Negative regulation of hepatitis B virus replication by cellular Hsp40/DnaJ proteins through destabilization of viral core and X proteins

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The hepatitis B virus core protein consists of an amino-terminal capsid-assembly domain and a carboxyl-terminal RNA-binding domain. By using the yeast two-hybrid system, two Hsp40/DnaJ chaperone-family proteins, Hdj1 and hTid1, that interact with the carboxyl-terminal region (aa 94–185) of the core protein were identified. Hdj1 is the prototype member of the family and hTid1 is the human homologue of the Drosophila tumour-suppressor protein Tid56. Binding of the viral core protein with the Hsp40 proteins was confirmed by affinity chromatography and immunoprecipitation of transiently expressed proteins. Moreover, in a sucrose gradient, the precursor form of hTid1 co-sedimented with capsid-like particles composed of the full-length core protein. Unlike the general perception of the role of the cellular chaperone proteins in assisting viral protein folding and thus enhancing virus replication, ectopic expression of Hdj1 and hTid1 suppressed replication of HBV in transfected human hepatoma cells. Conversely, RNA interference-mediated knock-down of hTid1 resulted in increased HBV replication. It was found that both Hsp40 proteins specifically accelerated degradation of the viral core and HBx proteins. Our results suggest that the cellular chaperones, through destabilization of viral proteins, exert inhibitory functions on virus replication and hence may play suppressive roles in hepatocellular carcinoma.

INTRODUCTION

Hepatitis B virus (HBV) is a member of the hepadnaviruses, small, hepatotropic DNA viruses replicating through reverse transcription of an RNA intermediate. HBV causes acute and chronic hepatitis and is associated strongly with the development of hepatocellular carcinoma (HCC) in humans. Virion particles contain an icosahedral capsid composed of the core protein, a partially double-stranded DNA of 3–2 kbp and a polymerase with the reverse transcriptase activity within an envelope carrying the surface glycoproteins (Ganem & Schneider, 2001). The amino-terminal two-thirds of the 21 kDa core protein are sufficient for capsid formation and this region is referred to as the assembly domain (Gallina et al., 1989). The carboxyl-terminal one-third contains four arginine clusters that are involved in the binding of the replication intermediate, called pre-genomic RNA, and newly synthesized viral DNA (Hatton et al., 1992). Several serine residues, including phosphorylation sites, are embedded in these arginine clusters (Roossinck & Siddiqui, 1987). In addition, this region contains a nuclear-localizing signal (Eckhardt et al., 1991).

To search for cellular factors that interact with the HBV core protein, we used the yeast two-hybrid system and identified two Hsp40/DnaJ family proteins, Hdj1 and hTid1, that associate with the carboxyl-terminal region of the core protein. Human Hsp40/DnaJ proteins comprise a large protein family, members of which feature the J domain (named after the bacterial DnaJ protein) and function as co-chaperones in stimulating the ATP-dependent activity of Hsp70 (Ohtsuka & Hata, 2000). Members of the Hsp40/DnaJ family play diverse roles in many cellular processes, such as folding, translocation, degradation and assembly of multi-protein complexes (Cyr et al., 1994). Hdj1, the first human Hsp40/DnaJ protein identified, plays important roles in protein translation and folding, as well as in the regulation of Hsp70 function. hTid1, the human homologue of the Drosophila tumour-suppressor protein Tid56, was initially identified by its interaction with the human papillomavirus oncoprotein E7 (Schilling et al., 1998).

Recent in vitro reconstitution studies showed that the duck hepatitis B virus (DHBV) reverse transcriptase can be activated by adding the purified Hsp40 protein (Beck & Nassal, 2003; Hu et al., 2002). Also, Hsp40 acts on the topological reorientation of the HBV large envelope protein (Lambert & Prange, 2003). Interestingly, hTid1 and other human Hsp40 proteins were identified as the human HCC-associated antigens in a serological analysis of cDNA expression libraries (SEREX) derived from four HCC patients whose tumour-tissue samples were positive for the HBV surface antigen.
Here, we show that the viral core protein interacts with two Hsp40 proteins (Hdj1 and hTid1) and that, in contrast to the enhancement expected from the requirement for Hsp40 in activating viral polymerase protein in vitro, the chaperones reduce virus replication in intact cells.

