HBXIP, Cellular Target of Hepatitis B Virus Oncoprotein, Is a Regulator of Centrosome Dynamics and Cytokinesis

Ryoji Fujii,1 Changjun Zhu,1 Yunfei Wen,1 Hiroyuki Marusawa,1 Beatrice Bailly-Maitre,1 Shu-ichi Matsuzawa,1 Hong Zhang,2 Youngsoo Kim,2 C. Frank Bennett,2 Wei Jiang,1 and John C. Reed1

1Burnham Institute for Medical Research, La Jolla, California and 2ISIS Pharmaceuticals, Inc., Carlsbad, California

Abstract

Hepatitis B virus accounts for more than 1 million cancer deaths annually, but the mechanism by which this virus promotes hepatocellular carcinoma remains unclear. The hepatitis B virus genome encodes an oncoprotein, HBx, which binds various cellular proteins including HBXIP. We show here that HBXIP is a regulator of centrosome duplication, required for bipolar spindle formation in HeLa human carcinoma cells and primary mouse embryonic fibroblast cells. We found that most cells deficient in HBXIP arrest in prometaphase with monopolar spindles whereas HBXIP overexpression causes tripolar or multipolar spindles due to excessive centrosome replication. Additionally, a defect in cytokinesis was seen in HBXIP-deficient HeLa cells, with most cells failing to complete division and succumbing eventually to apoptosis. Expression of viral HBx in HeLa cells mimicked the effects of HBXIP overexpression, causing excessive centrosome replication, resulting in tripolar and multipolar spindles and defective cytokinesis. Immunolocalization and fluorescent protein tagging experiments showed that HBXIP associates with microtubules of dividing cells and colocalizes with HBx on centrosomes. Thus, viral HBx and its cellular target HBXIP regulate centrosome dynamics and cytokinesis affecting genetic stability. In vivo experiments using antisense oligonucleotides targeting HBXIP in a mouse model of liver regeneration showed a requirement for HBXIP for growth and survival of replicating hepatocytes. Thus, HBXIP is a critical regulator of hepatocyte cell growth in vivo, making it a strong candidate for explaining the tumorigenic actions of viral HBx. (Cancer Res 2006; 66(18): 9099-107)

Introduction

Chronic hepatitis B virus infection affects ~400 million persons worldwide and is the single greatest risk factor for development of hepatocellular carcinoma (1–3). Hepatitis B virus–associated hepatocellular carcinoma kills more than 1 million people annually, ranking it among the most lethal cancers. Hepatitis B virus is a small ~3.4-kb DNA virus containing four partially overlapping open reading frames, encoding the C, S, and X proteins and a viral DNA polymerase (2). Among these four proteins, only the X protein (known as HBx) is clearly associated with tumorigenesis. Viral HBx is a multifunctional protein, which seems to dysregulate cell division and cell death through unclear mechanisms (reviewed in ref. 4). Although HBx lacks acute transforming activity, transgenic mice expressing HBx in the liver in susceptible strain backgrounds develop hepatocellular carcinoma (5).

Mammalian HBXIP is a conserved ~18 kDa protein of unknown function, which was originally identified because of its interaction with HBx (6). HBXIP sequences are well conserved among mammalian species, with close orthologues found in all vertebrate species where sequence data exist thus far. Overexpression of HBXIP suppresses hepatitis B virus replication in HepG2 cells, in addition to suppressing the transactivation phenotype of HBx (6).

Recently, we identified HBXIP as an interaction partner of Survivin, a BIR-family chromosomal passenger protein involved in controlling apoptosis and cell division (7), and showed that HBXIP collaborates with cytosolic Survivin to suppress apoptosis in interphase cells (8). In normal cells, the Survivin protein is produced only during mitosis, but in most transformed cells, Survivin is often present continuously at elevated levels (9, 10). The finding that HBXIP binds to and collaborates with Survivin in suppressing apoptosis prompted us to examine whether HBXIP also plays a role in cell division.

