Interaction of Hepatitis B Viral Oncoprotein with Cellular Target HBXIP Dysregulates Centrosome Dynamics and Mitotic Spindle Formation*5

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Hepatitis B virus infection is associated with hepatocellular carcinoma, claiming 1 million lives annually worldwide. To understand the carcinogenic mechanism of hepatitis B virus-encoded oncprotein HBx, we explored the function of HBx interaction with its cellular target HBXIP. Previously, we demonstrated that viral HBx and cellular HBXIP control mitotic spindle formation, regulating centrosome splitting. By using various fragments of HBx, we determined that residues 137CRHK140 within HBx are necessary for binding HBXIP. Mutation of the 137CRHK140 motif in HBx abolished its ability to bind HBXIP and to dysregulate centrosome dynamics in HeLa and immortal diploid RPE-1 cells. Unlike wild-type HBx, which targets to centrosomes as determined by subcellular fractionation and immunofluorescence microscopy, HBx mutants failed to localize to centrosomes. Overexpression of viral HBx wild-type protein and knockdown of endogenous HBXIP altered centrosome assembly and induced modifications of pericentrin and centrin-2, two essential proteins required for centrosome formation and function, whereas HBXIP nonbinding mutants of HBx did not. Overexpression of HBXIP or fragments of HBXIP that bind HBx neutralized the effects of viral HBx on centrosome dynamics and spindle formation. These results suggest that HBXIP is a critical target of viral HBx for promoting genetic instability through formation of defective spindles and subsequent aberrant chromosome segregation.

Chronic HBV infection affects ~400 million persons worldwide and is the single greatest risk factor for development of hepatocellular carcinoma (1–3). HBV-associated hepatocellular carcinoma kills over 1 million people annually, ranking it among the most lethal cancers. HBV 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 (1). Among these four proteins, only the X protein (known as HBx) is clearly associated with tumorigenesis. Viral HBx is a multifunctional protein, which appears 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 viral HBx (6). HBXIP sequences are well conserved among mammalian species, with close orthologs found in all vertebrate species where sequence data exist thus far. An overexpression of HBXIP suppresses HBV virus replication in HepG2 cells, in addition to suppressing the transacti

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2 The abbreviations used are: HBV, hepatitis B virus; WT, wild type; RNAi, RNA interference; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; PKA, cAMP-dependent protein kinase; GST, glutathione S-transferase; HA, hemagglutinin; siRNA, short interfering RNA; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; YFP, yellow fluorescent protein; CBP, CREB-binding protein.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) NM_006402 and U55762.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1 and Movies 1–4.

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interaction with HBxIP performing a mutagenesis analysis to identify the domains within cellular HBXIP and viral HBx viral proteins required for their interactions. Noninteracting mutants of HBx were then used to test the hypothesis that binding to HBXIP is critical for the dysregulatory effects of HBx on mitotic spindle formation.

**EXPERIMENTAL PROCEDURES**

**Plasmids and RNAi Reagents**—Full-length human HBXIP cDNA (GenBank accession number NM_006402) in pcDNA3 (8) was PCR-amplified using oligonucleotide primers 5’-AAATTTGAGTGGCAAGTCGTAGTCACTC-3’ (forward) and 3’-TCTGATAGGAGCCATTTTGTCGACTTCACCCTGT-3’ (reverse), and the resulting product was subcloned into XhoI/BamHI sites of the mammalian expression vector pEGFP-N1 (GenBank accession number U57562) or pGEX4T-1 (Invitrogen) for expression in bacteria. Various fragments of HBXIP cDNA were PCR-amplified from pGEX4T-1-HBXIP full-length plasmids. The gene encoding HBx was synthesized by PCR from DNA obtained from patients with hepatocellular carcinoma (10). HBx AAAA plasmids were PCR-amplified with oligonucleotide primers 5’-GTC TTT GTA CTA GGA GGC GCT GCT GCC TGG GTC TGC GCA CCA-3’ (forward) and 5’-TGG TGC GCA GAC CAA GGC GGC AGC AGC GCC TCC TAG TAC AAA GAC-3’ (reverse); HBx ADDD plasmids were PCR-amplified with oligonucleotide primers 5’-GTC TTT GTA CTA GGA GGC GCT GCT GCC TGG GTC TGC GCA CCA-3’ (forward) and 5’-TGG TGC GCA GAC CAA GGC GGC AGC AGC GCC TCC TAG TAC AAA GAC-3’ (reverse). The resulting products were subcloned into pCH expression vector derived from pcDNA3 (Invitrogen). Various fragments of pCHA-HBx cDNA were PCR-amplified from pCHA-HBx wild-type plasmid.