**METHODS**

**Plasmid construction.** The pHBCHA and pHBXHA plasmids, which encode haemagglutinin (HA) epitope-tagged HBV core and X gene sequences inserted into the pcDNA3 vector (Invitrogen), have been described previously (Kim et al., 2003). For the yeast two-hybrid screening, the DNA fragments encoding amino-terminal (aa 1–96) or carboxyl-terminal (aa 94–185) regions of the core protein were subcloned into the EcoRI site of pGBKKT7 (Clontech) in frame with the GAL4 DNA-binding domain. The complete coding sequences of hTid1L (the larger isoform encoded by an alternatively spliced transcript), hTid1S (the smaller isoform), Hdj1 and Hsp70 were amplified from the HeLa cDNA library and the amplicons were inserted into the EcoRI/XbaI sites of pcDNA6/V5-HisA (Invitrogen). HBV replication construct pHBV1.3, which carries a HindIII–SalI fragment (nt 1068–1982) of the genome of an HBV serotype ayw strain regulated by the cytomegalovirus (CMV) promoter of pcDNA3 (HindIII/EcoRV sites), was constructed by using the plasmid pT-HBV1.2 (Guidotti et al., 1995).

**Yeast two-hybrid screening.** For yeast two-hybrid assays, the Matchmaker GAL4 Two-Hybrid system 3 (Clontech) was used. Yeast cells (strain AH109) were transformed sequentially with pGBKKT7-coreC and a HeLa cDNA library of 1 × 10^6 colonies stored in the prey vector pACT2. Plasmids were rescued from colonies grown on SD/Tryptone/Leu/His/-Ade/-X/a–Gal plates and were sequenced after their activity was confirmed by retransformation of yeast with pGBKT7-coreC. Identity of the positive clones was confirmed by sequencing after their activity was confirmed by retransformation of yeast with pGBKT7-coreC. Identity of the positive clones was searched from databases with the BLAST algorithm developed by the National Center for Biotechnology Information.

**Protein expression and immunoblot analysis.** Human hepatoma Huh7 and HepG2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 units penicillin ml^{-1} and 100 μg streptomycin ml^{-1}. Huh7 cells were transfected with Lipofectamine PLUS (Invitrogen) and HepG2 cells were transfected with FuGene 6 (Roche). Typically, subconfluent cells in six-well plates were transfected with 2 μg expression plasmid, collected 36–48 h later and analysed by PAGE and immunoblot assay with the following antibodies: anti-HA (Sigma), anti-V5 (Invitrogen), anti-Tid1L (RS13; Neomarkers), anti-HBCAg (Dako), anti-enhanced green fluorescent protein (EGFP) (Santa Cruz) and anti-p53 (Calbiochem). The intensities of signals were determined with TINA 2.0 software (Raytest). In some experiments, for efficient expression of the recombinant proteins, cells were infected with the vaccinia virus vTF7-3 (Fuerst et al., 1986) for 1 h before DNA transfection (m.o.i. of 0.5). In other experiments, transfected cells were treated with protease inhibitors, starting from 24 h after transfection. In all transfections, appropriate empty vectors were added to keep the amount of transfected DNA equal.

**Immunoprecipitation and pull-down assays.** Huh7 cells grown in 60 mm dishes were co-transfected with 2 μg hTid1L–V5/His, hTid1S–V5/His or Hdj1–V5/His plasmids along with the core–HA or coreC–HA constructs. For immunoprecipitation, transfected cells were collected 36 h after transfection and resuspended in 400 μl lysis buffer [10 mM Tris/HCl (pH 7.4), 140 mM NaCl and 0.5% (v/v) NP-40] and the lysates were clarified by centrifugation at 12,000 g in a microfuge for 10 min at 4 °C. The HBV core protein was precipitated by adding 20 μl protein A–Sepharose 4 Fast Flow beads (Pharmacia) and 3 μl anti-HBV core antibody to the clarified lysates. After gentle rotation for 2 h at 4 °C, the beads were centrifuged briefly, collected and washed five times with 1 ml buffer [50 mM Tris/HCl (pH 7.4), 0.5% (v/v) NP-40, 150 mM NaCl and 5 mM EDTA]. The bead-bound proteins were analysed by electrophoresis and immunoblot assay with anti-HA or anti-V5 antibodies.