Our findings reveal a critical role for HBXIP at specific steps involved in mitosis and cell division, particularly in centrosome duplication to form a bipolar spindle during prometaphase and at telophase for cell splitting to form two daughter cells (cytokinesis). Overexpression of HBXIP promotes formation of tripolar and multipolar spindles. Similarly, transfection of viral oncoprotein HBx causes excessive centrosome formation, suggesting that HBx dysregulates the normal function of HBXIP during prometaphase. HBXIP and HBx also partially colocalize with centrosomes in mitotic cells. We hypothesize therefore that viral HBx dysregulates the function of cellular HBXIP, causing excessive centrosome production and multipolar mitotic spindles that lead to chromosome segregation defects, thereby creating genetic instability—a hallmark of malignancy (11).

Materials and Methods

Plasmids, siRNA, and antisense reagents. Full-length human HBXIP cDNA (NM 006402) in pcDNA3 (8) was PCR amplified using oligonucleotide primers 5'-AACATTTTCGTGAGATGGAGCCAGGTGCAGGTCACCTC-3' (forward) and 5'-TTGATCCAGAGGCCATTTTGTGCACTGCCACCGT-3' (reverse) and the resulting product was subcloned into XhoI/BamHI sites of the mammalian expression vector pmCherry-N3. The pEYFP-tubulin and pECFP-histone 2B plasmids have been described (12). EsiRNAs targeting the coding regions of Luciferase (538-983 bp) and HBXIP (NM 006402; 522 bp) were synthesized as previously described (13). The HBXIP-targeting siRNA (5'-CGGAAGCGCAGUGAUGUUUdTdT), Survivin(14), and control
double-strand RNA (dsRNA) sequence (siCONTROL; nontargeting siRNA #1) were purchased from Dharmacon (Lafayette, CO). Antisense oligodeoxynucleotides (AS-ODN) were based on 2′-O-methoxy-phosphorothioate chemistry and were synthesized and purified as described (15, 16); mouse HBXIP-antisense oligonucleotide (ASO; ISIS 352061), 5′-GGAATCCGGTG-3′; mouse Eg5-ASO (ISIS 285747), 5′-AGACTTTCAGTTC-3′; and scrambled group (ISIS 19125), 5′-CTTTCCCTGAAGGTTCCTCC-3′. The underlined nucleosides correspond to 2′-O-methoxymethyl-modified nucleosides. Oligonucleotides used for in vivo experiments were purified by anion exchange chromatography and desalted by reverse-phase chromatography (17). Oligonucleotides were analyzed for homogeneity and purity by liquid chromatography-mass spectrometry with an Agilent series 1100 spectrometer. Oligonucleotides were at least 90% full-length product and contained not more than 10% phosphodiester linkages.

**Cell culture and transfections.** HeLa cells in DMEM/10% fetal bovine serum (FBS) and mouse embryonic fibroblast (MEF) cells in DMEM/10% FBS and 100 μmol/L 2-meacaptoethanol were seeded at 20,000 per well in 24-well dishes (6-mm diameter) and transacted at 60% to 70% confluence with either 1 μL per well of Lipofectamine (4 μL Plus reagent) for plasmids, 1 μL of Oligofectamine for siRNA (InVitrogen), or 1 μL of Lipofectamine (4 μL Plus reagent) for ASO, using 0.4 of μg total plasmid DNA (40 μmol/L), 0.1 μg of ASO (100 nmol/L), or 0.6 μg of siRNA (20 μmol/L).

**Immunofluorescence and confocal microscopy.** Immunofluorescence staining was done as described (18) with rabbit polyclonal anti-HBXIP [1:200 dilution; affinity purified from the antisemum previously reported (8) using column-immobilized glutathione-S-transferase/HBXIP 83-173-amino-acid fusion protein], rabbit polyclonal anti-Survivin polyclonal antibody provided by Tony Hunter (Salk Institute, La Jolla, CA; 1:10,000 dilution), mouse monoclonal anti-α-tubulin (clone B-5-1-2, 1:1,000 dilution; Sigma, St. Louis, MO), rabbit anti-pericentrin antiserum (1:200 dilution; Abcam, Inc., Cambridge, MA), rat monoclonal anti-hemagglutinin high affinity antibodies (1 μg/mL; Roche Applied Science, Indianapolis, IN), followed by 1:200 dilution of various fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch, Soham, Cambridgeshire, United Kingdom and Southern Biotech, Birmingham, AL).

