, inhibition or down-regulation of Hsp90 reduced HBV production in HepG2.2.15 cells. These results showed that Hsp90 plays an important role in HBV capsid stabilization and HBV formation.

Keywords: Hepatitis B virus (HBV), core protein truncated at residue 149 (Cp149), host factor, Heat shock protein 90 (Hsp90), encapsidation, core assembly, capsid dissociation.

Student Number: 2007-22844

Content

Introduction

Human Hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family and has infected over two billion people worldwide (Vanlandschoot et al., 2003). Chronic HBV infection is associated with liver disease, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC), particularly in Asian and African cases (Zuckerman, 1999).

The HBV genome consists of a partially double-stranded 3.2-kb circular DNA that encodes four proteins: a surface protein (HBs), a core protein (HBc), a polymerase (HBV pol), and an X protein (HBx) (Seeger and Mason, 2000). The core protein plays an essential role in the HBV life cycle by packaging HBV pol, pre-genomic RNA (pgRNA), and other components, such as heat shock proteins and protein kinases (Seeger and Mason, 2000). Since the virus is generated by the assembly of core proteins with the viral genome and other proteins, core assembly is a critical step in HBV replication.

The core protein consists of 183–185 amino acids and is organized into two domains: an N-terminal domain (amino acids 1–149; Cp149), which is involved in core assembly, and a C-terminal domain (amino acids 150–183 or 185), which regulates viral replication (Seeger and Mason, 2000).

Cp149, a 34-residue, C-terminal truncated form, spontaneously forms a capsid under suitable conditions *in vitro* and *in vivo* (Kim et al., 2001). This protein has been used to study capsid assembly because it can be overexpressed in *Escherichia coli* more efficiently than the full-length core protein and is structurally similar to the HBV core protein (Biermer et al., 2003). Capsid formation is a key event in the viral life cycle because the capsid protects packaged viral and host factors. The identity of the host factors, such as heat shock proteins, that interact with the core protein and affect HBV capsid formation is unknown.

Heat shock protein 90 (Hsp90) is composed of three domains: an N-terminal ATP-binding domain, a middle domain, and a C-terminal dimerization domain (Prodromou et al., 1997; Stebbins et al., 1997; Terasawa et al., 2005). Hsp90, which functions in an ATP-dependent manner and often together with a co-chaperone, interacts with various proteins, including kinases and transcription factors, and controls biological processes by stabilizing protein folding (Cho et al., 2000a; Cho et al., 2000b; Clark et al., 2009; Ganem and Varmus, 1987; Hu et al., 2004; Hu et al., 2002). Geldanamycin (GA) binds directly to the ATP-binding domain of Hsp90, thereby preventing ATP binding and reducing its affinity for client proteins (Buchner, 1999; Ujino et al., 2009).

Hsp90 is known to be involved in duck HBV replication. During duck HBV replication, Hsp90 and the co-chaperone p23 bind together and affect HBV pol activity. Moreover, Hsp90 becomes encapsidated with the duck HBV pol/pgRNA complex (Clark et al., 2009; Hu et al., 1997). Human and duck HBV replication are similar in that Hsp90 binds to HBV pol and affects HBV pol activity in both strains of the virus (Cho et al., 2000a; Cho et al., 2000b; Hu et al., 2004). However, human HBV differs from duck HBV in that Hsp90 makes human HBV pol competent for *in vitro* priming rather than simply maintaining the human HBV pol/pgRNA complex (Gyoo Park et al., 2002).

HBV pol is known to bind to human HBV core proteins before being packaged into capsids (Lott et al., 2000), and also to Hsp90 and p23 (Hu et al., 1997). However, the relationship between Hsp90 and human HBV core proteins is unknown.

In this study, we established that Hsp90 binds to core protein dimers, but not to the capsid surface, and that Hsp90 is internalized into the human HBV capsid when Hsp90-bound core protein dimers form the capsid. This work demonstrates that Hsp90 can be packaged into the capsid by interacting not only with the HBV pol/pgRNA complex but also with core proteins. Furthermore, the presence of activated Hsp90 and HBV core protein dimers not only facilitated capsid formation but also

reduced capsid dissociation when subjected to various temperatures and detergent treatments.

Results

Hsp90 binds to HBV core protein dimers

To determine if Hsp90 binds to HBV core proteins, a co-immunoprecipitation (co-IP) analysis was performed. The results show that Hsp90 binds to the HBV core protein (Fig. 1A). The location of the interaction between the Hsp90 and the HBV core protein was investigated by co-IP and binding assays using purified proteins. Hsp90 was found to bind to Cp149 dimers but not to the capsid surface (Fig. 1B and 1C). In addition, each binding sites was identified (Fig. S1).

Hsp90 is incorporated into the HBV capsid through binding with Cp149 dimers

In ducks, Hsp90 is packaged into the capsid by interacting with the duck HBV pol/pgRNA complex (Hu et al., 1997; Nassal, 1999). We sought to determine whether an alternative pathway of Hsp90 packaging into human HBV capsid exists other than the pathway described for duck HBV. Since Hsp90 binds to Cp149 dimers, we assumed that this may be the

mechanism by which Hsp90 is packaged into the capsid. Sucrose density gradient analysis showed that when Hsp90 was mixed with Cp149 dimers, Hsp90 was present in fractions 8–10. The capsid was also detected in these fractions after the assembly reaction (Fig. 2A). When Hsp90 was mixed with BSA (control), it was detected in fractions 2–4 (data not shown). Two samples each from fractions 3 and 8 (Fig. 2A) were examined under non-denaturing and denaturing conditions to confirm that Hsp90 packaging into the capsid. SDS-PAGE revealed the presence of Hsp90 in fractions 3 and 8 (Fig. 2B, lanes 3 and 4, bottom panel). However, dot blot and native agarose gel analyses, which do not interfere with capsid formation, showed no detectable Hsp90 in the fractions 3 and 8 samples (Fig. 2B, top and middle panels). Hsp90 may be undetectable in the fraction 8 sample due to its localization inside the capsid. Since native agarose gel electrophoresis can only detect the capsid, Cp149 was only detectable in the fraction 8 sample (Fig. 2B, lane 4 of middle panel). In addition, dot blot analysis detected Hsp90 and Cp149 dimers (Fig. S2). From these results, we conclude an alternative packaging pathway where Hsp90 is packaged into the capsid by binding to Cp149 dimers.

Activated Hsp90 facilitates HBV core protein assembly

HBV core assembly begins with Cp149 homodimer formation through a cysteine disulfide bond between the 61st amino acid of each subunit (Nassal et al., 1992; Zheng et al., 1992). Hsp90 activity requires protein p23 binding and ATP, and its activity facilitates maturation of the client protein (Sullivan et al., 1997; Woo et al., 2009). To examine whether activated Hsp90 affects HBV core assembly, an assembly reaction with Cp149 dimers or a core protein point mutant (C61A) in the presence of Hsp90 was performed. The presence of activated Hsp90 facilitated capsid formation (Fig. 3A) not only for Cp149 dimers but also for C61A; however, the C61A capsid did not form in the absence of Hsp90 or in the presence of Hsp90 inactivated by GA treatment (Fig. 3B). In addition, sucrose density gradient analysis showed that capsid formation increased in the presence of ATP-activated Hsp90 but was unaffected by BSA or Hsp90 inactivated by GA (Fig. 3C). To confirm that activated Hsp90 facilitates HBV core assembly, activated Hsp90, an Hsp90 mutant (N190Δ, in which the ATPase site was deleted), and a BSA (control) were independently added to Cp149 dimers and capsid formation was assessed in a time-course experiment. Capsid formation in the presence of activated Hsp90 was faster than in the presence of the Hsp90 mutant or BSA. The Hsp90 deletion mutant (N190Δ) slowed the saturation of capsid formation to 120 min, as did the absence of Hsp90 (i.e., the BSA control) (Fig. 3D). The

intensity of the Cp149 dimer band at 15 min after the assembly reaction was set to 1, and the relative intensities of the other bands were analyzed in comparison to this band. Identical amounts of Cp149 were added to each group. Thus, capsid levels in all samples were saturated at 120 min (Fig. 3D, right graph).