For the resin pull-down assay, transfected cells were collected and lysed by sonication in 300 μl lysis buffer [20 mM Tris/HCl (pH 8.0), 100 mM NaCl and 1% (v/v) NP-40]. After lysates were clarified, supernatants were mixed with Talon affinity resin (Clontech) at room temperature for 30 min. Proteins bound to the resin were precipitated by centrifugation at 8000 g in a microfuge for 3 min. After several washes in 20 mM Tris/HCl (pH 8.0) and 100 mM NaCl, the resin-bound proteins were separated by 12% PAGE and analysed by immunoblot assay.

**Sucrose-gradient ultracentrifugation.** Huh7 cells grown in 100 mm dishes were co-transfected with pHBCHA and hTid1L–V5/His or Hdj1–V5/His plasmids, collected 48 h later and resuspended in 1 ml lysis buffer [10% (v/v) glycerol, 0.5% (v/v) NP-40, 1 mM EDTA, 10 μg leupeptin ml^{-1} and 10 μg aprotinin ml^{-1} in PBS]. Lysates were clarified as described in previous sections, layered on top of a 10–50% continuous sucrose gradient in an SW41 Ti rotor (Beckman) and centrifuged at 200,000 g for 2 h at 4 °C. Each fraction was collected through a hole at the bottom of the gradient tube and an 80 μl aliquot was used for electrophoresis and immunoblot assay.

**Endogenous polymerase assay (EPA).** The EPA has been described previously by Koschel et al. (2000). For purification of HBV core particles, Huh7 cells in 60 mm dishes were transfected with 2 μg pHBCHA and hTid1L–V5/His or Hdj1–V5/His plasmids, collected 48 h later and resuspended in 1 ml lysis buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl2 and 0.2% (v/v) NP-40]. After 15 min incubation on ice, lysates were clarified by centrifugation and mixed for 6 h at 4 °C with 20 μl protein A–Sepharose CL-4B beads (Pharmacia) that had been pre-incubated with 3 μl anti-HBC antibody. After washing the beads, 50 μl reaction buffer [50 mM Tris/HCl (pH 7.5), 75 mM NH4Cl, 1 mM EDTA, 20 mM MgCl2, 0.1% (v/v) β-mercaptoethanol, 0.5% (v/v) NP-40, 0.4 mM dATP, 0.4 mM dGTP, 0.4 mM dTTP and 10 μCi (370 kBq) [α-32P]dCTP (300 Ci mmol^{-1}; Perkin-Elmer)] was added and these mixtures were incubated at 37 °C overnight. To degrade contaminating DNA, 0.25 μg DNase I ml^{-1} was added and mixtures were incubated at 37 °C for 30 min. For disruption of the core particles, 50 μl 1% (w/v) SDS, 10 mM Tris/HCl (pH 7.5), 10 mM EDTA, 0.6 mg proteinase K ml^{-1} and 0.8 mg yeast RNA ml^{-1} was added and incubation was continued at 37 °C for 30 min. Viral DNA was extracted and electrophoresed through 1% agarose gel. Gels were dried in a vacuum and exposed to X-ray film. As a control for the inhibition of HBV replication, cells were treated with 1000 U alpha interferon ml^{-1} (IFN-α; Research Diagnostics), starting 2 h after transfection. To monitor consistency in transfection, pCMV-β-galactosidase under the control of the CMV promoter, was co-transfected. Data were disregarded if the difference in the reporter activity among samples within a set of transfections was > 5%.

**Small interfering RNA (siRNA)-mediated knock-down of hTid1.** For stable expression of siRNA targeting hTid1, a pair of oligonucleotides was annealed and inserted into the pSilencer2.1-U6 vector according to the manufacturer’s protocol (Ambion). The sequences of the oligonucleotides were 5’-GATCCGACGCTCTATACGAGTT-GCCCTTAAAGAGCCAGTATAGGGCTTTTTTGGAAA-3’ and 5’-GATCCGACGCTCTATACGAGTT-GCCCTTAAAGAGCCAGTATAGGGCTTTTTTGGAAA-3’.
Southern blot analysis of viral DNA. Huh7 cells, stably expressing hTid1-siRNA or negative-control siRNA, were transfected with 2 μg ptT-HBV1.2, harvested 4 days later and resuspended in 1 ml lysis buffer [50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 % (v/v) NP-40 and 1 mM EDTA]. After 20 min incubation on ice, lysates were clarified by centrifugation at 15000 g and supernatant was digested with 100 μg DNase I ml⁻¹ and 100 μg RNase A ml⁻¹ (Promega) at 37 °C for 2 h. Core particles were precipitated with 7 % (w/v) polyethylene glycol and digested with proteinase K at 50 °C for 3 h. Viral DNA was extracted and analysed by Southern blot using a 32P-labelled HBV DNA probe.