The pEYFP-tubulin and pECFP-histone 2B plasmids have been described (11). RNAi reagents targeting the coding regions of luciferase (538–983 bp) and HBXIP (GenBank accession number NM_006402; 522 bp) were synthesized by in vitro transcription of plasmids, as described previously (9). The plasmids encoding full-length p300 were provided by Neuveut and co-workers (12). The pCRE-Luc reporter containing four consensus CBP-responsive element sites and the plasmids encoding protein kinase A (PKA) were purchased from Stratagene. The pCRE-Luc reporter plasmids (Stratagene) by Lipofectamine 2000 (Invitrogen). Luciferase activity was determined 24 h after transfection, and the results are the average of six independent repeats. Luciferase activities in cell lysates were measured by a dual luciferase reporter assay kit (Promega), and luciferase activity was normalized with firefly activity or Renilla luciferase activity.

**Preparation of GST and GST Fusion Proteins**—Transformants of Escherichia coli BL21 carrying plasmid pGEX-4T-1 (containing GST) or derivatives containing various GST-HBXIP fusion proteins were cultured at 37 °C in LB medium with 100 μg/ml ampicillin for 4–6 h until the absorbance at 600 nm reached 0.5. Isopropyl β-D-thiogalactopyranoside (Sigma) was added to a final concentration of 1.0 mM and incubated at 24 °C for 2–3 h to induce the expression of GST and GST fusion proteins. Cells were harvested with CelllyticTM B cell lysis reagent (Sigma) with 0.1 mM PMSF, 0.1 mM dithiothreitol, and 0.5 ml/10 ml (per volume) of protease inhibitor mixture (Sigma). GST and GST fusion proteins were purified from bacterial extracts using glutathione-agarose beads as described previously (14), with the modification that after the protein-containing glutathione-Sepharose 4B beads were washed, the proteins were eluted with elution buffer (100 mM Tris-HCl, pH 8.0, and 100 mM glutathione), dialyzed against buffer containing 20 mM Tris-HCl, pH 8.0, and stored at −80 °C.

**In Vitro Transcription and Translation and GST Binding Assays**—In vitro transcription and translation with 35S-labeled methionine were carried out by use of the TNTTM T7-coupled reticulocyte lysate system (Promega) according to the vendor’s instructions. In vitro translated HBx WT and mutant viral proteins were incubated with the GST-HBXIP fusion proteins immobilized on glutathione-Sepharose 4B beads. The beads were washed with 1X phosphate-buffered saline containing 0.05% SDS, 0.1% Nonidet P-40, 0.1 mM PMSF, 0.1 mM dithiothreitol, and 1 tablet of protease inhibitor mixture (Roche Applied Science) and resuspended in the same buffer. Then 15 μl (Fig. 1B, top panel) or 30 μl (Fig. 1B, bottom panel) of in vitro translated 35S-labeled HBx WT or mutant proteins prepared as described above for binding assays were added to beads (10-μl bed volume) containing 20 μg of bound GST fusion proteins, and the volume was increased to 200 μl with washing buffer. The mixtures were incubated at 4 °C for 2 h or overnight with thorough mixing. The beads were collected by brief centrifugation at 1,000 rpm, 4 °C for 5 min, washed three times with 800 μl...
of washing buffer, resuspended in 50 μl of 2× SDS-PAGE sample buffer (Invitrogen), and fractionated by SDS-PAGE.

For detecting 35S-labeled proteins, the gels were fixed with destaining buffer (25% isopropl alcohol and 10% acetic acid), treated with NAMP100 (Amersham Biosciences) for 15–30 min, and subjected to fluorography at −80 °C. The gels also were stained with Coomassie Blue to assess loading of each GST protein applied in each reaction.