For immunostaining, cells were grown on 13-mm² glass coverslips (Carolina Microscope cover glass: 19843) at 4.5 × 10⁴/well for 12 hours. Cells were washed once in serum-free DMEM, then twice in PBS (pH 7.4), followed by fixation for 10 minutes in PBS with 3% formaldehyde (formalin) and 2% sucrose. Residual formaldehyde was neutralized with 0.1 mol/L glycine in PBS for 10 minutes. Cells were permeabilized and blocked for 30 minutes by incubation in PBS containing 10% serum (goat serum or FBS) and 0.4% Triton X-100. After washing with PBS, cells were incubated for 2 hours with primary antibodies diluted in DMEM and for 1 hour with secondary antibodies diluted in PBS containing 0.1% Triton X-100. For imaging, cells were mounted in FluoroGuard Antifade solution (Bio-Rad, Hercules, CA) and coverslips were sealed with Cytoseal-60. For some experiments, HeLa cells were transfected with plasmids encoding fusion proteins tagged with green fluorescent protein (GFP), cyanin fluorescent protein (CFP), yellow fluorescent protein (YFP), or cherry red fluorescent protein (CRP), fixed, stained with 4′,6-diamidino-2-phenylindole (DAPI; 5 μg/mL in H₂O), then processed as above. Cell imaging was done with a Zeiss Axiovert 100M microscope.

**Time-lapse microscopy.** HeLa cells were transfected with 0.5 μg of pEYFP-α-tubulin and pECFP-histone 2B plasmids together with 200 nmol/L of siRNAs using Oligofectamine. Alternatively, cells were transfected with 50 ng of pEYFP-α-tubulin and pECFP-histone 2B plasmids together with 0.5 μg of HBx expression plasmid (HBxD; refs. 19, 20) using Lipofectamine 2000. After 24 hours, cells were cultured with CO₂-independent medium (Life Technologies, Inc., Gaithersburg, MD) containing 10% FBS overnight. Then, dishes were transferred to a heated stage (37°C) on a Zeiss Axiovert 100M microscope. Phase-contrast and fluorescence images of live cells were collected at 2-minute intervals for 7 to 10 hours.

**Liver regeneration model.** Male mice on a C57BL/6 background at 8 to 10 weeks of age were anesthetized with methoxyflurane and subjected to midventral laparotomy with a two-third liver resection. The left and right median and left lateral lobes were removed without injuring the remaining liver tissue. For ASO experiments, mice received i.p. injections of 30 mg/kg × 4 doses before the hepatectomy; then, mice received two additional doses of ASO at 30 mg/kg, 12 to 24 hours after surgery. Mice were divided into
four groups: control group (injected with saline solution); HBXIP-ASO treatment group (ISIS 352061); Eg5-ASO treatment group (ISIS 285747); and scrambled-ASO group (ISIS 141923; 4-6 mice per treatment group). Mice received 50 μg/g (body weight) bromodeoxyuridine (BrdUrd) i.p. in 0.2% solution in PBS 2 hours before sacrificing. Mice were sacrificed 36 hours after surgery. The ASO were dissolved in 200 μL of 0.9% sodium chloride injection (USP from Baxter, Deerfield, IL).

Tissue analysis. The remnant liver was harvested and the wet weight was measured. A portion of liver was snap frozen in liquid nitrogen for protein and RNA isolation whereas another portion was embedded in optimum cutting temperature compound for preparation of cryosections (5 μm thick) using a microtome, followed by mounting on glass slides. For BrdUrd staining, liver sections were analyzed by immunofluorescence using Cell Proliferation kit (Amersham Biosciences Corp., Piscataway, NJ) and counterstained with DAPI at 1.5 μg/mL. The percentage of BrdUrd-labeled nuclei was determined by counting positively stained nuclei in 10 high-power fields (×400), counting a minimum of 3,000 total nuclei.

For terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis, 5-μm liver sections were processed with commercial kit employing 3,3'-diaminobenzidine peroxidase substrate (Boehringer Mannheim Co., Indianapolis, IN) and counterstained with DAPI at 1.5 μg/mL. The percentage of BrdUrd-labeled nuclei was determined by counting positively stained nuclei in 10 high-power fields (×400) counting a minimum of 3,000 total nuclei.

For terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis, 5-μm liver sections were processed with commercial kit employing 3,3'-diaminobenzidine peroxidase substrate (Boehringer Mannheim Co., Indianapolis, IN) and counterstained with 0.5% (w/v) methyl green. Specimens were evaluated by UV-microscopy at high-power magnification (×400) in a blinded fashion. A total of 20 random fields were counted for each TUNEL-stained tissue sample and the percentage of TUNEL-positive nuclei was determined from a minimum of 500 counted.