HBV capsid formation facilitated by activated Hsp90 is regulated by temperature changes

The above results clearly demonstrate that activated Hsp90 facilitates capsid formation (Fig. 3). Hsp90 activity is known to suppress thermal stress-protein aggregation and protect unfolded proteins from fully unfolding while promoting rapid recovery (Freeman and Morimoto, 1996; Yonehara et al., 1996). The HBV capsid formation from HBV core protein dimers is temperature-dependent (Hilmer et al., 2008). To determine how activated Hsp90 affects HBV capsid formation at varying temperature, capsid formation *in vitro* was assessed over a range of temperatures in the presence of (1) Cp149 dimers and BSA (control), (2) Cp149 dimers and activated Hsp90, and (Huang et al.) Cp149 dimers and GA-treated Hsp90. In the control experiment, capsid formation peaked at 37°C (Fig. 4A, left

panel). In Cp149 dimer and activated Hsp90 experiment, capsid formation peaked over a wide range of temperatures, from 30°C to 43°C, and at levels that were twice the control levels (Fig. 4A, middle panel). To demonstrate that capsid assembly was stabilized exclusively by activated Hsp90, GA was added to Hsp90 and Cp149 dimers. GA-treatment reversed the effect of Hsp90 and resulted in the same pattern as when using Cp149 and BSA (Fig. 4A, right panel). To assess the effect of Hsp90 on HBV capsid formation in cells at various temperatures, pCMV/Flag-core was transfected into Huh7 cells, and the cells were treated with GA. After heat shock, HBV capsid levels were measured by immunoblot analysis. In contrast to results obtained using purified proteins (Fig. 4A, middle panel), capsid formation levels in cells not treated with GA remained constant, regardless of the temperature (Fig. 4B, left panel). This is because capsid formation saturated during the 48 h incubation after transfection. Heat shock after treatment with GA, however, affected capsid formation; the levels decreased relative to capsid formation levels in cells not treated with GA at all temperatures except 37°C (Fig. 4B, right panel). A similar result was obtained for the experiment conducted only in the presence of Hsp90 (data not shown). Hsp90 inactivated by GA cannot facilitate temperature-dependent capsid formation, reflecting the same pattern as the experiment using purified proteins. These results support the hypothesis that activated

Hsp90 stabilizes capsid formation under temperatures ranging from 30°C to 43°C.

Activated Hsp90 decreases detergent-mediated dissociation of the HBV capsid

Next, we assessed the protective effect of Hsp90 on capsid dissociation in the presence of denaturing agents. Capsids were treated with varying detergent concentrations, including urea and SDS, at 37°C for 30 min. When capsids were treated with 3 M urea, activated Hsp90 (Fig. 5A, middle panel) maintained capsid levels 2.7-fold higher than in the absence of Hsp90 (Fig. 5A, left panel) or in the presence of Hsp90 treated with GA (Fig. 5A, right panel). Capsid levels after 0.05% SDS treatment were 2.4-fold higher in the presence of activated Hsp90 (Fig. 5B, middle panel) than in the absence of Hsp90 (Fig. 5B, left panel) or in the presence of Hsp90 treated with GA (Fig. 5B, right panel). The shielding effect of Hsp90 on capsid dissociation disappeared as SDS concentration exceeded 0.1%, and all capsids dissociated beyond this point (Fig. 5B). To monitor HBV capsid dissociation in response to detergent treatment, Cp149 dimers assembled *in vitro* and treated with detergents were observed by

transmission electron microscopy. In a previous report, the diameter of Cp149 was determined to be approximately 30 nm (Newman et al., 2003). TEM data showed that, in the presence of activated Hsp90 and in the untreated control, compact spherical capsids were formed with an average diameter of 30 nm (Figs. 5C, 5D and 5F; average diameters, 30.5 nm, 30.75 nm, and 30.87 nm). However, in the presence of GA-inactivated Hsp90, 95% of capsids formed were irregular complexes or “shrinking capsids” (Fig. 5E) and 42% were “inflating capsids” (Fig. 5G). These results show that activated Hsp90 reduces the HBV capsid dissociation degree induced by detergents.

Both inhibition and downregulation of Hsp90 reduce the amount of extracellular HBV DNA

HepG2.2.15 cells derived from HepG2 cells produce HBV (Liu et al., 2009). To measure the amount of extracellular HBV DNA following Hsp90 inhibition and down-regulation, HepG2.2.15 cells were treated with GA and transfected with shRNA-Hsp90. After 24 h, HBV DNA isolated from the culture medium was quantified using real-time PCR (qRT-PCR). Compared with untreated HepG2.2.15 cells, the amount of intracellular

capsid formed in GA- and shRNA-Hsp90-treated cells was reduced by 53% and 49%, respectively (Fig. 6A, lanes 3 and 4); extracellular HBV DNA was also reduced (Fig. 6B, lanes 3 and 4, 50% and 48%, respectively). Next, HepG2.2.15 cells were treated with lamivudine (3TC) to inhibit HBV pol activity for 24 h. Extracellular HBV DNA was reduced by 83% (Fig. 6B, lane 5), but there was no change in capsid assembly (Fig. 6A, lane 5). Consistent with previous studies' results (Bouchard et al., 2003; Severini et al., 1995), this result indicates that inhibition of polymerase activity by 3TC decreases extracellular HBV DNA levels through inhibition of HBV DNA synthesis in the cytoplasm; however, it does not decrease capsid assembly. Importantly, HBV pol inhibition does not affect intracellular capsid formation. Furthermore, when HepG2.2.15 cells were treated with 3TC and GA at the same time, the amounts of intracellular capsid and extracellular HBV DNA were reduced by 62% and 63%, respectively, compared to cells treated with 3TC only (Fig. 6B, lane 6). This means that, on the premise that polymerase activity is inhibited by 3TC, the reduced intracellular capsid levels due to Hsp90 inhibition affects the amount of extracellular HBV DNA. Hsp90 levels, except for the shRNA-Hsp90 transfected sample, were constant in all samples. B-actin was used as a control (Fig. 6C). These results suggest that Hsp90 is a host factor that contributes to the amount of extracellular HBV DNA not

only through HBV pol activity but also through capsid assembly.

Discussion

HBV capsid assembly is crucial to HBV replication in the HBV life cycle. Host factors, such as heat shock proteins and protein kinases, which are packaged into the HBV capsid with the HBV pol/pgRNA complex, contribute to HBV replication in ducks and humans (Bartenschlager and Schaller, 1992; Hirsch et al., 1990; Hu et al., 2004; Hu et al., 1997; Kann and Gerlich, 1994; Nassal, 1999). Hsp90,one of the host factors, affects polymerase priming and HBV pol/pgRNA complex formation by interacting with the HBV pol (Cho et al., 2000a; Cho et al., 2000b; Gyoo Park et al., 2002; Hu et al., 2004; Hu et al., 2002; Wang et al., 2002). Nonetheless, the relationship between Hsp90 and the HBV core protein has never been studied.