**Immunofluorescence and Confocal Microscopy**—Immunofluorescence and confocal microscopy analysis of cells were performed after 48 h of transfection and stained as protocol described previously (9) with rabbit polyclonal anti-HBXIP (1:200 dilution, affinity-purified from the antiserum reported previously (8) using column-immobilized glutathione S-transferase/HBXIP-(83–173) fusion protein; mouse monoclonal anti-α-tubulin (clone B-5-1-2, 1:1000 dilution; Sigma); rabbit anti-pericentrin antiserum (1:2000 dilution; Abcam Inc.); and rat monoclonal anti-HA high affinity antibodies (1 μg/ml; Roche Applied Science) followed by 1:200 dilution of various fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch and Southern Biotech). Cell imaging was performed using a Zeiss Axiovert 100M microscope. Data acquisition for quantification of immunofluorescence channels was performed using Simple PCI version 6.2 image software system (Compix Inc.) and a Radiance 2100/AGR-3Q Bio-Rad multiphoton laser point scanning confocal microscope. Images were analyzed by MetaMorph/MetaFluor version 7.0.

**Time-lapse Microscopy**—HeLa cells were transfected with 0.5 μg of pEYFP-α-tubulin and pECFP-Histone2B plasmids together with 200 nM of siRNAs using OligofectamineTM. Alternatively, cells were transfected with 50 ng of pEYFP-α-tubulin and pECFP-Histone2B plasmids together with 0.5 μg of HBx expression plasmid (HBx WT) (15, 16) using LipofectamineTM 2000. After 24 h, cells were cultured with CO2-independent medium (Invitrogen) containing 10% FBS overnight. The dishes were then transferred to a heated stage (37 °C) on a Zeiss Axiovert 100M microscope and observed under a ×63 lens. Phase contrast and fluorescence images of live cells were collected at 1-min intervals for 7–10 h.

**Isolation of Centrosomes**—Centrosomes were isolated from HeLa cells using an established protocol (17) with minor modifications. Cells were grown in 10 150-mm plastic dishes until ~60% confluent and then treated with nocodazole (0.1 μg/ml) for 12 h to enrich the population of metaphase cells. Mitotic cells were collected by a mitotic shake off procedure and incubated with nocodazole (10 μg/ml) and cytochalasin D (1 μg/ml) for 90 min to disrupt tubulin and actin cytoskeleton. Cells were lysed in 5 mM Tris-HCl, pH 8.0, containing 1% Triton X-100, proteinase inhibitor mixture (Sigma), 1 mM PMSF, 5 mM MgSO4, 5 mM EGTA, 1 μg/ml nocodazole, and 1 μg/ml cytochalasin D. Cell lysates were sedimented at 1500 × g to pellet the nuclei and cell fragments. The supernatant fraction was filtered through a nylon filter (40-μm pore size) and centrifuged on a 20% w/w Ficoll-400 cushion at 10,000 × g for 1 h. The crude centrosomal fraction (localized at the Ficoll-water interface) was collected, diluted 3-fold in cell lysis buffer, and further purified by a 20–70% sucrose gradient (1 ml each) centrifugation at 100,000 × g for 1 h. The individual 1-ml fractions, including the centrosome fraction (at ~55–60% sucrose), were collected, diluted 3-fold in cell lysis buffer, and centrifuged at 14,000 rpm in a tabletop Eppendorf centrifuge to pellet the centrosomes. The collected samples were immediately subjected for SDS-PAGE separation. Monoclonal anti-γ-tubulin antibody was purchased as mouse IgG from Sigma. Anti-centrin-2 (S-19) affinity-purified goat polyclonal antibody was purchased from Santa Cruz Biotechnology.