Results

HBXIP colocalizes with microtubules in dividing cells. Using two different antibodies, we localized endogenous HBXIP to microtubules in synchronized dividing HeLa cells (Fig. IA and B, and data not shown). In contrast, Survivin was associated with the centromere region of chromosomes (kinetochore) in prometaphase and metaphase, then resided with the spindle midzone microtubules in anaphase, followed by localization to the cleavage furrow and midbody microtubules of telophase cells (Fig. 1B). Thus, HBXIP extensively colocalized with p17 Survivin only during telophase, when both proteins were predominantly associated with midbody microtubules and the cleavage furrow, and during interphase, when both proteins resided diffusely in the cytosol and nucleus. Therefore, the endogenous HBXIP and Survivin proteins colocalize on mitotic structures during late stages, but not during early stages, of mitosis. Expression of endogenous HBXIP protein in relation to the cell cycle also differed from Survivin, with HBXIP protein levels reaching peak levels immediately after release from thymidine block, and declining at approximately the time Survivin protein levels reached their peak after release from thymidine block (Supplementary Fig. S1).

We used fluorescent protein tagging technology to confirm these protein localization studies by an independent method. In experiments where HBXIP was expressed fused to CRP and α-tubulin was expressed fused to GFP, extensive colocalization of these tagged proteins was found in mitotic cells (Fig. 2A). In contrast, when CRP-HBXIP was coexpressed with Survivin fused to GFP, colocalization was observed in late anaphase and during telophase, where the tagged protein associated with midbody microtubules (Fig. 2B).

HBXIP is required for mitosis and cell division. To study the function of HBXIP in human tumor cells, we used the technique of

![Figure 2. Localization of HBXIP and Survivin by fluorescent protein tagging. HeLa cells were transfected with plasmids encoding CRP-HBXIP and GFP-α-tubulin (A) or GFP-Survivin (B). After 2 to 3 days, cells were fixed, stained with DAPI, and imaged by confocal microscopy using appropriate filters to display red, green, or combined (merged) fluorescence. Photomicrographs are representative of at least 20 cells examined at each of the designated phases of the cell cycle.](www.aacrjournals.org)
RNA interference (RNAi; refs. 21, 22). Two RNAi techniques were employed—one in which short 21-mer dsRNAs were chemically synthesized [“small interfering RNA” (siRNA); Fig. 2] and another in which a HBXIP cDNA was prepared enzymatically by in vitro transcription and then cleaved by RNase III to create a pool of short dsRNAs [“enzymatic siRNA” (esiRNA; Supplementary Fig. S2)]. As controls, cells were treated with either chemically synthesized or enzymatically synthesized dsRNAs (see Materials and Methods for details). Immunoblot analysis confirmed reduced levels of HBXIP protein in HeLa cells treated with HBXIP siRNA compared with cells transfected with control dsRNAs (Fig. 3). HBXIP esiRNA produced an even more profound reduction in HBXIP protein levels (Supplementary Fig. S2). Thus, the esiRNA method was more effective but also has the risk of nonspecific effects because a pool of dsRNAs is used.

We compared the effects on cell division of transfecting HeLa cells with HBXIP-targeting siRNA or esiRNA, versus control dsRNAs, using time-lapse video microscopy to monitor division of HeLa cells expressing CFP-tagged histone H2B (CFP-H2B) to mark chromosomes and YFP-tagged tubulin (YFP-tubulin) microtubules (23). Whereas control siRNA–transfected HeLa cells completed mitosis and cytokinesis in a timely fashion (Fig. 3A, movie 1), HBXIP siRNA–treated cells experienced one of two cell division defects. First, ~80% of cells arrested in prometaphase with monopolar spindles for several hours, and then underwent apoptosis (Fig. 3B, movie 2) or remained in protracted prometaphase with monopolar spindles for the duration of filming (~10 hours; Fig. 3C, movie 3). Second, other cells (~20%) completed mitosis after a delay, then arrested in telophase, failing to complete cytokinesis and thereby producing binucleated cells (Fig. 3D, movie 4). Similar observations were made for HeLa cells in which HBXIP expression was ablated using the esiRNA method, except an additional phenotype emerged in which ~15% of the cells developed tripolar or multipolar spindles, followed by either cell death with morphologic characteristics of apoptosis or failure to complete cytokinesis, resulting in trinucleated cells or cells with multiple micronuclei (Supplementary Fig. S2). DNA content analysis using flow cytometry confirmed that apoptosis, with accumulation of cells with subdiploid DNA content (<2n, where n = 12 haploid genome), was the dominant phenotype observed in cultures of HBXIP-siRNA treated HeLa cells (Supplementary Fig. S3).

