Antisense Oligodeoxynucleotides Targeted against Molecular Chaperonin Hsp60 Block Human Hepatitis B Virus Replication*

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The major role of hepatitis B virus polymerase (HBV pol) is polymerization of nucleotides, but it also participates in protein priming and the packaging of its own genome into capsids. Therefore, HBV pol may require many assistance factors for its roles. Previous reports have shown that Hsp60, a molecular chaperone, activates HBV pol both in vitro and ex vivo, such as inside insect cells. Moreover, HBV pol binds to Hsp60 in the HepG2 host cell line. In this report, we show that Hsp60 plays a role in the in vitro replication of HBV. Antisense oligodeoxynucleotides (A-ODNs) specifically directed against Hsp60 induced its down-regulation, severely reducing the level of replication-competent HBV without influencing cell proliferation and capsid assembly under these conditions. Furthermore, we found that Hsp60 did not encapsidate into nucleocapsids. Our results indicate that Hsp60 is important for HBV replication in vivo, presumably through activation of HBV pol before encapsidation of HBV pol into HBV core particle. In addition, A-ODNs specific for Hsp60 also inhibit replication of a mutant HBV strain that is resistant to the nucleoside analogue 3TC, which is the main drug used for HBV treatment, and we suggest that A-ODNs directed against Hsp60 are possible reagents as anti-HBV drugs. Conclusively, this report shows that the host factor, Hsp60, is essential for in vivo HBV replication and that mechanism of Hsp60 is probably through an activation of HBV pol by Hsp60.

Hepadnavirus is a small hepatotropic DNA-containing virus that replicates through an RNA intermediate (1). Human hepatitis B virus (HBV1), a member of the hepadnavirus family, infects the liver acutely and chronically. Acute infections can produce serious illness, and ~0.5% of patients terminate with fatal, fulminant hepatitis. Chronic HBV infection causes liver diseases, such as liver cirrhosis, hepatocellular carcinoma, as well as other serious consequences. Nearly 25% of patients with chronic infections terminate in untreatable liver cancer (2). Annual deaths from liver cancer caused by HBV infection exceed one million worldwide. An effective vaccine has been available for 20 years, and attempts for universal vaccination are now underway in developed countries (3). However, vaccination is not effective for established infections and only prevents transmission, such as from mother to newborn (4).

Following infection of hepatocytes, the partially double-stranded DNA genome of hepadnavirus is converted into covalently closed circular DNA in the nucleus (5). Hepadnavirus produces four RNA transcripts for replication in the infected cells (1). Among the transcripts, pregenomic RNA (pgRNA) acts as a mRNA that encodes the HBV core protein and the polymerase (pol); pgRNA also acts as the template for genome synthesis after it is packaged into a capsid. Binding of pol to a stem-loop structure, ε, which is close to the 5' end of pgRNA, is necessary for the nucleocapsid assembly and replication initiation (6, 7). This binding of pol to the 5’-ε stem-loop region is important because, after binding, to initiate replication of the HBV genome pol incorporates three to four nucleotides at tyrosine residue 63, a process called “priming” (8, 9).

To study the biochemical aspects of HBV pol, many researchers have tried to produce in vitro HBV pol systems. However, HBV pol is not active in cell-free systems, and producing HBV pol in Escherichia coli is difficult. Only HBV pol fusions with large proteins, such as maltose binding protein, can be expressed in E. coli (10, 11). However, pol is very unstable and highly degradable; therefore, reconstitution experiments of pol are complicated. Mechanistic questions have become tractable only after the establishment of a model system, the duck HBV model, which provides active pol in a cell-free system (12). During priming in vitro, duck HBV pol requires a chaperone complex, such as the Hsp90 complex, to change its conformational states (13–15). These changes enable duck HBV pol to bind to the ε stem loop region in pgRNA, after which pol shows priming activity. Although much information has been obtained from the duck HBV pol model, many mechanistic details of HBV pol are still unknown.

Recently, HBV pol was shown to bind to Hsp60 and activate pol in vitro (16). In addition, HBV pol was shown to bind to Hsp60 in HepG2 cells, which are human hepatocytes (17). These results suggest that Hsp60 assists HBV pol in vivo. To probe the role of Hsp60 for HBV pol in vivo, we used antisense oligodeoxynucleotides (A-ODNs) to down-regulate Hsp60. Generally, A-ODNs targeting a specific gene are used to knock-out a gene without exerting other effects (18, 19). For example, it was shown that protein kinase C α and RAF1 are important in the translational regulation of lipoprotein lipase in adipocytes through down-regulation of protein kinase C α and RAF1 by using A-ODNs (19). The most commonly used ODNs are phosphorothioate ODNs. These oligonucleotide analogues are very resistant to nuclease (20) but hybridize with a slightly lower efficiency than the binding efficiency of unmodified ODNs (21).