**RESULTS**

**Molecular Basis of Interaction between HBx Viral Protein and HBXIP**—To map the region within HBXIP required for binding viral HBx, we conducted in vitro protein binding assays with GST fusion proteins composed of full-length HBXIP or truncated mutants of HBXIP constructed with guidance from the predicted secondary structure of this cellular protein (18) (Fig. 1A). These GST fusion proteins were tested for binding with in vitro translated 35S-labeled HBx. Full-length GST-HBXIP-(1–173) and N-terminal fragments composed of residues 1–99, 1–81, and 1–64 bound to HBx in vitro, thus suggesting that the N-terminal region of HBXIP is sufficient for HBx binding. In contrast, C-terminal and interior fragments of HBXIP comprising residues 23–99, 46–99, 82–144, and 82–163 failed to bind HBx viral (Fig. 1B).

We reasoned that if HBXIP is the cellular target of HBx that is relevant to HBx-mediated spindle defects (9), then overexpression of HBXIP should neutralize HBx, as should fragments of HBXIP that bind HBx. To test this hypothesis, we transfected HeLa cells with HBx in combination with full-length HBXIP, a fragment of HBXIP composed of residues 1–64 that retains HBx binding activity, or a pcFLAG vector then determined the percentage of cells with aberrant spindles among mitotic cells. Expression of FLAG epitope-tagged HBXIP full-length and the HBXIP-(1–64) fragment were confirmed by immunoblotting with anti-FLAG monoclonal antibody (Fig. 1C). To test the effects of HBXIP and HBXIP-(1–64) on spindle formation, HeLa cells were fixed at 48 h after transfection with plasmids encoding HBx WT viral protein, in combination with FLAG-tagged HBXIP full-length FLAG-HBXIP-(1–64) fragment, or pcFLAG vector and stained with anti-α-tubulin (green) anti-pericentrin (red) antibodies, and with DNA-binding fluorochrome (DAPI). The percentages of cells with multipolar or unipolar or bipolar spindles were enumerated among mitotic cells, counting the number of centrosomes per cell (Fig. 1D).

Untransfected control HeLa cells rarely demonstrated any spindle abnormalities (≥98% bipolar spindles (data not shown)). In contrast, cells transfected with HBx showed marked spindle abnormalities, with approximately one-third of prometaphase/metaphase cells having multipolar spindles with three or more centrosomes, as well as an increase in cells with unipolar spindles (Fig. 1D) consistent with previous reports (9, 19). Co-expressing HBXIP full-length protein or the HBXIP-(1–64) fragment with HBx viral protein reduced the percentage of cells with abnormal spindles, thus confirming our predictions. By themselves, in the absence of HBx, overexpression of HBXIP or HBXIP-(1–64) induced ~5% multipolar/unipolar spindles (data not shown), as published previously (9, 19). Representative images of cells are presented in Fig. 1E, showing an
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HBx-expressing cell with tripolar spindle and bipolar spindle in cells expressing HBx and in combination with HBXIP full-length or HBXIP-(1–64).

Next we determined which residues within hepatitis X virus oncoprotein (HBx) are necessary for binding to HBXIP using the same approach. Using the predicted secondary structure (13, 18) to guide construction of HBx truncation mutants (Fig. 2A), we compared full-length and truncated HBx proteins (Fig. 2B) for binding to HBXIP in vitro. For these experiments, in vitro protein-binding assays were performed with single-form GST fusions containing HBXIP full-length protein or the HBXIP N-terminal 1–64 fragment (Fig. 2C), testing binding to $^{35}$S-labeled full-length HBx and various C-terminal truncation mutants of HBx. As expected, the $^{35}$S-labeled full-length HBx protein bound to both GST-HBXIP-(1–173) and GST-HBXIP-(1–64) at levels greater than observed for GST control protein (Fig. 2D). Similarly, $^{35}$S-HBx-(1–140) maintained its binding activity. In contrast, $^{35}$S-HBx-(1–136), which has just four amino acids fewer than HBx-(1–140) displayed weak binding to GST-HBXIP (FL) and no binding to GST-HBXIP-(1–64), suggesting this portion of HBx makes important contributions to HBXIP binding. The HBx-(1–117) truncation mutant completely lost its ability to bind to either GST-HBXIP (FL) or GST-HBXIP-(1–64) (Fig. 2D).