Expression of viral HBx in HeLa cells also mimicked HBXIP deficiency, in that (a) approximately half of the cells arrested in prometaphase, failing to complete mitosis within the time allowed for filming (Fig. 3E, movie 5); (b) about a third of the cells completed mitosis after a delay but failed to complete cytokinesis, thus producing binucleated cells (Fig. 3F, movie 6); and (c) ~10 to 15% of HBx-expressing cells developed multipolar spindles.

Figure 3. HBXIP is required for mitosis and cytokinesis. Time-lapse photomicroscopy was used to study HeLa cells that had been transfected with control siRNA (A), siRNAs targeting HBXIP (B-D), or plasmid encoding HBx (E-G), together with YFP-tubulin and CFP-H2B plasmids. Representative photomicrographs of merged images of YFP-tubulin and CFP-H2B (pseudocolored red and green, respectively) in phase contrast, and indicating the approximate time cells remained at each phase (see accompanying movies). Immunoblot analysis of HBXIP (top) and α-tubulin (bottom) protein levels is shown in the inset (right), done using lysates (50 μg protein) from HeLa cells transfected 2 days prior with control or HBXIP siRNA.

Cancer Res 2006; 66: (18). September 15, 2006 9102 www.aacrjournals.org
followed by aberrant chromosome segregation during attempted mitosis (Fig. 3G, movie 7), consistent with a prior report (24). Unlike HBXIP deficiency, however, apoptosis was rare among HBx-expressing cells (< 10%). Thus, HBx elicits spindle and cytokinesis defects resembling HBXIP deficiency, but differs from HBXIP with respect to apoptosis induction.

**HBXIP regulates centrosome duplication.** During prophase to prometaphase, duplicated centrosomes split and migrate to opposite poles of the cell (25). To explore the reason for prometaphase arrest in HBXIP siRNA–treated cells, we examined in more detail the apparent failure of HBXIP-deficient HeLa cells to form bipolar spindles by immunofluorescence-based monitoring of a centrosome marker, pericentrin. At 48 hours after transfection with control dsRNA versus HBXIP siRNA, HeLa cells were fixed and stained with anti-pericentrin (green) and anti-α-tubulin (red) antibodies and with DNA-binding fluorochrome DAPI to identify prometaphase cells with condensed chromosomes. The percentage of cells with monopolar versus bipolar spindles was then enumerated. Figure 4A shows photomicrographic examples of the cells and Fig. 4B quantifies the data. Compared with control dsRNA–treated cells, prometaphase cells with monopolar spindles were five to six times more frequent, constituting more than half of the cells. The monopolar spindles of HBXIP-deficient cells included cells where a single centrosome was present and cells where the centrosome had split but failed to migrate to opposite poles and succumb to mitotic catastrophe. We conclude therefore that HBXIP is required for centrosome splitting and perhaps centrosome migration to opposite poles to form the bipolar spindle, a step necessary for cell cycle progression from prometaphase to metaphase. Furthermore, the failure to complete this transition frequently precipitated apoptosis, which might also be interpreted as mitotic catastrophe (26) and which requires further investigation with respect to the mechanisms involved.

The effect of viral HBx and overexpression of cellular HBXIP on centrosome duplication in dividing cells was examined using the same experimental approach, where transfected cells were stained with anti-pericentrin antibody. In contrast to HBXIP siRNA–treated cells where monopolar spindles predominated (Fig. 4B), overexpression of HBXIP caused a relative increase in the frequency of cells with tripolar or multipolar spindles (Fig. 4C), roughly triple the frequency seen in cultures of control transfected cells.
HBx caused an even more striking increase in the frequency of cells with tripolar or multipolar spindles, representing an ~6-fold increase above baseline (Fig. 4C). Photomicrograph examples of multipolar cells observed in cultures of HeLa cells transfected with plasmids encoding HBXIP or HBx are shown in Fig. 5.