Both Hsp90 and another host factor, DD3X DEAD-box RNA helicase, which unwinds RNA in an ATPase-dependent manner, are encapsidated by binding the HBV pol (Hu et al., 2004; Hu et al., 2002; Wang et al., 2009). However, we discovered a relationship between Hsp90 and the HBV core protein and determined that Hsp90 interacts with HBV core protein dimers to be packaged into the capsid (Fig. 1 and Fig. 2). These results differ from current hypotheses on the encapsidation pathway of Hsp90. Most

importantly, our study presents a new mechanism by which Hsp90 is packaged into the HBV capsid. In further experiments, we identified that Hsp90, HBV core protein, and HBV pol form a complex, although HBV pol and core protein were identified by only a two-peptide sequence using MS/MS analysis (Thermo Fisher Scientific, Inc) (Table S1). Combined with the results of previous studies that showed HBV pol binding to Hsp90 (Cho et al., 2000a; Cho et al., 2000b) and HBV core protein (Lott et al., 2000), our results indicate Hsp90, HBV core dimer, and HBV pol may form a complex. Therefore, a more extensive study on the relationship among Hsp90, HBV core protein, and HBV pol is required to fully understand the process of HBV replication.

The effect of molecular chaperones on capsid formation during HBV replication is not clear. However, protein kinase A (PKA) and protein kinase C (PKC) activities increase HBV core assembly and capsid stability (Kang et al., 2008; Kang et al., 2006), but a host factor, Hsp40, decreases HBV replication and capsid formation by binding to the HBV core protein (Sohn et al., 2006). Additionally, Hsp90 binds P1, the core protein of picornaviruses, along with p23 (Geller et al., 2007), but the effect of Hsp90 is different. In contrast to our finding that Hsp90 facilitates HBV core assembly by binding to the HBV core protein (Fig. 3), Hsp90 binds P1 to prevent capsid degradation caused by the viral-encoded protease in

picornaviruses (Geller et al., 2007).

HBV Cp149 is an arginine-rich domain (ARD), a truncated form of full-length core protein (Newman et al., 2009). In addition, HBV Cp149 assembles spontaneously to form the capsid under optimal conditions (Wingfield et al., 1995), but it can dissociate in some external environments (Newman et al., 2003). Because activated Hsp90 suppresses HBV capsid dissociation in detergents (Fig. 5), we conclude that Hsp90 not only facilitates HBV capsid formation but also decreases HBV capsid dissociation.

On the basis of these results, we hypothesize that Hsp90 increases the affinity between core protein dimers to induce HBV capsid formation and maintains the core-core interaction to suppress HBV capsid dissociation by binding the HBV core protein.

Inhibition of Hsp90 affects the ability of various viruses to replicate. Inhibition of Hsp90 down-regulates RNA replication in the flock house virus (Kampmueller and Miller, 2005) and suppresses replication of the hepatitis C virus by blocking the function of nonstructural protein 5A (NS5A), a viral replicase, and Hsp90 complex (Okamoto et al., 2006). Our results show that both inhibition and downregulation of Hsp90 decrease HBV titers and capsid levels (Fig. 6). These results indicate that Hsp90 contributes to the production of HBV particles through both polymerase activity and capsid

assembly.

Expression levels of Hsp90 increase in tumorous tissues (Lim et al., 2002), and up-regulation of Hsp90 facilitates tumor cell invasion induced by HBx to affect HBV-related tumor progression (Li et al., 2010). Moreover, inhibition of Hsp90 induces the inactivation and degradation of hepatocarcinogenesis-driving factors, suggesting that Hsp90 may play an important role in the development of HCC (Breinig et al., 2009).

In conclusion, our study reveals that Hsp90 encapsidates into the HBV capsid and increases HBV capsid stability by interacting with HBV core protein dimers and that Hsp90 is related to the production of HBV particles in HepG2.2.15 cells. These results provide new insights into the mechanism of viral assembly and HBV replication, which are associated with host factors. Furthermore, Hsp90 may be a new drug development target for the treatment of HCC.

Materials and Methods

Cell culture and transfection

Human hepatoma cell lines, Huh7 and HepG2.2.15, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Welgene, Daegu, Republic of Korea) and 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and maintained at 37°C in 5% CO_2. The cells were transfected with Fugene 6 (Roche, Mannheim, Germany) transfection reagent as instructed by the manufacturer.

Expression and purification of Cp149, Cp149 point mutants, and p23 protein

Cp149 was cloned directly using a pET30a vector (Novagen, Madison, WI, U.S.A.). Constructs were transformed into BL21(DE3) + pLysS *E. coli* (Novagen, Madison, WI, U.S.A.) and purified as described in a previous study (Choi et al., 2005). The core protein point mutant, C61A, was constructed using a site-directed mutagenesis protocol (Qiagen GmbH, Hilden, Germany). The following forward (F) and reverse (R) primers were

used, and pCMV/Flag-core (Kang et al., 2008) was used as a template: C61A, F-5′-CAGGCAAGCTATTCTGGCTTGGGGTGAGTTGATG-3′ and R-5′- CATCAACTCACCCCAAGCCAGAATAGCTTGCCTG-3′. Purified core protein dimers were stored in 100 mM glycine (pH 9.5). At this pH, core protein dimers are stable (Wingfield et al., 1995). For Cp149 assembly, the reaction buffer (50 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 15 mM NaCl, and 10 mM $CaCl_2$) was mixed with Cp149 and incubated at 37°C for 30 min (Choi et al., 2005). The p23 protein was directly cloned from a pET28b vector (Novagen). Constructs were transformed into BL21(DE3)+ pLysS *E.coli* (Novagen) and purified as described in a previous study (Choi et al., 2005).

Expression and purification of Hsp90 and Hsp90 deletion mutant

Human Hsp90β was cloned into the expression vector pET28b (Cho et al., 2000a). The Hsp90 mutant was amplified by PCR using forward (F) and reverse (R) primers with pET28b-Hsp90β as the template: N190Δ (191–724), F-5′-GCGGTCGACCAGAGTACCTAGAAGAGAGG-3′ and R-5′-CCGCGGCCGCCTAATCGACTTCTTCC-3′. This construct has a hexa-

histidine tag added to the N-terminus of Hsp90 (Cho et al., 2000b) and the Hsp90 deletion mutant, which were overexpressed in *E. coli* BL21(DE3) cells cultured in 2 × YT medium. Protein expression was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were incubated for an additional 20 h at 22°C. The cells were lysed by sonication in lysis buffer (20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 5% (v/v) glycerol). The crude lysate was centrifuged at 18,000 × *g* for 60 min. The supernatant was applied to an affinity chromatography column of nickel-nitrilotriacetic acid (Ni-NTA)-agarose (Qiagen). The protein was eluted with lysis buffer containing 500 mM imidazole.

Co-immunoprecipitation of the HBV core protein and Hsp90

For co-immunoprecipitation (co-IP) of Hsp90 with the HBV core protein, Huh7 cells were transfected with pCMV/Flag-core. After 48 h, cell lysates were co-immunoprecipitated with a mouse monoclonal anti-HBV core antibody (Ab) (Santa Cruz, sc-23945) and a goat polyclonal anti-Hsp90 Ab (Santa Cruz, sc-1057) at 4°C for 2 h. Immunoprecipitated lysates were analyzed by 15% SDS-PAGE followed by immunoblot analysis with a mouse monoclonal anti-FLAG M2 Ab (1:5000; Sigma, St. Louis, Mo, F3165) and a goat polyclonal anti-Hsp90 Ab (1:1000; Santa Cruz, sc-

1057). For co-IP of Hsp90 with Cp149 dimers or capsid, 20 μM of Cp149 dimers or capsid was added to 20 μM of Hsp90 and the samples were incubated at 30°C for 1 h. Cp149 dimers (or capsid) and Hsp90 were co-immunoprecipitated as described above. Immunoblot analysis was performed using a rabbit polyclonal anti-HBV core Ab (1:4000; Dako, B0586) and a goat polyclonal anti-Hsp90 Ab, 1:1000 (Santa Cruz, sc-1057).