Because these results implicated the four residues corresponding to amino acids 137–140 in the interaction of HBx with HBXIP, we produced an alanine substitution mutant replacing $^{137}$CRHK$^{140}$ with AAAA. Also, because the $^{137}$CRHK$^{140}$ sequence is rich in basic amino acids, we generated a mutant containing acidic residue replacements, namely ADDD (Fig. 3A). In vitro protein binding assays demonstrated that the AAAA and ADDD mutants failed to bind GST-HBXIP or GST-HBXIP-(1–64) (Fig. 3B).

To determine whether residues 137–140 are also important for HBx binding to HBXIP in cells, we performed co-immunoprecipitation experiments using transfected HeLa cells. Accordingly, cDNAs encoding HBx WT or mutant HBx proteins were subcloned into a mammalian expression plasmid with HA epitope tags and co-expressed in HeLa cells with GFP-tagged HBXIP. Immunoprecipitations were performed with either control mouse IgG or antibody directed against the HA-epitope tagg on HBx, and the immune complexes were analyzed by SDS-PAGE/immunoblotting using anti-GFP to detect GFP-tagged HBXIP. These co-immunoprecipitation studies showed that only HA-HBx WT protein associated with GFP-HBXIP, but not the two HA-HBx mutants (Fig. 3C).

Immunoblotting analysis of the lysates showed that all of the HBx variants were expressed at comparable levels in HeLa cells (see below), thus excluding a trivial explanation for the differential recovery of GFP-HBXIP with immunoprecipitates containing WT versus mutants of HBx. We therefore conclude that residues 137–140 of HBx are critical for interactions with HBXIP in vitro and in cells.

Finally, we performed additional co-immunoprecipitation experiments using an alternative tag on HBXIP (FLAG instead of GFP) and synchronizing the cells in G2/M-phase to enrich for the phase of the cell cycle where HBXIP plays its intrinsic role in bipolar spindle formation (9). Moreover, in addition to full-length HBXIP, the ability of HBx (wild type), HBx (ADDD), and HBx (AAAA) to bind the HBXIP-(1–64) N-terminal frag-
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Time-lapse Video Microscopy of Cells Expressing Wild-type Versus Mutant Viral HBx—To extend the studies of the effects of HBx WT and mutants on the cell division process, we used time-lapse video microscopy to monitor division of HeLa cells expressing cyanine fluorescent protein-tagged histone H2B (CFP-H2B) to mark chromosomes and yellow fluorescent protein (YFP)-tagged α-tubulin (YFP-tubulin) for microtubules (22). Whereas HeLa cells transfected with control pc-HA vector completed mitosis and cytokinesis in a timely fashion (Fig. 6 and supplemental movie 1), one-third of the cells transfected with an HBx-encoding plasmid developed tri- or multipolar spindles and experienced a marked delay in attempting to reach metaphase, followed either by a failure to undergo cytokinesis or a marked delay (Fig. 6 and supplemental movie 2). In contrast, HeLa cells transfected with the HBx-(AAAA) and HBx-(ADDD) mutants, respectively (p < 0.05 for each mutant compared with wild type by unpaired t test) (Fig. 5A). Increased frequencies of cells with unipolar spindles were also found in cultures transfected with WT HBx compared with the mutants (Fig. 5A).

We initially worked with HeLa cells for studying the effects of the HBx-HBXIP interaction on centrosome dynamics and spindle formation. However, these cells are known to have impaired p53 and retinoblastoma proteins and defective cell cycle checkpoint regulation (20). To extend these studies, we also determined the phenotype of WT and mutant HBX in human diploid epithelial cells (RPE-1), a telomerase-immortalized human retinal epithelium cell line with normal p53 function (21). Similar results were obtained for RPE-1 cells transfected with HBx versus HBx mutants, showing that WT but not the HBXIP nonbinding mutants of HBx caused spindle abnormalities (Fig. 5B). We therefore conclude that mutations of the 137CRHK140 motif within the HBx viral protein that impact HBXIP binding largely abolished its ability to induce abnormal spindles in dividing epithelial cells.