Capsid analysis by native agarose-gel electrophoresis. Cell lysates were prepared as described above. Lysate (20 μl) was mixed with 10× loading buffer and resolved on a 1.2 % agarose gel in TAE buffer. The gel was transferred to nitrocellulose membrane in 20× SSC buffer and analysed by immunoblotting with a polyclonal anti-Hbc antibody (Dako; 1 : 5000).

RNA extraction and RT-PCR. Total RNA was isolated from transfected cells by using TRI Reagent (Molecular Research Center) and treated with DNase I to remove residual DNA. Reverse transcription was performed with gene-specific primers and SuperScript II RNase H−RT (Invitrogen). The resulting cDNA was amplified by PCR.

RESULTS

Two Hsp40 proteins bind HBV core protein

To identify cellular proteins that interact with the HBV core protein, we screened a HeLa cDNA library by using the yeast two-hybrid system. For this analysis, the amino-terminal (named coreN) or carboxy-terminal (coreC) regions of the HBV core protein were fused to the GAL4 DNA-binding domain. As a bait protein often exhibits an intrinsic trans-activating property, we checked the basal level of activation by the coreN or coreC fusion proteins before screening the library. Yeast transformants expressing coreN demonstrated growth and colour formation on the SD/Trp⁺/His⁺/Ade⁺/X−/Gal plates, whereas coreC conferred no growth. Thus, coreC was used as bait to screen 1 × 10⁶ cDNA library clones. Among the 11 independent clones with yeast phenotypes that suggested a positive interaction with coreC were the two Hsp40 family proteins Hdj1 (clone 2-20) and hTid1 (clone 5-1) (Fig. 1a).

Fig. 1. Hdj1 and hTid1 bind to the HBV core protein. The carboxy-terminal region of the core protein (coreC, aa 94–185) was used to screen 1 × 10⁶ clones of a HeLa cell cDNA library. Clones 2-20 and 5-1, which correspond to the Hsp40/DnaJ chaperone family proteins Hdj1 and hTid1, respectively, were among those that activated the reporter in association with coreC. (a) Yeast growth on His−, Trp−, Leu−, Ade−, X−/Gal plates. (b) Domains of the Hsp40/DnaJ proteins. Hdj1 has the J and G/F-rich domains, whereas hTid1 has no G/F-rich domain, whereas hTid1 has three domains. hTid1L and hTid1S, products of alternatively spliced transcripts, differ in their carboxy-terminal sequences. The residues required for mitochondrial targeting are indicated by an arrowhead and the cleavage site is marked with an asterisk. (c) Huh7 cells were infected with recombinant vaccinia virus carrying T7 RNA polymerase followed by transfection with the core−/HA and Hdj1−/V5/His constructs. Proteins in lysates were electrophoresed and immunoblotted with anti-V5 antibody before (left four lanes) and after (right four lanes) immunoprecipitation (IP) with the anti-core antibody. (d) Cells were transfected as described in (c) with plasmids encoding coreC and hTid1L−/V5/His or hTid1S−/V5/His. The proteins in lysates were electrophoresed and immunoblotted with anti-HA antibody before (left six lanes) and after (right six lanes) affinity chromatography with Talon resin.
Hsp40 family members have three distinct domains: a highly conserved J domain that mediates interaction with Hsp70 and regulates its ATPase activity, a Gly/Phe-rich (G/F) region that probably acts as a flexible linker and a Cys-rich region that resembles a zinc-finger domain (Fig. 1b). To date, 44 Hsp40/DnaJ homologues have been identified in mammals and can be categorized into three groups: type I has all three domains, type II contains the J and G/F domains, but not the Cys-rich domain and type III has the J domain only (Cheetham & Caplan, 1998; Ohtsuka & Hata, 2000). Hdj1 is the best-known member of type II. hTid1, a human homologue of the Drosophila tumour-suppressor protein Tid56, belongs to type I. hTid1 was initially identified by yeast two-hybrid analysis as a cellular partner of the human papillomavirus oncoprotein E7 (Schilling et al., 1998). hTid1S and hTid1L, small and large products, respectively, of alternatively spliced transcripts of hTid1, localize predominantly in the mitochondrial matrix and are involved in apoptosis (Edwards & Münger, 2004; Syken et al., 1999, 2003; Trentin et al., 2004), IFN (Sarkar et al., 2001) and NF-κB (Cheng et al., 2002) signalling pathways and interaction with viral proteins such as HTLV Tax (Cheng et al., 2001) and herpes simplex virus UL9 (Eom & Lehman, 2002).