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Using A-ODNs directed against Hsp60 (22), we reduced the level of cytoplasmic Hsp60 in HepG2 cells, thereby blocking HBV replication.

In this report, it was shown that Hsp60 is essential for HBV replication in vivo. This aspect is indicated by the loss of interaction between HBV pol and Hsp60 that is due directly to lowered Hsp60 levels by the A-ODNs directed against Hsp60 with phosphorothioate modification. In addition, HepG2 cells are targeted for anti-HBV drug development, because down-regulation of Hsp60 in infected cells blocks replication of HBV and mutant HBV (M550V) that is resistant to the nucleoside analogue 2′-deoxy-3′-thiacytidine (3TC), the main drug for HBV treatment (23).

MATERIALS AND METHODS

Constructions and Oligonucleotides—The pHBV1.2× construct is a previously described construct (24) that supports HBV replication by providing all required HBV transcripts. Briefly, one copy of the HBV genome having sequences of subtype adr (25) was inserted into the pUC18 plasmid at the BamHI site (nucleotide 1397). A fragment spanning the BamHI site (nucleotide 1397) to the end of the pgRNA (nucleotide 1987) was added to this plasmid (Fig. 1A). The pCMVCore plasmid was constructed by inserting the HBV core open reading frame (ORF) in the pCMV vector (Fig. 1A) (Invitrogen). The phosphorothioate ODNs, A-ODN #1, A-ODN #2, sense ODN (S-ODN), FITC/A-ODN #1, and FITC/S-ODN, were synthesized by Hokkaido System Sciences Co. (Hokkaido, Japan) (Fig. 1B).

Cells and Transfections—HepG2 cells were cultured in minimal essential medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen). Cells were transfected by using the FuGENE 6 transfection reagent (Roche Applied Science) as instructed by the manufacturer. The transfection efficiency in each sample was normalized by cotransfection with a type B pestle (20 slow strokes). Cell debris and nuclei were removed from the lysed cells by centrifugation at 1,500 × g for 15 min at 4 °C. Postnuclear supernatants were separated by centrifugation at 100,000 × g for 1 h into pellets (mitochondria fraction) and supernatants (cytosol fraction). The postmitochondrial supernatants were separated by centrifugation at 10,000 × g for 1 h into pellets (microsome fraction) and supernatants (cytosol fraction). The pellets were dissolved in homogenization buffer containing 0.25 mM sucrose, 10 mM Tris, pH 7.5, and 2 mM EDTA and then homogenized by using a Dounce homogenizer with a type B pestle (20 slow strokes). Cell debris and nuclei were removed from the lysed cells by centrifugation at 1,500 × g for 15 min at 4 °C. The postnuclear supernatants were separated by centrifugation at 30,000 × g for 1 h into pellets (mitochondria fraction) and supernatants (postmitochondrial fraction). The postmitochondrial supernatants were separated by centrifugation at 100,000 × g for 1 h into pellets (microsome fraction) and supernatants (cytosol fraction). The proteins in the supernatants were used for metabolic labeling of HepG2 cells with 1-l-[35S]methionine (1000 Ci/mmol, PerkinElmer Life Sciences) was added to the culture media and incubated for 3 h. Cells were treated with 1 μM A-ODN #1 or 1 μM S-ODN at 24 h prior to l-methionine starvation, during l-methionine starvation, and during the metabolic labeling period.

Cell Proliferation and Apoptosis Assays—MTS assay for cell proliferation was performed with the CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega). Caspase-3 activity for detection of cell apoptosis was performed with the CaspACE™ assay system, colorimetric kit (Promega). The above two assays were performed as instructed by the manufacturer.