Binding assay of Hsp90 with the HBV core protein

To assay the binding of Hsp90 with the HBV Cp149 dimers and capsid, 5 μg of His-tagged Hsp90 was bound to 100 μl of Ni-NTA agarose at 4°C for 1 h. The Hsp90-Ni-NTA mixture was loaded onto a column with the bottom outlet capped. The bottom cap was removed, and the column was drained. Next, 5 μg of each Cp149 dimers and capsid were added to the column. Ni-NTA agarose-coupled proteins were washed with TN buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl) 4 times and eluted with TN buffer containing 500 mM imidazole. Eluates were analyzed on a 15 % SDS-PAGE followed by immunoblot analysis with a goat polyclonal anti-Hsp90 Ab (1:1000; Santa Cruz, sc-1057) and a rabbit polyclonal anti-HBV core Ab (1:4000; Dako, B0586).

Sucrose density gradient analysis and dot blot assay

The assembly reaction was performed at 37°C for 30 min in reaction buffer. After the assembly reaction, sucrose density gradient analysis was performed by ultra-centrifugation at 20°C for 4 h 30 min and 160,000 × *g* using a P55ST2 rotor of CP-100α (Hitachi Koki Co., Ltd, Tokyo, Japan), and included gradient ranges of 10–50% (w/v) sucrose in 50 mM Hepes (pH 7.5). Fractions were collected, loaded onto a 15% SDS-PAGE, and subjected to immunoblot analysis with rabbit polyclonal anti-HBV core Ab (1:4000; Dako, B0586) and goat polyclonal anti-Hsp90 Ab (1:1000; Santa Cruz, sc-1057). Dot blot analysis was performed according to the instructions in the Bio-Dot Microfiltration Apparatus manual (Bio-Rad Laboratories Inc., Hercules, California, USA).

Detection of the HBV capsid by native agarose gel electrophoresis

To study the effect of Hsp90 on HBV capsid formation, 20 μM Cp149 dimer and 20 μM Hsp90 were incubated in reaction buffer containing 20 mM Na_2MoO_4, 1 mM DTT, 0.01% NP-40, and 0.5 mM adenosine 5′-*O*-(3-

thiotriphosphate) (ATP-γ-S) (Sullivan et al., 1997). Samples were incubated at 37°C for 30 min, separated by electrophoresis on a 0.9% native agarose gel, and subjected to immunoblot analysis with rabbit polyclonal anti-HBV core Ab (1:4000; Dako, B0586) as described in a previous study (Kang et al., 2006). Capsid bands were analyzed by ImageMaster 2D Elite software 4.01 (Amersham Pharmacia Biotechnology).

Treatment with Hsp90 inhibitor and temperature changes in Huh7 cells

An Hsp90 inhibitor, geldanamycin (GA; 4 μM, A.G. Scientific, Inc., San Diego, CA, USA), was added to Huh7 cells. Cells were transfected with pCMV/Flag-core to express the core protein. Cells were transferred to a CO_2 incubator at 30, 35, 37, 40, and 43°C for 2 h. After heat shock, cells were lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40) and centrifuged at 16,000 × *g* for 15 min to remove debris. Cleared lysates were separated by electrophoresis on a 0.9% native agarose gel, followed by immunoblot analysis with a mouse monoclonal anti-FLAG M2 Ab (1:5000; Sigma, F3165).

Detection of HBV capsid stability following urea and SDS treatment

Assembled Cp149 and Hsp90 were incubated at the indicated concentrations of urea (0–3 M) or SDS (0–1%). Samples were incubated at 37°C for 30 min, separated by electrophoresis on a 0.9% native agarose gel, and subjected to immunoblot analysis with a rabbit polyclonal anti-HBV core Ab (1:4000; Dako, B0586).

Transmission electron microscopy for HBV capsid dissociation following urea and SDS treatment

In vitro assembly of Cp149 was performed and treated with urea and SDS. For negative staining, 10 μl of a solution containing assembled Cp149 was applied to a carbon-coated grid, and the grid was incubated for 1 min. The grid was stained with 2% uranyl acetate for 1 min and washed with water. Transmission electron micrographs were taken on a JEM 1010 (JEOL, Tokyo, Japan) operating at 80 kV at the NICEM (National Instrumentation Center for Environmental Management).

Quantification of HBV DNA by real-time quantitative PCR

HepG2.2.15 cells were treated with 4 μM GA or 85 μM lamivudine (3TC) (HBV pol inhibitor) (Allen et al., 1998; Doong et al., 1991; Severini et al., 1995), and transfected with shRNA-Hsp90 (Sigma, MISSION shRNA NM_007355) and negative control plasmid pLK0.1. After 24 h, the medium was collected and the released HBV virus was harvested. After extraction with phenol, the DNA was precipitated in ethanol. The DNA was added to a real-time PCR SYBR-Green reaction mixture (Qiagen, Hilden, Germany) containing HotStarTaq polymerase, which was included to avoid false positives in the quantitative PCR. The primers, F-5′-GTGTCTGCGGCGTTTTATCA-3′ and R-5′-GACAAACGGGCAACATACCTT-3′, were designed to amplify a 98-base pair product from positions 379 to 476 of the HBV genome (Garson et al., 2005). The reaction conditions were 95°C for 15 min, followed by 40 cycles at 94°C for 15 s, 55°C for 30 s and 72°C for 30 s. The level of HBV in the medium was quantified relative to a standard curve of serial dilutions (5.28×10^6 copies per milliliter (cpm), 5.28×10^5 cpm, 5.28×10^4 cpm, 5.28×10^3cpm, 5.28×10^2 cpm) of pHBV 1.2 ×, which is similar to a previously described construct (Guidotti et al., 1995).