We determined and HBXIP-(64–173) C-terminal fragment was compared. Immunoprecipitation of wild-type HA-tagged HBx revealed the presence of associated full-length HBXIP and HBXIP-(1–64), but not HBXIP-(64–173) C-terminal fragment (Fig. 4). In contrast, the HBx (AAAA) and HBx (ADDD) proteins did not associate with HBXIP or HBXIP fragments. Immunoblotting analysis confirmed production of all HBX mutants and all HBXIP fragments (Fig. 4, A and B). Thus, the N-terminal 1–64 region of HBXIP is necessary and sufficient for binding HBx, and the 137CRHK140 motif of HBx is required for binding to the N-terminal domain of HBXIP.

Abnormal Spindle Formation Induced by HBx Viral Protein Depends on the 137CRHK140 Motif—Previously we showed by RNAi experiments that HBXIP is essential for bipolar spindle formation in dividing HaCaT cells and mouse embryo fibroblast cells (9). If the interaction of HBx with cellular HBXIP is important for the ability of HBx to disrupt normal centrosome dynamics and bipolar spindle formation, we predicted that the HBx mutants that fail to bind HBXIP would display a loss of function phenotype. To test this hypothesis, we expressed WT versus mutant versions of HBx in HeLa cells or RPE-1 cells by transient transfection, and we fixed the cells 48 h later, staining the cells for immunofluorescence microscopy by staining with anti-α-tubulin (green) and anti-pericentrin (red) followed by DAPI (blue). The mitotic cells were identified by condensed chromosomes. Representative images of the predominant phenotypes of spindles for each group are shown in Fig. 5. Cultures of HeLa cells transfected with WT HBx contained many cells with tripolar or multipolar spindles, whereas such cells with aberrant mitotic spindles were rarely seen in cultures transfected with HBx (AAAA) or HBx (ADDD). Quantification of the results showed 26.7 ± 4.7% cells with multipolar or tripolar spindles in cultures transfected with WT HBx, compared with only 1.8 ± 1.3 and 3.8 ± 2.7% in cultures transfected with HBx (AAAA) and HBx (ADDD) mutants, respectively (p < 0.05 for each mutant compared with wild type by unpaired t test) (Fig. 5A). Increased frequencies of cells with unipolar spindles were also found in cultures transfected with WT HBx compared with the mutants (Fig. 5A).

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mutants that fail to bind HBXIP have a substantial decrease in their ability to alter spindle formation and cytokinesis compared with the WT viral HBx protein in HeLa cells.

Wild-type HBx (but Not HBx Mutants) Co-localize with Centrosomes in Dividing RPE-1 Cells—We investigated the location of the viral HBx and mutant HBx proteins in human diploid epithelial cells (RPE-1), in which WT but not mutant HBx induces spindle defects (Fig. 5B). Previous studies reported expression of the HBx protein in cytosol and nucleus, which we confirmed, but we wondered whether a portion of the intracellular HBx protein associates with centrosomes in dividing cells. To address this question, we used anti-HA antibody (green) to localize HA-tagged wild-type or mutant HBx viral proteins and anti-pericentrin antibody (red) to mark centrosomes in immunofluorescence experiments (Fig. 7A). Quantitative analysis of two-color immunofluorescence confocal images measuring the co-localization of red and green fluorescence (“Scattergram values”) showed greater co-localization of pericentrin with wild-type HBx compared with the AAAA and ADDD mutants of HBx. Statistical analysis of the quantitative measurement of the intensity of overlapping green and red fluorescence confirmed significantly more co-localization of pericentrin with wild-type HBx compared with the HBXIP nonbinding HBx mutants (Fig. 7B, p = 0.005 by unpaired t test). Thus, we concluded that the 137CRHK140 motif is not only necessary for HBx binding to HBXIP but also required for targeting of HBx to centrosomes in dividing human epithelial cells.

Next, we used subcellular fractionation to explore the question of HBx association with centrosomes. First, expression of WT and mutant (AAAA and ADDD) proteins in HeLa cells was compared by immunoblotting, confirming comparable levels of these proteins in whole cell lysates (Fig. 8A). Second, centrosome-containing fractions were prepared from HeLa cells transfected with HBx viral protein WT or mutants that were synchronized by nocodazole using an established procedure that included discontinuous sucrose density gradient centrifugation (23–25). Aliquots from the gradients were analyzed by immunoblotting to localize HA-tagged HBx proteins (Fig. 8B) and the centrosomal marker protein γ-tubulin (Fig. 8C). Whereas WT HBx was readily detected in centrosome-containing sucrose gradient fractions, little or no HBx AAAA or HBx ADDD mutant proteins were found (Fig. 8B). These experiments thus provide corroborating evidence for immunofluorescence co-localization data that a portion of WT HBx but not non-HBXIP-binding HBx mutants associates with centrosomes (Fig. 7).