RESULTS

Hsp60 Antisense ODNs Block HBV Replication—To determine whether Hsp60 is necessary for HBV pol function in vivo, we used A-ODN #1 for down-regulation of the Hsp60 level in HepG2 cells. The ODNs had been chemically modified to phosphorothioate ODNs by substituting the oxygen molecules of the phosphate backbone with sulfur for longer half-lives. To exclude nonspecific effects of the A-ODNs, we used two controls, S-ODN and A-ODN #2. The S-ODN is against A-ODN #1 and is used to determine the effect of phosphorothioate ODNs on cells (Fig. 1B). A-ODN #2 (Fig. 1B) was directed against a sequence...
that has only four overlapping bases with A-ODN #1. It was used to test whether the sequence of A-ODN #1 nonspecifically affects the cells. Treatment of HepG2 cells with 1 μM A-ODN #1 to down-regulate Hsp60 severely reduced the endogenous polymerase assay activity of HBV, similar to treatment with 1 μM 3TC. However, treatment with 1 μM S-ODN did not affect endogenous polymerase assay activity (Fig. 2A). This concentration for A-ODN #1 was obtained during our experiment in which 1 μM A-ODN #1 was sufficient for almost complete reduction of endogenous polymerase assay activity (Fig. 3A).

Treatment of HepG2 cells with A-ODN #2 also resulted in reduced endogenous polymerase assay activity, but this reduction was not as efficient as the reduction seen with A-ODN #1. This result might be due to A-ODN #1 having a higher affinity (48°C) than the Tm (46°C) of A-ODN #2. In addition to the above result found after 2.5 days, HBV replication was also blocked by A-ODNs #1 treatment for 5 and 7.5 days (Fig. 2A).

Down-regulation of Hsp60 Does Not Affect Capsid Assembly—After A-ODN #1, A-ODN #2, and 3TC treatment, levels of the assembled HBV core particles were lower than that of the control (Fig. 2B, top panel). To test whether down-regulation of Hsp60 affected capsid formation, we examined capsid formation in HepG2 cells, where Hsp60 was down-regulated by treatment of A-ODN #1. To express the HBV core, we transfected HepG2 cells with the pCMV/Core plasmid and then treated the transfected with A-ODN #1. Assembled capsid particles were separated by ultracentrifugation 2.5 days after transfection. In Fig. 2B, the bottom panel clearly shows that, although Hsp60 was down-regulated, the level of HBV capsid formation was the same for both A-ODN #1-treated and untreated pCMV/Core-transfected cells. These data indicate that loss of replication-competent HBV nucleocapsids by A-ODN #1 treatment is not due to inhibition of HBV core assembly and HBV core protein expression, but that the loss might be due to inactivation of HBV pol. We also examined expression of HBV core proteins in insect cells and found that HBV core proteins did not bind to Hsp60 (data not shown).

Hsp60 Does Not Encapsidate in the Nucleocapsid—A previous report showed that ATP synergistically activates HBV pol (16). This result indicates that HBV pol might be liberated from the HBV complex after activation, because the HBV complex requires ATP for the release of the substrate (28). To determine whether Hsp60 associates with capsid particles, we quantified nucleocapsids where pol is found.

If one HBV complex (composed of 14 subunits) associates with one nucleocapsid, 10 fmol of Hsp60 complexes (~8 ng) should be present in 10 fmol of nucleocapsid (~50 ng). Also, it is reported that HBV pol can be encapsidated into capsids without pgRNA (29). Even if Hsp60 associates with HBV pol in capsids without pgRNA, at least 10 fmol of Hsp60 complex should be present in the capsid. We were unable to detect Hsp60 in ~10 fmol of nucleocapsids, even though 0.6 fmol of
the control Hsp60 complexes (~0.5 ng) was detected in the analysis (Fig. 2C). In addition, we were unable to detect Hsp60 by an immunoblot analysis with anti-Hsp60 (N-20) antibodies on the stripped nylon membrane, which was used previously for detecting native nucleocapsid particles (about 10 fmol; data not shown). Therefore, we concluded that Hsp60 does not encapsidate in the nucleocapsid.

**Down-regulation of Hsp60 Blocks Mutant HBV Resistant to 3TC—**To test the potential of using A-ODN #1 as an anti-HBV drug, we tested A-ODN #1 in a mutant, 3TC-resistant HBV (M550V), which has mutations of HBV pol that change the YMDD motif to YVDD. This mutant virus produces severe problems during 3TC treatment, because it is highly resistant to 3TC and thus is the primary cause of treatment failure. To test the effect of down-regulation of Hsp60 by A-ODN #1 treatment in this mutant virus, we constructed the pHBV1.2× (M550V) plasmid for producing a 3TC-resistant virus in HepG2 cells. In a previous report, the activity of this mutant HBV pol was shown to be lower than the activity of wild-type pol (30). As shown in Fig. 2D, the replication efficiency of the mutant virus was also lower than the efficiency of the wild-type virus (Fig. 2D). Treatment with A-ODN #1 blocked replication of the mutant virus, but treatment with 3TC did not block its replication. This result indicates that inhibition of HBV replication by A-ODN #1 appears to be effective against the 3TC-resistant mutant virus.