Because HBx targets HBXIP and presumably alters its function, we reasoned that coexpressing excess HBXIP in combination with viral HBx might neutralize the effect of the viral protein by competing with endogenous HBXIP for binding to HBx. Indeed, when HeLa cells were cotransfected with plasmids encoding HBXIP and viral HBx, the frequency of cells with tripolar and multipolar spindles was greatly reduced (Fig. 4C). Interestingly, however, coexpressing HBXIP with HBx caused an increase in cells with monopolar spindles, suggesting that a precise amount of unperturbed HBXIP is required for proper centrosome dynamics.

Immunoblot analysis indicated that HBXIP did not interfere with HBx expression in transfected cells (ref. 8, and data not shown).

HBXIP and HBx colocalize with centrosomes. We investigated the location of the viral HBx and cellular HBXIP proteins in mitotic cells by fluorescent protein tagging and immunofluorescence microscopy (Fig. 5). HBXIP tagged either with GFP or CRP (data not shown) was not associated predominantly with microtubules but also colocalized with centrosomes in mitotic cells, colocalizing with centrosome marker pericentrin in mitotic cells (Fig. 5B). Hemagglutinin-tagged HBx associated extensively with centrosomes, extending from centrosomes outward along the attached microtubule bundles, as determined by immunofluorescence studies (Fig. 5C). HBx and HBXIP proteins also colocalized on centrosomes and the microtubule network attached to these structures (Fig. 5D). Control transfections using pEGFP vector (for GFP-HBXIP) or pHA vector (for HBx) confirmed the specificity of these results (data not shown).

HBXIP is also required for bipolar spindle formation in normal cells. To extend these studies beyond HeLa cells (a human tumor cell line) to normal cells, we generated ASOs targeting mouse HBXIP mRNA. From a screen of 96 oligodeoxynucleotides (ODN) designed to target various sites along the murine HBXIP mRNA, we identified an active ODN that potently reduced HBXIP mRNA levels when transfected into an indicator mouse cancer liver cell line (Fig. 6A). The effects of this ASO (ISIS 352061) and a scrambled control ODN on HBXIP mRNA levels were then confirmed by transfecting mouse tumor cells with various amounts of these synthetic DNAs, showing dose-dependent reductions in HBXIP mRNA by the ASO but not the control ODN (Fig. 6B). In contrast, neither ASO nor control ODN affected expression of control RNAs (Supplementary Fig. S4).

We applied the HBXIP-targeting ASO for studying the role of HBXIP in normal MEFs. First, the ability of the ASO but not of control ODN to reduce HBXIP protein expression in MEFs was confirmed by immunoblotting (Fig. 6C). Then, the status of centrosomes in cultures of ASO-transfected and control ODN–transfected MEFs was evaluated by fixing cells at 24 hours after ODN transfection and staining the cells with anti-pericentrin antibody and DNA-binding fluorochrome DAPI. Compared with...
MEFs transfected with control ODN, cultures of MEFs treated with HBXIP ASO contained significantly fewer cells with bipolar spindles and more cells with monopolar or tripolar spindles (Fig. 6D). We conclude therefore that HBXIP is required for normal centrosome dynamics in normal dividing cells in culture.

**HBXIP is required for hepatocyte replication in vivo.** To explore the role of HBXIP in cell division in vivo in cells relevant to the biology of HBx, we used the ASO targeting mouse HBXIP to knock down HBXIP expression in the liver of mice and subjected animals to partial hepatectomy, assessing the effects of HBXIP deficiency on liver regeneration. In this model where two thirds of the liver are surgically removed, liver weight returns to normal within 2 to 3 days due to massive hepatocyte proliferation. The frequency of replicating hepatocytes was assessed by BrdUrd incorporation and apoptosis was monitored by the TUNEL method, which detects cells with fragmented DNA. As a positive control, animals were also dosed with ASO targeting the mitotic kinesin, Eg5, deficiency of which has been shown to produce a phenotype similar to HBXIP deficiency, with prometaphase arrest and failure to achieve centrosome duplication, followed by apoptosis (12, 16, 27). Compared with regenerating liver, HBXIP ASO did not induce apoptosis of quiescent hepatocytes in livers of mice (data not shown).