HBXIP Knockdown and HBx Expression Alter Pericentrin and Centrin-2 Modification—We further used cell fractionation and discontinuous sucrose gradient centrifugation to characterize centrosomal proteins pericentrin and centrin-2 in cells expressing WT versus HBXIP nonbinding mutants of HBx. Previous studies have reported that pericentrin undergoes proteolytic processing in connection with centrosome assembly and centrosome dynamics (23–25). In cells expressing WT HBx, two predominant forms of pericentrin (~250 and ~150 kDa) were present (Fig. 9A). In contrast, cells expressing HBXIP nonbinding mutants of HBx contained predominantly the larger ~250-kDa form of pericentrin (Fig. 9A), similar to con-

FIGURE 3. Analysis of site-specific mutations of HBx viral protein. A, residues in HBx subjected to mutagenesis are depicted. B, binding of wild-type (WT) versus mutants (AAAA or ADDD) HBx to GST-HBXIP or GST-HBXIP-1–64 was compared by in vitro protein binding assays. 35S-Labeled HBx WT and HBx mutants (AAAA and ADDD) were incubated with GST control, GST-HBXIP, or GST-HBXIP-1–64. Bound proteins were detected by SDS-PAGE/autoradiography. C, comparison of WT and mutant HBx protein interaction with HBXIP by co-immunoprecipitation. GFP-tagged HBXIP and HA-tagged HBx WT or mutant HBx (AAAA and ADDD) proteins were expressed by co-transfection in HeLa cells. After 24 h, cell lysates were prepared and used for immunoprecipitation with IgG-control or anti-HA-conjugated agarose beads. Immune complexes were analyzed by SDS-PAGE/immunoblotting using anti-GFP antibody. As a control, 20% of cell lysate was run directly in gels.

FIGURE 4. Association of FLAG-tagged HBXIP mutants and HA-tagged HBx mutants in cells synchronized in G2/M phase. A, expression of HBXIP full-length, C-terminal fragment (64–173) and N-terminal fragment (1–64) proteins from HeLa S cells was measured by immunoblotting (IB) using lysates normally used for total protein content (50 μg). B, G2/M synchronized HeLa cells expressing HA-tagged HBx WT or mutants and FLAG-tagged HBXIP full-length or fragments were lysed, and immunoprecipitation (IP) was performed using anti-HA antibody and followed by immunoblotting with anti-FLAG antibody (top panel). Lysates were also analyzed directly by immunoblotting using anti-HA antibody (bottom panel).

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Immunoblot analysis showed that only an ∼20-kDa form of centrin-2 was detected in centrosome-containing fractions from WT HBx-expressing cells, whereas both an ∼20 and a smaller ∼16-kDa form of centrin-2 were found in cells expressing HBXIP nonbinding mutants (Fig. 9B), similar to control untransfected cells (Fig. 9C). These results suggest that HBx alters centrosome assembly, maturation, or stability, unlike HBXIP nonbinding mutants of HBx.

Next, to assess the contribution of HBXIP to centrosome structure, we used previously characterized RNAi reagents to specifically knockdown expression of endogenous HBXIP (9). Immunoblotting comparison of control and HBXIP siRNA-treated HeLa cells confirmed a lack of HBXIP protein in association with isolated centrosomes from HBXIP-siRNA-treated cells (Fig. 9D). Analysis of HBXIP-deficient centrosomes by density gradient centrifugation indicated that much of the pericentrin in HBXIP-deficient cells appeared as an ∼150-kDa form (Fig. 9, C and D) rather than the usual 250-kDa form seen in the control cells (Fig. 9C), whereas centrin-2 was present mostly as an ∼20-kDa isof orm (Fig. 9D) rather than the usual pair of ∼20- and ∼16-kDa isoforms seen in control cells (Fig. 9C). Density gradient analysis also suggested that centrosomal protein complexes from HBXIP-deficient cells are larger, sedimenting at lower sucrose concentrations in discontinuous gradients (shift from 55 to 65% fractions to the 0–40% fractions), as determined by comparison of the sedimentation of γ-tubulin, pericentrin, and centrin-2 (Fig. 9, C and D). These results implicate endogenous HBXIP in the proper assembly, maturation, or stability of centrosomes in dividing cells.