**Reduction of HBV Replication by A-ODN #1 Treatment Is Dose-dependent—**HepG2 cells harboring the pHBV1.2× plasmid were treated with serial dilutions of A-ODN #1 or 3TC and secreted HBV was estimated by HBV DNA quantification. From samples of culture media, HBV nucleic acids were isolated after DNase I treatment (10 mM MgCl2 and 500 µg/ml DNase I) for 1 h at 37 °C. HBV DNA was quantified by using “real-time” fluorescence-based PCR. This experiment shows the average values of data from duplicate or triplicate reactions.

**HepG2 Cells Uptake FITC-conjugated Hsp60 A-ODN #1—**To test whether HepG2 cells uptake A-ODN #1, we used FITC-conjugated A-ODN #1. Previously, FITC conjugation was shown to have no affect on uptake of ODNs in cells (31, 32). HepG2 cells were treated with 0.5 µM FITC-conjugated A-ODN #1 for 3 h. Fig. 4 clearly shows that HepG2 cells uptake both A-ODN #1 and S-ODN. For the control, HepG2 cells were treated with FITC-conjugated A-ODN #1 for 15 s. No signal was observed in this control (data not shown), indicating that the uptake shown in Fig. 4 is specific. Fig. 4 also shows that
Hsp60 Is Essential for HBV Replication

Fig. 5. Down-regulation of levels of cytoplasmic Hsp60 in HepG2 cells by A-ODN #1 without degradation of Hsp60 mRNA. A, newly synthesized Hsp60 was analyzed by metabolic labeling with L-[35S]-methionine. After labeling, Hsp60 was immunoprecipitated from labeled cells with the anti-Hsp60 (N-20) antibody and analyzed by SDS-PAGE and autoradiography. β-Actin was coimmunoprecipitated with the anti-β-actin antibody as an internal standard. B, extracts of HepG2 cells were treated with 1 μM of A-ODN #1, A-ODN #2, S-ODN, or 3TC and fractionated by differential pelleting. Fractionated samples (cytosol, microsome, and mitochondria fractions) were quantified, and equivalent amounts were removed from samples for analysis by immunoblotting with the anti-Hsp60 (N-20) antibody. C, determining whether down-regulation of Hsp60 was a result of degradation of Hsp60 mRNA was tested by examining levels of Hsp60 mRNA in HepG2 cells treated with A-ODN #1, A-ODN #2, S-ODN, or 3TC by Northern blot analysis. For Northern blot analysis, 20-μg samples of total HepG2 cell RNA were size-fractionated using 1.2% formaldehyde agarose gels and transferred to nylon membranes (PerkinElmer Life Sciences). The probe used for Northern hybridization analysis was a 1722-bp cDNA fragment of the Hsp60 full open reading frame labeled with [α-32P]dCTP (3000 Ci/mmol). Glyceraldehyde-3-phosphate dehydrogenase was used as the internal standard for hybridization.

HBV pol participates in its genome replication, but mechanistic details have not yet been fully discovered. The obstacle for studying HBV pol has been its low expression level in HBV-infected hepatocytes and in heterologous expression systems. However, when pol was expressed in insect cells, it was very resistant to degradation and showed priming activity. Host factors for HBV pol in insect cells might be similar to the factors in hepatocytes, enabling stabilization and function of Hsp60 protein is not due to mRNA degradation by RNase H activation but due to inhibition of Hsp60 translation. Therefore, Hsp60 down-regulation is not irreversible, and the blocking of translation might be controlled by the concentration of A-ODNs.

To check Hsp60 levels in different regions of the cell, we treated extracts of cells with A-ODN #1 and fractionated treated extracts through differential pelleting by ultracentrifugation. Such methods have been well defined in many studies. Three fractions of mitochondria, microsome, and cytoplasm were fractionated by pelleting. Equal amounts of fractionated proteins were analyzed by SDS-PAGE and by immunoblotting with the anti-Hsp60 (N-20) antibody. Fig. 5B clearly shows that reduction of newly synthesized Hsp60 mainly affected the level of Hsp60 in cytoplasm, which is the site of HBV replication. This treatment did not affect the Hsp60 level in mitochondria, the main site of Hsp60 activity. These results suggest that down-regulation of Hsp60 in cytoplasm strongly affects HBV replication.