**Figure 6.** HBXIP is required for normal centrosome dynamics in normal mouse embryonic fibroblasts. A, identification of an effective HBXIP ASO. Mouse END cells were transfected in 96-well plates with 96 different 2-OMe–based ASOs targeted different sites along the mouse HBXIP mRNA, using methods previously described (16). After 2 days, RNA was isolated and analyzed by quantitative reverse transcription-PCR (RT-PCR) using HBXIP-specific 3′ primers, normalizing results relative to total levels of RNA determined by Ribogreen. Columns, mean percent control relative to untreated cells (n = 3); bars, SE. The most active of the ASO is indicated (352061), which was used for subsequent experiments. B, HBXIP ASO induces concentration-dependent reduction in HBXIP mRNA in mouse liver cells. Murine bEND cells grown in 96-well plates were transfected with various concentrations of HBXIP ASO 352061. RNA was isolated 24 hours later and analyzed by quantitative RT-PCR using HBXIP-specific primers or by Ribogreen-based fluorescence to compare total RNA levels (not shown). Points, mean percent control relative to cells transfected with cationic lipid alone (n = 3); bars, SD. (See Supplementary Fig. S4 for total RNA control.) C, HBXIP ASO reduces HBXIP protein levels. MEFs were transfected with 0.1 μg of control ODN or ASO 352061. After 24 hours, cells were fixed and stained with anti-pericentrin antibody to enumerate centrosomes and with DAPI to localize chromosomes. The proportion of cells with bipolar (gray columns), unipolar (black columns), or multipolar spindles (white columns) was determined from n > 30 prometaphase cells examined (columns, mean (n = 3); bars, SE).
Figure 7. HBXIP is required for hepatocyte replication and survival in vivo in regenerating liver. Mice were injected daily i.p. with saline (untreated) or with 30 mg/kg of HBXIP, negative control (“scrambled”), or Eg5 (positive control) ASOs, then subjected to partial hepatectomy and injected with BrdUrd 2 hours before sacrifice at 36 hours post partial hepatectomy. A, the remnant liver was fixed, sectioned, and stained with anti-BrdUrd and DNA-binding fluorochrome DAPI. Columns, mean percentage of BrdUrd-positive nuclei per 1,000 nuclei (n = 3-6 livers); bars, SE. B, apoptotic cells were detected in liver sections by the TUNEL method. Columns, mean percentage TUNEL-positive nuclei (n = 3-6 livers); bars, SE. C, HBXIP mRNA levels in mouse liver were measured by quantitative RT-PCR at 1 day after dosing with HBXIP ASO. Columns, mean percent relative to saline-injected control mice (n = 5 livers); bars, SD. Statistical significance was determined by t test. HBXIP AS-ODN treatment was significantly different from control ODN at the P < 0.05, P < 0.005, and P < 0.01 levels (A-C, respectively).

Discussion

Based on RNAi knockdown and gene transfection overexpression experiments, we show here that HBXIP regulates centrosome dynamics in dividing cells, with deficiency of HBXIP causing failure of centrosome duplication during prometaphase (resulting in monopolar spindles) and HBXIP overexpression causing excessive centrosome production (resulting in tripolar and multipolar spindles). The viral oncoprotein HBx also alters centrosome dynamics in a manner that is modulated by the levels of HBXIP, implying that HBx alters HBXIP activity to create genetic instability. Both cellular HBXIP and viral HBx were localized to microtubules in mitotic cells, and they partially colocalized with centrosomes, based on microscopy studies, suggesting they may directly control the process of centrosome cleavage. Although the precise mechanism by which HBXIP and viral HBx control centrosome dynamics remains to be clarified, we have detected an interaction of HBXIP with dynein light chains by yeast two-hybrid cDNA library screening and have confirmed by immunoprecipitation and other protein interaction assays that HBXIP can bind dynein light chains, suggesting perhaps that HBXIP is a regulator of the motoric protein complex responsible for movement of centrosomes along microtubule tracks.