To determine whether Hdj1 and hTid1 interact with the viral core protein, we expressed the proteins transiently in cultured human Huh7 hepatoma cells and tested their binding by immunoprecipitation and affinity chromatography. Immunoblot analysis indicated that Hdj1 co-precipitated with the core protein (Fig. 1c). Similarly, binding of the core protein with the small and large isoforms of hTid1 was confirmed by the resin-binding assay with coreC (Fig. 1d). Hdj1 was also co-purified with coreC in the same assay (data not shown). These results demonstrated binding of Hsp40/DnaJ proteins with core protein in cultured human hepatoma cells and confirmed the yeast two-hybrid screening result. These results also indicated that the carboxy-terminal end (aa 447–480) of hTid1L is not required for binding core protein, as it is missing in hTid1S (Fig. 1b).

**hTid1 associates with viral capsid-like particles**

As the HBV core protein assembles into empty capsid particles, we tested the physical association of Hsp40 proteins with viral capsid particles. Either hTid1S or Hdj1 was co-expressed with the core protein and cell lysates were sedimented in a 10–50 % linear sucrose gradient. Immunoblot analysis of the fractions revealed that the majority of the hTid1S precursor co-sedimented with capsid particles, although some precursor was also found in the upper fractions (Fig. 2a). In the absence of core, hTid1S was detected only in the upper fractions of the gradient (data not shown). These results provided direct evidence for the interaction of the viral capsid-like particles with hTid1. As hTid1S is the major form present in the cell (shown below in Fig. 4a), we did not investigate the effect of hTid1L. Unlike hTid1, Hdj1 was not associated with the capsid particles and was found in the upper fractions of the gradient (Fig. 2b). These results suggested that the two

![Fig. 2. Association of the hTid1 precursor with the viral capsid-like particles. Huh7 cells were co-transfected with core–HA and either hTid1S–V5/His (a) or Hdj1–V5/His (b) constructs. Cell lysates were sedimented in 10–50 % linear sucrose gradients for 2 h at 200 000 g and fractions were collected from the bottom of tubes. Proteins in the fractions were resolved by PAGE (10 % gel) and immunoblotted with anti-core (upper panels) or anti-V5 (lower panels) antibodies. Positions of core, precursor (p) and mature (m) forms of hTid1S and Hdj1 are indicated.](image)

Hsp40 proteins differ in their mode of interaction with the core protein. Perhaps hTid1S may associate with residues on the surface of the assembled capsids, whereas Hdj1 may interact with core polypeptide.

Whilst these results provided strong evidence for the physical binding of hTid1S with capsid particles, our attempt to investigate such interactions with replicative nucleocapsids (produced from pHBV1.3) was not conclusive. Some hTid1S seemed to co-sediment with nucleocapsids in ultracentrifugation, but migrated slower than the nucleocapsids in a native agarose gel (data not shown). The discrepancy may reflect the different nature of ‘empty’ capsids and nucleocapsids. The dynamic nature of viral nucleocapsids, in assembly as well as in disintegration, is not understood fully. Also, the information on the factors that might affect the processes, including the involvement of cellular chaperones at various stages (Lingappa et al., 1994), is not sufficient. Our data suggest that hTid1S might bind core polypeptides and intermediate forms of capsids in the early stage of virus assembly, but dissociate from the mature nucleocapsids in the later stage.