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Figure

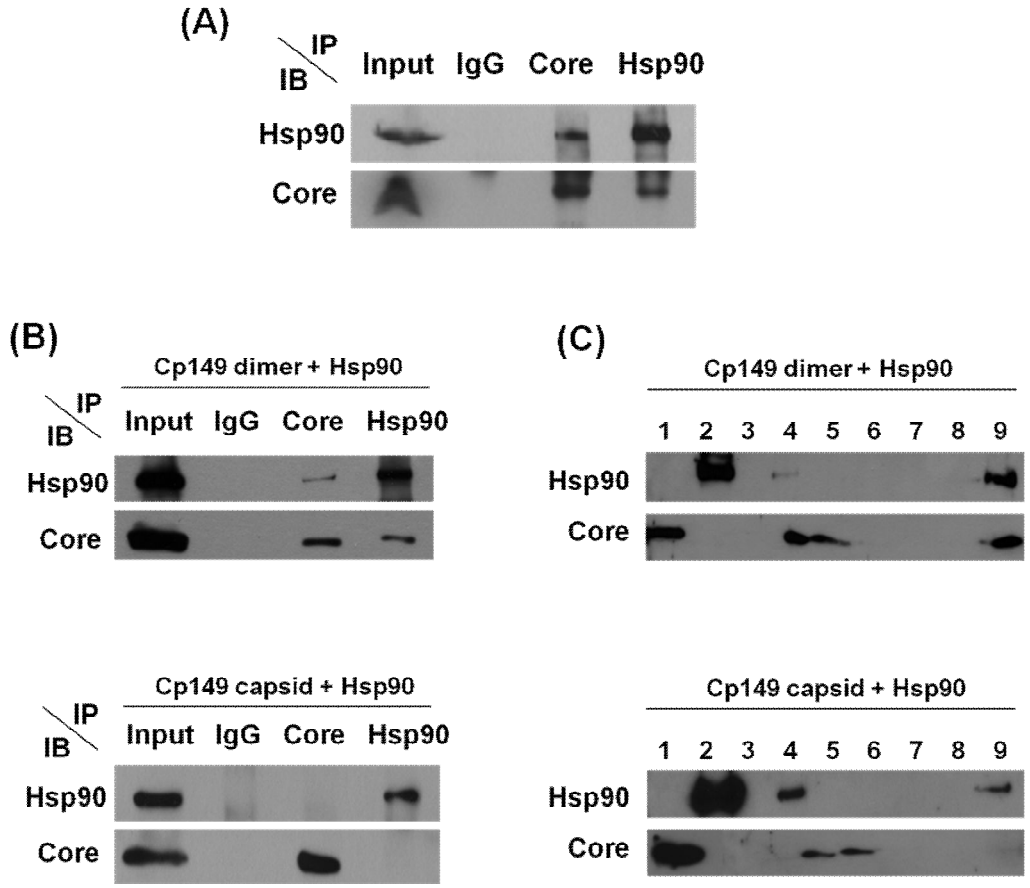


Fig. 1. Hsp90 binds to the HBV core protein

(A) The human hepatoma cell line, Huh7, was transfected with pCMV/Flag-core to express the HBV core protein. Lysates from transfected cells were co-immunoprecipitated using anti-HBV core Ab and anti-Hsp90 Ab. Immunoblot analysis was performed with anti-Flag M2 Ab and anti-Hsp90 Ab. An input of 4% of the total protein mixture was used as

the positive control. Non-specific IgG was used as the negative control. (B) Cp149 dimers were mixed with Hsp90 and incubated at 30°C for 1 h in reaction buffer. The mixture was co-immunoprecipitated using anti-HBV core Ab and anti-Hsp90 Ab and analyzed by 15% SDS-PAGE. Immunoblot analysis was performed using anti-HBV core Ab and anti-Hsp90 Ab (top panel). Capsid was formed in an assembly reaction with 20 μM Cp149 dimers and Hsp90. Co-immunoprecipitation was performed as above (bottom panel). An input of 5% of the total protein mixture was used as the positive control. Non-specific IgG was used as the negative control. (C) His-tagged Hsp90 was immobilized on Ni-NTA agarose, and Cp149 dimers (top panel) and capsid (bottom panel) were independently loaded on the agarose. Lane 1, purified Cp149; lane 2, purified Hsp90; lane 3, Ni-NTA agarose alone; lane 4, Hsp90 immobilized on agarose (3%); lane 5, flow-through; lanes 6–8, 50 mM imidazole washes to show protein that did not bind to agarose; and lane 9, 500 mM imidazole elution.

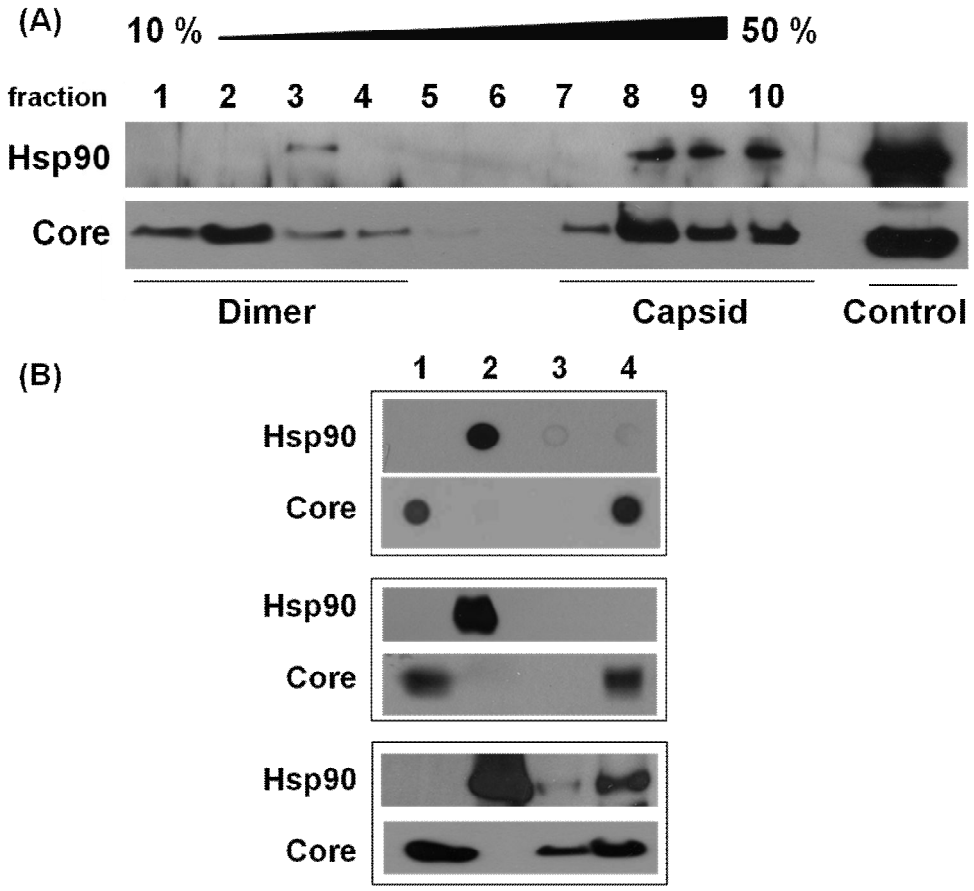


Fig. 2. Hsp90 is incorporated into the HBV capsid by interaction with Cp149 dimers

(A) To perform the assembly reaction, Hsp90 was mixed with Cp149 dimers and assembly was tracked by sucrose density gradient analysis. Cp149 and Hsp90 were analyzed by immunoblot analysis using anti-HBV core Ab and anti-Hsp90 Ab, respectively. The positive control was 5% of the total loading volume. (B) Non-denaturing conditions were applied to

samples from fractions 3 and 8 of Fig. 2A, and the reactions were analyzed by dot blot analysis (top panel) and native agarose gel electrophoresis on a 0.9% agarose gel (middle panel). The two samples were analyzed by 15% SDS-PAGE under denaturing conditions (bottom panel). Then, each sample, under various conditions, was probed with the anti-HBV core Ab and anti-Hsp90 Ab. Both Cp149 (lane 1) and Hsp90 (lane 2) were purified from E. coli and used as markers. Samples from fractions 3 and 8 of Fig. 2A (lanes 3 and 4 of Fig. 2B, respectively) were included in the analysis.