In addition to the prominent effects of cellular HBXIP and viral HBx on centrosome dynamics, these proteins also regulate cytokinesis, with HBXIP-deficient cells and HBx-expressing HeLa cells that managed to complete mitosis with bipolar spindles subsequently arresting in telophase. In these arrested cells, the midbody microtubules that join the two forming daughter cells remained bundled instead of dissociating; then, these cells in the act of splitting rejoined and merged to produce binucleated cells (see accompanying movies). This phenotype is highly reminiscent of the effect of Survivin deficiency and correlates with colocalization of HBXIP with Survivin on microtubules of the midzone during late anaphase and on microtubules of the midbody and cleavage furrow during telophase. Given that we have previously shown that HBXIP is capable of interacting with Survivin (8), we hypothesize that HBXIP and Survivin collaborate in the execution of cytokinesis. Interestingly, several proteins previously localized to centrosomes that are known to be involved in centrosome duplication and bipolar spindle formation have also been localized to the midbody of dividing cells and implicated in cytokinesis, including Eg5, γ-tubulin, dynamin 2, dynein, dynactin, KIFC5A, and NudC (28-32). Thus, HBXIP joins this list of proteins implicated in both centrosome dynamics and cytokinesis.

The mechanisms that account for the apoptosis phenotype elicited by HBXIP deficiency remain to be elucidated but perturbations in centrosome dynamics that result in aberrant spindle formation have previously been associated with cell death. For example, it has been reported that chemical compounds and RNAi or antisense reagents targeting the kinesin-family protein Eg5 result in monopolar spindles, followed by apoptosis (12, 16, 27). Thus, failure to form a bipolar spindle may be a signal for apoptosis. In this regard, HBXIP has been reported to bind phosphorylated Survivin and to collaborate with Survivin in suppressing caspase-9 activation, at least in interphase cells where Survivin was overexpressed in the cytosol (8). However, in dividing cells, HBXIP and Survivin extensively colocalized only during telophase, and an apoptosis-inducing phenotype was not observed for either HBXIP or Survivin deficiency at that final phase (i.e., telophase) of the cell division process.

HBXIP is widely expressed in adult tissues in vivo, but its functions have heretofore not been explored. Using a model of partial hepatectomy, we investigated the requirement for HBXIP in dividing cells in vivo, employing a modified AS-ODN targeting the HBXIP mRNA. In this regard, modified AS-ODNs have been shown to efficiently knock down expression of target genes in the liver of animals, suggesting that liver is among the most responsive tissues to antisense-mediated target mRNA modulation (15, 33). Recent

Manuscript in preparation.
results from human clinical trials also support this notion. Antisense-mediated knockdown of HBXIP expression severely impaired liver regeneration, causing extensive apoptosis of dividing cells and reduced percentages of replicating cells. In contrast, quiescent liver was unaffected by AS-ODN targeting HBXIP. These observations thus show that HBXIP is required for survival and replication of hepatocytes induced to enter cell cycle but not quiescent hepatocytes. Thus, these studies document that HBXIP is a critical regulator of hepatocyte growth in vivo, thus making it a strong candidate for explaining the tumorigenic actions of HBx.

Acknowledgments

Received 5/23/2006; revised 7/6/2006; accepted 7/20/2006.

Grant support: The Edward Mallinckrodt, Jr. Foundation (W. Jiang), Lisa U Pardee Foundation (W. Jiang), NIH grants CA112053 (J.C. Reed) and AG15402 (J.C. Reed), Fondation Pour la Recherche Medicale (B. Bailly-Maire), California Breast Cancer Research Program (B. Bailly-Maire), and Uehara Memorial Foundation, Japan (R. Fujii).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank M. Hanai and J. Vailos for manuscript preparation; M. Farbirouz for technical assistance; T. Kanda (Chiba University, Japan) and J. Miyazaki (Osaka University, Japan) for HBx plasmids; E. Koller (Eis Pharmaceuticals, Carlsbad, CA) for the murine Egr5 ASO; R. Tsien for microscope access.

References

5. Kim CM, Koike K, Saito I, Miyamura T, Jay G. HBX gene expression in vivo that HBXIP is a critical regulator of hepatocyte growth but not quiescent hepatocytes. Thus, these studies document that HBXIP is required for HBXIP. These observations thus show that HBXIP is essential for midzone formation and cytokinesis. Proc Natl Acad Sci U S A 2005;102:3433–8.
HBXIP, Cellular Target of Hepatitis B Virus Oncoprotein, Is a Regulator of Centrosome Dynamics and Cytokinesis

Ryoji Fujii, Changjun Zhu, Yunfei Wen, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/18/9099

Cited articles
This article cites 33 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/18/9099.full.html#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
/content/66/18/9099.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.