**Ectopic expression of Hsp40 inhibits HBV replication**

As the finding above raised the possibility that Hsp40/DnaJ proteins may influence virus replication through interaction with the core protein, we tested their effect on HBV
replication in cultured cells. Human hepatoma HepG2 cells were transfected with a replication-competent, pHBV1.3 construct together with Hdj1 or hTid1 expression plasmids. Four days later, cytoplasmic nucleocapsid particles were immunoprecipitated with anti-HBc antibody and analysed by EPA. Radiolabelled HBV DNA was electrophoresed through 1% agarose and the gel was dried and exposed to X-ray film. The nicked circular (NC) and linear (L) forms of HBV DNA are indicated on the right, with their relative intensity estimated at the bottom. (b) As a control, pHBV1.3-transfected cells were treated with IFN-α (1000 U ml⁻¹). (c) Huh7 cells were transfected with 1 μg pHBV1.3 and an increased amount of Hdj1 or hTid1 expression plasmid as indicated. Four days later, lysates were analysed by native agarose-gel electrophoresis and immunoblotting with anti-core antibody.

Fig. 3. Inhibition of HBV replication by Hsp40/DnaJ proteins. (a) HepG2 cells were transfected with 2 μg pHBV1.3 and 0.5, 1 or 2 μg of either Hdj1 (lanes 2–4) or hTid1S (lanes 5–7) expression plasmids. Four days after transfection, cytoplasmic nucleocapsids were immunoprecipitated with anti-HBc antibody and analysed by EPA. Radiolabelled HBV DNA was electrophoresed through 1% agarose and the gel was dried and exposed to X-ray film. The nicked circular (NC) and linear (L) forms of HBV DNA are indicated on the right, with their relative intensity estimated at the bottom. (b) As a control, pHBV1.3-transfected cells were treated with IFN-α (1000 U ml⁻¹). (c) Huh7 cells were transfected with 1 μg pHBV1.3 and an increased amount of Hdj1 or hTid1S expression plasmid as indicated. Four days later, lysates were analysed by native agarose-gel electrophoresis and immunoblotting with anti-core antibody.

Fig. 4. Suppression of hTid1 increases HBV replication. (a) Endogenous hTid1 was knocked down in Huh7 cells by siRNA. Huh7 cells were transfected stably with the plasmid encoding hTid1-specific siRNA (hTid1-si) or a control hairpin RNA of unrelated sequence (Control-si). hTid1 expression levels were analysed by immunoblotting with the anti-hTid1 antibody. Actin was used as a loading control. (b) Huh7-hTid-si and Huh7-Control-si cells were transfected with 2 μg pHBV1.2 construct. Four days later, cytoplasmic nucleocapsids were isolated and the level of replicated DNA was measured by Southern blotting. The relaxed circular (RC), double-stranded linear (DL) and single-stranded (SS) forms of HBV DNA are indicated on the right, with their relative intensity estimated at the bottom. (c) Equal amounts of lysate were analysed by native agarose-gel electrophoresis and immunoblotting using anti-core antibody.

Hsp40 proteins in the transfected cell was no greater than five times that of the endogenous proteins and comparable with that induced by heat shock (data not shown).

Suppression of hTid1 increases HBV replication

To substantiate the negative effect of hTid1 expression on HBV replication observed above, we used RNA-interference technology and generated the hTid1-si cell as described in Methods. Immunoblot analysis showed that the level of both hTid1L and hTid1S isoforms was reduced significantly in this stably transfected cell (Fig. 4a).

We next examined the effect of hTid1 knock-down on HBV replication. The hTid1-si cell was transfected with pHBV1.2 and the replicated viral DNA was analysed by Southern blotting. A significant increase (83%) in HBV replication was observed in hTid1-si cells (Fig. 4b). The result demonstrated that hTid1 has an inhibitory effect on HBV replication. To
test which step of virus replication was affected by hTid1, intracellular viral capsid was separated in agarose gel and quantified by immunoblot with anti-core antibody (Fig. 4c). The level of viral capsid increased accordingly in hTid1-si cells, suggesting that hTid1 inhibits virus replication through limiting the formation of viral nucleocapsids.