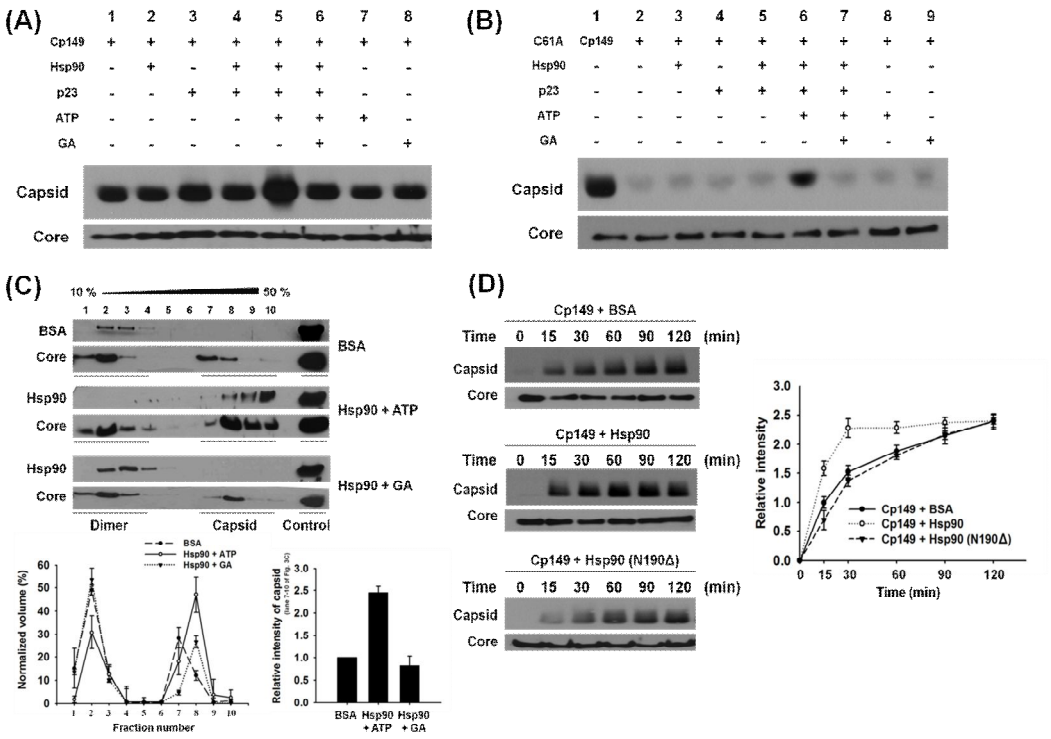


Fig. 3. Hsp90 facilitates HBV core assembly

(A) Cp149 dimers and (B) the core protein point mutant (C61A) were independently combined with Hsp90, p23, 0.5 mM ATP-γ-S, and 2 μM GA, and subjected to the assembly reaction. Samples were analyzed by 0.9% agarose gel electrophoresis and evaluated by immunoblot analysis using anti-HBV core Ab to detect the HBV capsid. Core, the total amount of Cp149 dimers, and C61A were analyzed by 15% SDS-PAGE. (C) Cp149 dimers and ATP-activated Hsp90 were mixed and incubated under conditions permitting capsid assembly. Sucrose density gradient analysis

was conducted (middle panel). Furthermore, Cp149 dimers were allowed to react with GA-inactivated Hsp90 (bottom panel). BSA was used as a control (top panel). Each sample was assayed by immunoblot analysis using an anti-HBV core Ab with 5% of the total loading volume used as the positive control. The bottom left graph shows the band pattern of the fractions on the top panel in comparison with the control. The bottom right graph compares the amount of capsid formation in the presence of BSA, ATP-activated Hsp90, and Hsp90 inactivated by GA (fractions 7–10). The core intensity resulting from sucrose density gradient analysis of Cp149 dimers with BSA was used as a standard. (D) Capsid formation was detected in an in vitro time-course experiment (0–120 min) by immunoblot analysis. Top panel, Cp149 dimers were incubated without Hsp90, using BSA as a control. Middle panel, Cp149 dimers were incubated in the presence of ATP-activated Hsp90. Bottom panel, Cp149 dimers were incubated with the ATPase-site-deleted Hsp90 mutant (N190Δ). The right graph shows the band intensity pattern. Capsid intensity resulting from incubation of Cp149 dimers with BSA for 15 min was used as a standard and assigned a value of 1. Each experiment was carried out three times, and the error bars represent standard deviation from the three experiments.

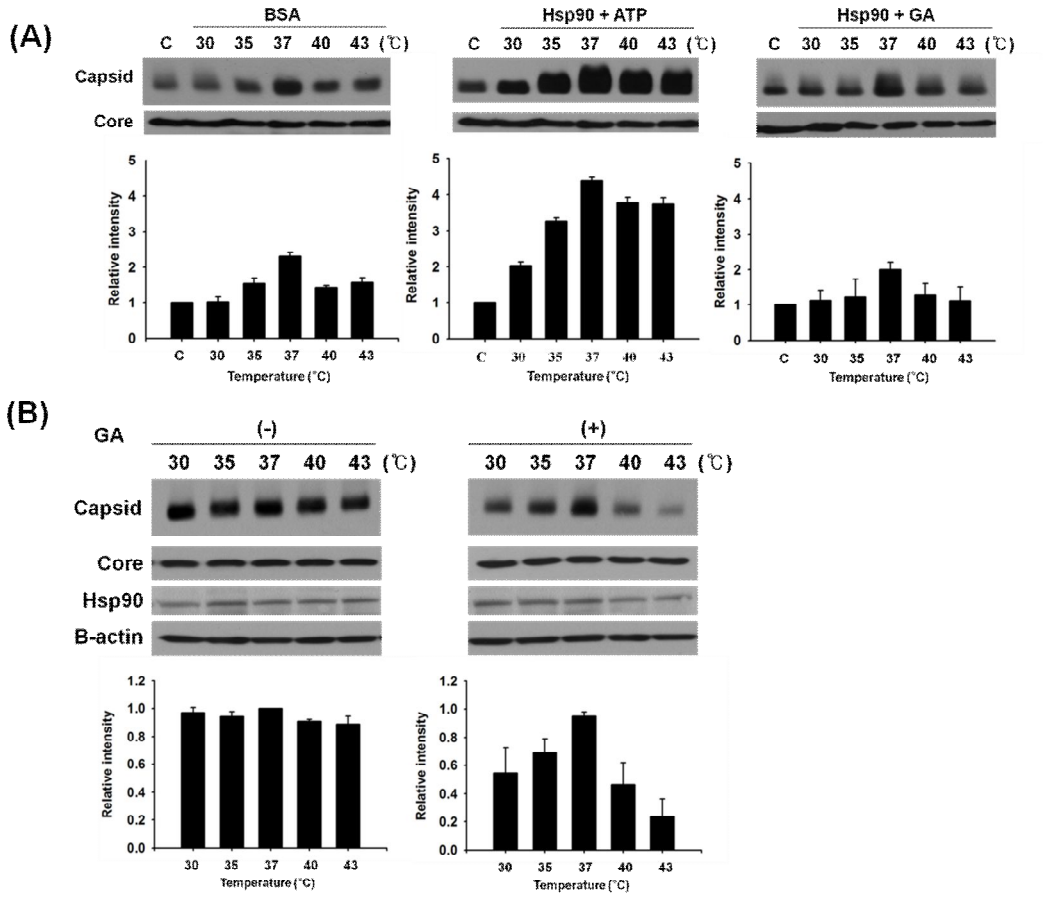
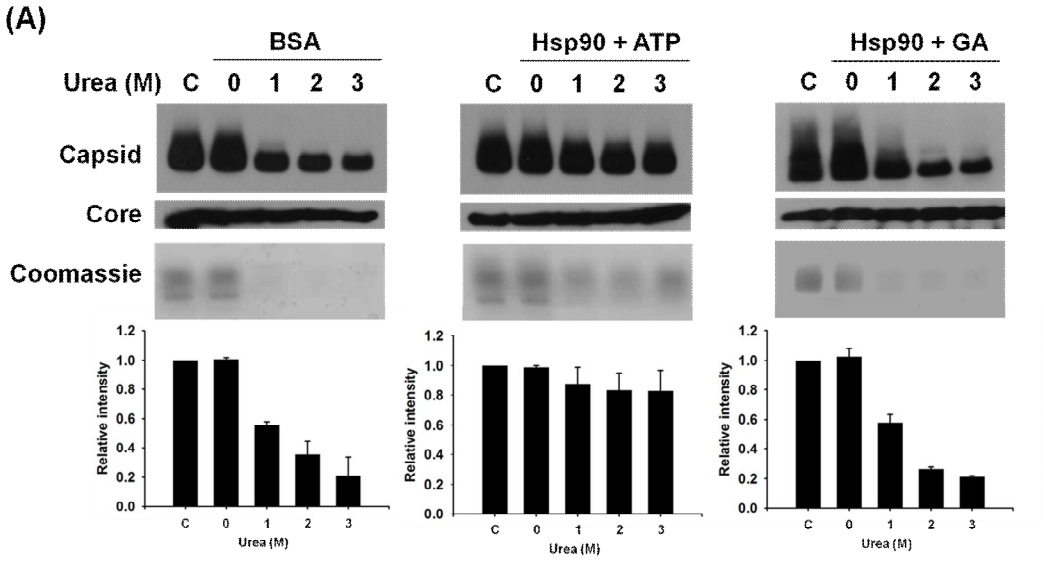