To test whether down-regulation of Hsp60 is due to mRNA degradation, we examined levels of Hsp60 mRNA by Northern blot analysis. Some A-ODNs are able to activate RNase H and induce target mRNA degradation (33, 34). In this experiment, we found that the level of Hsp60 mRNAs in cells was not affected by treatment with A-ODN #1 (1, 0.1, and 0.01 μM A-ODN #1). This result indicates that down-regulation of the Hsp60 protein is due to mRNA degradation by RNase H but not induced by A-ODN #1. Therefore, Hsp60 down-regulation is not irreversible, and the blocking of translation might be controlled by the concentration of A-ODNs.

DISCUSSION

HBV pol participates in its genome replication, but mechanistic details have not yet been fully discovered. The obstacle for studying HBV pol has been its low expression level in HBV-infected hepatocytes and in heterologous expression systems. However, when pol was expressed in insect cells, it was very resistant to degradation and showed priming activity. Host factors for HBV pol in insect cells might be similar to the factors in hepatocytes, enabling stabilization and function of Hsp60.
pol. Previous reports have indicated that Hsp60 activates HBV pol in insect cells and binds to HBV pol in hepatocytes (16, 17). Such results suggest the possibility that Hsp60 might have an actual role in HBV replication. To probe the effects of Hsp60 on HBV replication in vitro, we used phosphorothioate A-ODNs to down-regulate the Hsp60 level in cells. With A-ODNs directed against Hsp60, we were able to show that Hsp60 participates in HBV replication and is essential for replication because down-regulation of Hsp60 severely reduced the levels of replication-competent HBV nucleocapsids but did not inhibit capsid formation. Furthermore, Hsp60 synthesis was reduced by treatment with A-ODN #1, and Hsp60 levels were reduced mainly in the cytoplasm, which is the site of encapsidation of HBV pol and pgRNA into capsids.

To determine that the result is not caused by other effect such as viability and A-ODNs #1-cellular protein interaction, some experiments are carried. In these experiments, viability of cells treated with A-ODN #1 did not decrease, and apoptosis was not induced by treatment. Despite the requirement of Hsp60 for cell function, inhibition of Hsp60 synthesis did not affect cell viability, because Hsp60 localized in mitochondria is more than 95% in HepG2 cells and mitochondria are its main functional region. Even though HepG2 cells were treated with a high concentration of A-ODN #1 (10 μM), viability was not affected. This result means that the reduction of replication-competent HBV nucleocapsids was not caused by an abnormality of cell functions. At each transfection, the transfection efficiencies that were normalized by measuring β-galactosidase activity were not affected by treatment with A-ODN #1. This result indicates that down-regulation of Hsp60 in the cytoplasm affects neither protein synthesis nor the function of other proteins. Sense phosphorothioate ODNs did not affect HBV replication. A-ODN #2, which is directed against another site of the Hsp60 gene, also blocks HBV replication, indicating that blocking is Hsp60-specific.

Under in vitro conditions, ATP synergizes Hsp60 function for HBV pol (16). Generally, ATP is required for release of substrate from the Hsp60 complex (28). This requirement indicates that Hsp60 might bind to HBV pol transiently and that bound Hsp60 might be liberated from the HBV pol-Hsp60 complex after activation. In this report, we found that Hsp60 is not encapsidated in the HBV nucleocapsid, meaning that HBV pol detaches from the Hsp60 complex before encapsidation, a result that coincides with our in vitro data.

Confirming Hsp60 function in vivo, our report also suggests A-ODN #1 as a drug for HBV treatment. Established drugs for HBV treatment have problems. Treatment of patients with chronic HBV infections with interferon α (35) is limited by side effects, incomplete efficacy, restriction to patients with compensated disease, and the requirement for parenteral administration (36, 37). Whereas direct administration of ODNs in vitro is not an effective delivery method, phosphorothioate ODNs administered intravenously without delivery reagents in animal models have shown effective and specific antisense inhibition. These surprising results helped revive antisense technology and have encouraged researchers to perform more clinical trials (38, 39). The use of A-ODNs as antiviral agents has also emerged as a powerful new approach (40, 41). Many A-ODN drugs are now being widely developed. Forvirsen, an A-ODN used against cytomegalovirus, is already approved by the Food and Drug Administration (42). In this report, replication inhibition of the HBV mutant by Hsp60-directed A-ODNs implicates Hsp60 as a possible novel target for combinational antiviral therapy with 3TC.

In conclusion, A-ODNs targeted against Hsp60 blocks HBV replication without HBV core assembly. This result shows that Hsp60 is required for HBV replication in vivo, possibly through activation of HBV pol. Our study also found that the HBV-HBV pol interaction is required before HBV pol encapsidation into HBV nucleocapsid.

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