**Hsp40 proteins destabilize HBx and core proteins**

To understand the mechanism by which the Hsp40 proteins reduce levels of HBx and core proteins. Some molecular chaperones facilitate protein turnover. In some cases, they simply prevent substrate proteins from forming massive aggregates that are partially shielded from proteolytic attack. In other cases, they function in more specific targeting of substrate proteins to particular proteolytic pathways (Hayes & Dice, 1996). To determine the effect of Hsp40 proteins on the viral proteins, we co-expressed HBx or core proteins with hTid1 or Hdj1 and analysed the protein levels by immunoblot assay. The levels of core and HBx proteins were reduced significantly by co-expression of Hdj1 (Fig. 5a), hTid1S (Fig. 5b) and hTid1L (data not shown), whereas that of EGFP was not affected by the chaperones. This effect was not due to transcriptional control, because expression of each protein was driven by the same promoter element. Also, RT-PCR revealed that core and HBx mRNA levels did not change significantly in the presence of Hsp40 proteins. Thus, these results indicated that Hdj1 and hTid1 promoted the degradation of core and HBx proteins and suggested that the Hsp40 proteins regulate virus replication negatively, probably through destabilization of viral core and HBx proteins.

**Fig. 5.** Expression of Hsp40/DnaJ proteins reduces levels of HBx and core proteins. HA-tagged HBx or core constructs were used to transfect Huh7 cells with Hdj1 (a) or hTid1S (b) constructs. After 48 h, cells were harvested and proteins in the lysates were electrophoresed and immunoblotted with the anti-HA antibody. HBx and core mRNA was analysed by RT-PCR. Amounts of transfected DNAs are indicated above each lane. A plasmid expressing EGFP was used as a control in (b). (c) Hdj1 or hTid1S proteins were co-expressed with the Hantaan virus nucleocapsid protein Np or the cellular p53 protein. The proteins were detected by immunoblotting with anti-Np or anti-p53 antibodies. EGFP was used as a control. In the lower panel, HBx was co-expressed with different amounts of actinin-1a. Amounts of transfected DNAs are indicated above each lane.

![Fig. 5](image-url)
Inhibitory effects of Hsp40 proteins are specific for HBx and core proteins

Although EGFP served as a control, we tested whether the inhibitory effects of Hsp40 proteins were specific for HBV core and HBx proteins by using the cellular protein p53 and the nucleocapsid protein Np of Hantaan virus as additional controls. Huh7 cells were transfected with each gene construct and the target proteins were detected by immunoblotting. Conversely, HBx was co-expressed with different amounts of actinin-1a, a cellular actin-binding protein unrelated to HBx. The expression level of Np or p53 was not affected by Hsp40 proteins, nor was the expression of HBx affected by actinin-1a (Fig. 5c). These results provided evidence that Hdj1 and hTid1 enhance proteolytic degradation of HBx and core proteins specifically.

Hsp70 stimulates degradation of viral proteins

Hsp40 is known to assist Hsp70 as a co-chaperone and regulate complex formation between Hsp70 and its client proteins (Tsai & Douglas, 1996; Wall et al., 1995). Therefore, we tested whether Hsp70 has any additional effect on Hsp40-mediated degradation of HBx and core proteins. HBx or core protein was co-expressed with either Hsp70 or Hsp40, or with both. Again, the HBx level was reduced considerably by Hdj1 or hTid1S, although the additional effect of Hsp70 was barely seen in this experiment due to the nearly complete disappearance of HBx signal by overexpression of each chaperone (Fig. 6a). However, for core protein, an additive inhibitory effect of Hsp40 and Hsp70 was easily detectable (Fig. 6b, lanes 4 and 6).

Enhanced degradation of HBx by Hsp40 is through stimulation of proteasome activity