Fig. 4. Hsp90 promotes HBV core assembly over a wide range of temperatures

(A) Cp149 dimers were incubated with BSA (control protein; left panel) at 30°C for 30 min and then at varying temperatures for 30 min. ATP-γ-S (0.5 mM) was added to Hsp90 at 30°C for 30 min (middle panel) and GA (2 μM) was added to Hsp90 at 30°C for 30 min (right panel), and each mixture was incubated with Cp149 dimers at varying temperatures for 30

min. The amount of capsid formation was measured by immunoblot analysis using anti-HBV core antibody at each temperature. Core, the total amount of Cp149 analyzed on 15% SDS-PAGE. C, total capsid formed by incubating Cp149 dimers alone at 30°C for 30 min. Bottom graph, intensity of the bands in the gel above, using the intensity of C as a standard (set to 1). These experiments were repeated three times and the error bars represent the standard deviation from the three experiments. (B) Huh7 cells were transfected with pCMV/Flag-core. Cells were incubated at 30, 35, 37, 40, and 43°C for 2 h with no treatment (left panel) or 4 μM GA-treatment (right panel). Capsids were analyzed by 0.9% agarose gel electrophoresis and detected by immunoblot analysis with anti-FLAG M2 Ab. Hsp90, β-actin, and HBV core protein expression were assessed by immunoblot analysis on 15% SDS-PAGE. Bottom graph, band intensity of the sample incubated at 37°C of Fig. 4B (left panel) was used as a standard (set to 1), and the intensity of the other bands was compared to this value. These experiments were repeated three times, and the error bars represent the standard deviation of the three experiments.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Capsid
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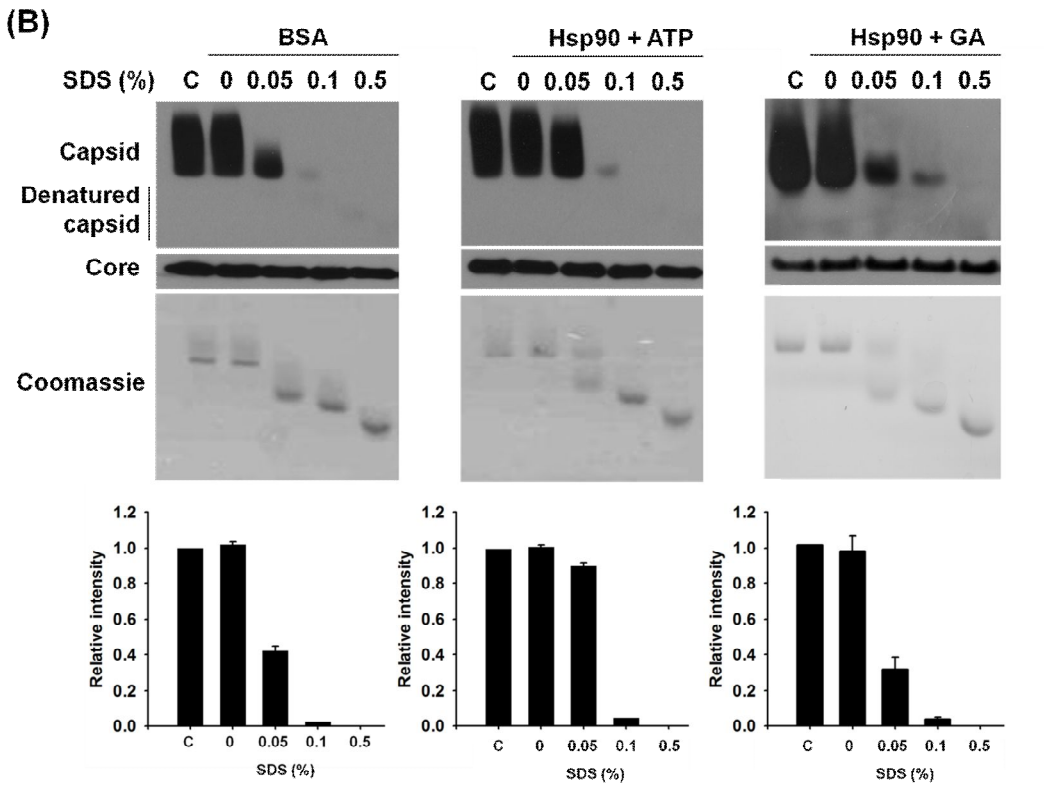
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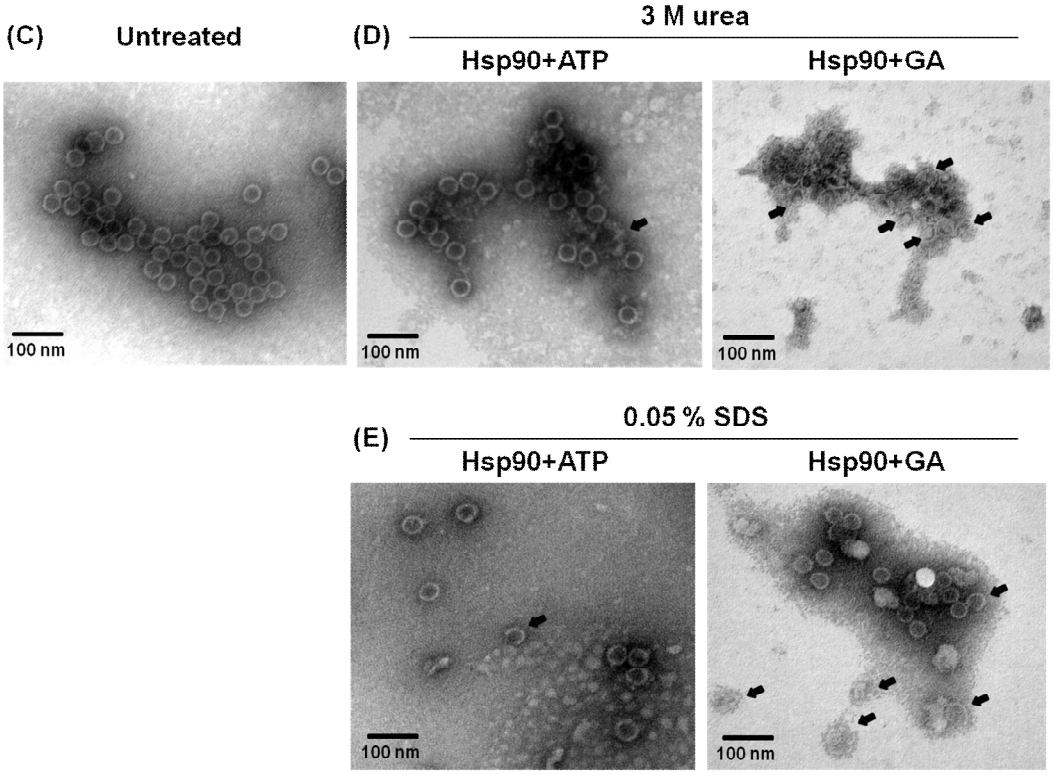

Fig. 5. Hsp90 inhibits HBV capsid dissociation under detergent treatment

(A) Cp149 dimers were incubated at 37°C for 30 min. After capsid formation, varying urea concentrations (0–3 M) were added and the capsids were incubated with BSA, ATP-activated Hsp90, or GA-treated Hsp90 at 37°C for 30 min. Mixtures were analyzed by 0.9% agarose gel electrophoresis, and proteins were detected by immunoblot analysis using anti-HBV core Ab (top panel) and Coomassie blue (bottom panel). (B) Capsids were treated with different concentrations of SDS (0–0.5%) in the presence of BSA, ATP-activated Hsp90, or GA-treated Hsp90 at 37°C for

30 min. Mixtures were then immediately analyzed by 0.9% agarose gel electrophoresis as described above. Coomassie staining was used to measure the amount of capsid and denatured capsid. Core, the total amount of Cp149 analyzed on 15 % SDS-PAGE. C, the amount of capsid not treated with detergents. Bottom graph, C was used as the standard (set to 1), and the intensity of the other bands was compared to this value. These experiments were repeated three times, and the error bars represent standard deviation of the three experiments. Capsids were examined by electron microscopy in the presence of detergent; (C) Capsids not exposed to detergents (urea or SDS) were used as the control. (D) Capsids and ATP-activated Hsp90 or GA-inactivated Hsp90 in the presence of 3 M urea. (E) Capsids and ATP-activated Hsp90 or GA-inactivated Hsp90 in the presence of 0.05% SDS. The arrows indicate the detergent-dissociated capsid (urea or SDS).

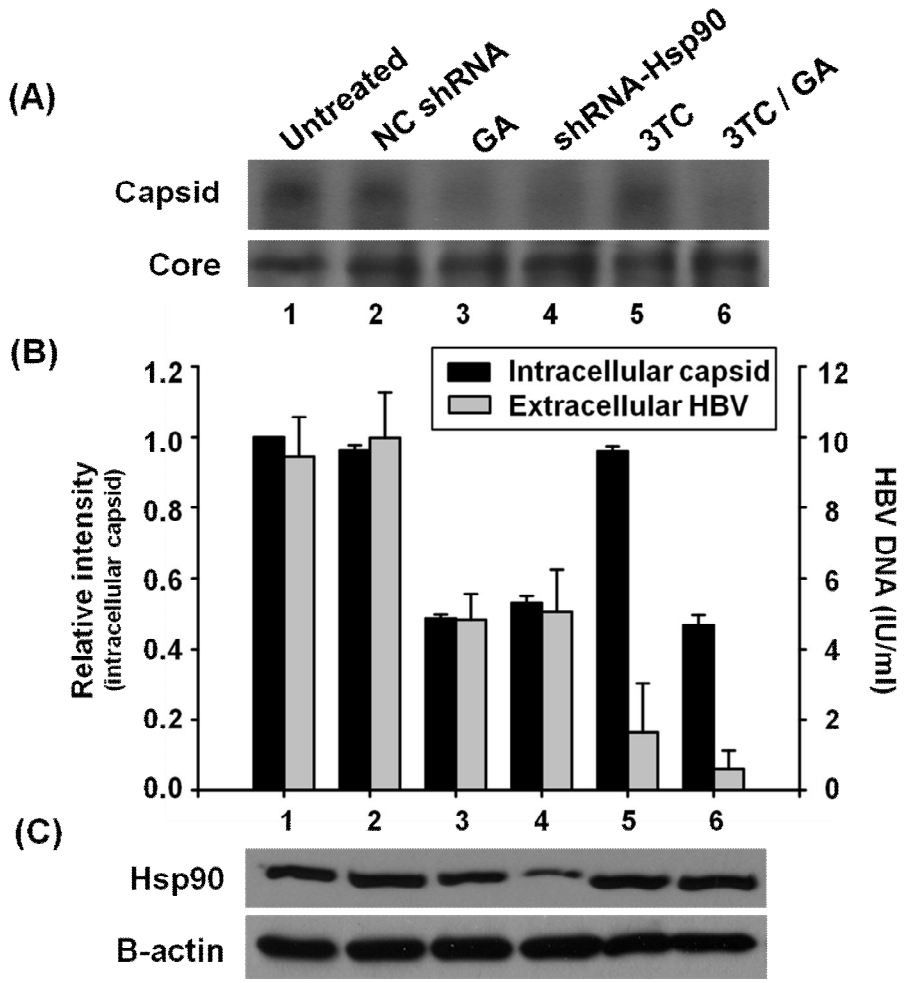

Fig. 6. Both inhibition and down-regulation of Hsp90 reduce the amount of extracellular HBV DNA in HepG2.2.15

(A) After HepG2.2.15 cells were treated with GA or 3TC and transfected with shRNA-Hsp90 for 24 h, cells were lysed in lysis buffer and analyzed by 0.9% native agarose gel electrophoresis to detect HBV capsid (top panel) as well as 15% SDS-PAGE to detect HBV core protein (bottom

panel). (B) HBV capsid bands were analyzed by ImageMaster 2D Elite software 4.01 (black bars) and HBV DNA in the media from cell cultures was measured by qRT-PCR (ABI 7300, Applied Biosystems) (grey bars). (C) Cell lysates were analyzed by 15% SDS-PAGE to detect Hsp90. B-actin was used as a control. Immunoblot analysis was performed using the anti-HBV core and anti-Hsp90 Abs. These experiments were repeated three times, and the error bars represent standard deviation of the three experiments. NC shRNA, the negative control of shRNA (pLK0.1-scramble vector).

국문초록

B 형 간염바이러스 캡시드 형성에 관여하는 숙주요인에 의한 메커니즘이 불확실하게 남아 있습니다. 이 연구는 숙주요인인 열 충격 단백질 90 과 B 형 간염바이러스 코어 단백질 간의 상호작용을 분석하였습니다. 그 결과 열 충격 단백질 90 은 B 형 간염바이러스 코어 단백질 다이머와 결합하며 B 형 간염바이러스 캡시드 내로 들어가는 것을 확인하였습니다. 또한, 활성화된 열 충격 단백질 90 은 코어 단백질 조립을 촉진시키며, 생체 내외에서 다양한 온도와 약물처리에 따른 캡시드 분리의 정도를 감소시키는 것을 알 수 있었습니다. 게다가, 열 충격 단백질 90 을 억제하거나 발현을 저해할 경우 HepG2.2.15 세포에서 생성되는 B 형 간염바이러스가 감소하는 것을 알 수 있었습니다. 이러한 결과들을 통해 열 충격 단백질 90 이 B 형 간염바이러스 캡시드의 안정화와 형성에 중요한 역할을 한다는 것을 확인할 수 있었습니다.

주요어: B형 간염바이러스, 끝을 자른 코어 149 단백질, 숙주요인, 열 충격 단백질90, 바이러스 입자를 단백질 막으로 둘러싸기, 코어 조립, 캡시드 분리

학번: 2007-22844