HBx is degraded rapidly in cells through ubiquitin-mediated proteasome activity (Hu et al., 1999). To address the possibility that the enhanced degradation of the viral proteins by Hsp40 is through stimulation of proteasome activity, we assessed the effect of proteasome inhibition in this process. Cells that had been transfected with HBx or core proteins in combination with Hsp40 were treated with the proteasome inhibitor MG132 and the level of HBx and core proteins was assessed by immunoblot analysis. HBx degradation, stimulated by co-expression of Hdj1 or hTid1S, was blocked effectively by the treatment, whereas degradation of the core protein was not affected (Fig. 7a, b). Whilst similar results were obtained with other proteasome inhibitors, such as LC-β-lactone and ALLN, no protective effect was seen with the calcium chelator BAPTA-AM, the lysosomal protease inhibitors E64 and leupeptin, lysosomal acidification inhibitor NH4Cl or aspartate protease inhibitor pepstatin A (Fig. 7c, d). These results indicated that...
stimulation of proteasome activity by Hsp40/DnaJ led to the facilitated degradation of HBx. In contrast, enhanced degradation of core by Hsp40 was not affected by any of the protease inhibitors. Thus, degradation of core seems to occur through a mechanism not involving proteasomal or lysosomal pathways.

**DISCUSSION**

Several molecular chaperones are involved in the replication of hepadnaviruses: Hsp40, Hsp70, Hsp90, p50/CDC37, Hop and p23 in the activation of DHBV polymerase (Beck & Nassal, 2001, 2003; Hu & Seeger, 1996; Hu et al., 1997, 2002; Wang et al., 2002a), Hsp60 and Hsp90 in the activation of HBV polymerase (Cho et al., 2000; Park & Jung, 2001), Hsp70 and Hsp40 in the topological reorientation of the HBV large envelope protein (Lambert & Prange, 2003; Prange et al., 1999) and Hsp60 in HBx-mediated apoptosis (Tanaka et al., 2004). In this study, we have identified Hdj1 and hTid1 as novel cellular partners of the viral core protein. They are members of the Hsp40/DnaJ chaperone family, which is known to recruit specific substrates to Hsp70, bind to Hsp70 and stimulate its ATPase activity.

In general, molecular chaperones are considered to be beneficial for virus replication (Sullivan & Pipas, 2001). One recent demonstration in that regard is the role of Hsp40 in the replication of avian adenovirus CELO (Glotzer et al., 2000). In CELO infection, the Hsp40 and Hsp70 proteins were upregulated, which was attributed to the function of the virus-encoded Gamm1 protein. This function was considered essential for the virus, as the Gamm1-negative virus was replication-defective. Replication of the Gamm1-negative virus, however, could be partially restored by either heat shock or exogenous expression of Hsp40. We initially expected that the Hsp40/DnaJ proteins would assist in viral assembly and thus would probably be advantageous for HBV replication. Instead, our results demonstrate that Hsp40 proteins inhibit HBV replication. Our analysis pointed out that Hdj1 and hTid1 facilitate degradation of core and HBx proteins. Moreover, combined expression of Hsp40 and Hsp70 enhanced the degradation of core and HBx proteins in an additive manner, although Hsp70 alone could induce the degradation to some extent.

Chaperones are involved in various proteolytic pathways, such as chaperone-mediated autophagy and ubiquitin-dependent proteasomal degradation (Hayes & Dice, 1996). Our data implicate the Hsp40/DnaJ proteins in suppression of oncogenesis through degradation of the viral proteins in this process. Proteasomal degradation is considered important in regulating the intracellular level of HBx, the key regulator of virus replication and oncogenesis (Hu et al., 1999; Kim et al., 2003). Human Hsp40/DnaJ proteins, including hTid1, were recently identified as HCC-associated antigens in a SEREX analysis of HBsAg-positive HCC patients (Wang et al., 2002b). Several studies have implicated hTid1 in the regulation of oncogenic proteins (Cheng et al., 2001, 2002; Schaal et al., 2004; Schilling et al., 1998) and suppression of tumour development in animal models (Canamasas et al., 2003; Lo et al., 2004). In the latest study, the hTid1 protein was shown to interact with the receptor tyrosine kinase ErbB-2 and promote its degradation, leading to subsequent attenuation of oncogenic signalling in carcinoma cells (Kim et al., 2004). Whilst our results demonstrate clearly that proteasomal activity is the key process enhanced by Hsp40 proteins for HBx, it is not understood what other proteolytic pathway might be operative for the core protein.

Viral infection poses an enormous threat to host cells. The data presented here suggest that molecular chaperones might perform important antiviral functions for host cells, yet, at the same time, are the targets exploitable by viral-survival strategies. Further study is needed to understand the molecular mechanisms of Hsp40-mediated degradation of viral proteins and the significance of the regulation in virus replication and oncogenesis.

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