Dissociation of Mitotic Spindle Phenotype of HBx from Transcriptional Activity—HBx is reported to have transcriptional activity in some contexts (26). For example, HBx control untransfected cells (Fig. 9C). Immunoblot analysis showed that only an ∼20-kDa form of centrin-2 was detected in centrosome-containing fractions from WT HBx-expressing cells, whereas both an ∼20 and a smaller ∼16-kDa form of centrin-2 were found in cells expressing HBXIP nonbinding mutants (Fig. 9B), similar to control untransfected cells (Fig. 9C). These results suggest that HBx alters centrosome assembly, maturation, or stability, unlike HBXIP nonbinding mutants of HBx.

FIGURE 5. Spindle defects induced by WT but not mutants of HBx. A, HeLa cells were transfected with plasmids encoding HBx wild-type (WT) or AAAA and ADDD mutant HBx proteins. After 48 h, cells were fixed and stained using antibodies recognizing centrosome marker pericentrin (red) or α-tubulin (green), followed by DAPI for detecting DNA (blue). Representative photomicrographs are presented, showing a cell with tripolar spindle in cultures transfected with wild-type HBx and examples of cells with bipolar spindles in cultures transfected with 137AAAA and 137ADDD mutants of HBx, white bars represent 5 μm (right). The proportion of cells with the proportion of cells with unipolar (white bars) or multipolar (black bars) spindles was enumerated (mean ± S.E.; n = 144) based on examination of 144 mitotic cells per condition. B, representative photomicrographs of RPE-1 cells were treated and analyzed as above. Shown are examples of a cell with monopolar spindle in cultures transfected with HBx WT and cells with bipolar spindles in cultures transfected with HBx mutants. The proportion of cells with unipolar or multipolar spindles was enumerated based on examination of 86 mitotic cells per condition (mean ± S.D.; n = 86).

FIGURE 6. Comparison of WT and mutant HBx proteins by time-lapsed video microscopy. Time-lapse video microscopy was used to study HeLa cells that had been transfected with control pCtH-vector or pCtH-HBx (WT or mutant) plasmids, together with enhanced YFP-tubulin and ECFP-H2B plasmids. Representative photomicrographs taken during filming are shown in the panels, representing merged images of YFP-tubulin and CFP-H2B (pseudo-colored red and green, respectively) in phase contrast, and indicating the approximate time elapsed (minutes). CTR, control transfected cells with normal mitosis; HBx-WT showing tripolar spindle formation, followed by delayed cytokinesis; and HBx (AAAA) and HBx (ADDD) showing essentially normal mitosis with metaphase delay.
HBx Dysregulates HBXIP Control of Mitotic Spindle Formation

**DISCUSSION**

The data presented here provide evidence that the viral oncoprotein HBx alters centrosome dynamics through a mechanism requiring its interaction with cellular protein HBXIP, a regulator of centrosome dynamics and cytokinesis. Specifically, mutants of viral HBx that failed to bind HBXIP failed to alter spindle formation and cell division, unlike wild-type HBx. Also, full-length HBXIP as well as an HBx-binding fragment of HBXIP functionally neutralized viral HBx, presumably competing for interaction with endogenous HBXIP protein. Thus, we propose that viral HBx dysregulates the normal role of HBXIP in centrosome biology, leading to aberrant tripoli and multipolar spindles, and thus contributing to genetic instability and tumorigenesis. Moreover, the different phenotypes of wild-type HBx and HBXIP nonbinding mutants of HBx were documented in both HeLa and RPE-1 cells. RPE-1 cells are immortal diploid cells with normal p53 checkpoints (27), whereas HeLa cells have defective p53 regulation. Thus, HBx dysregulation of HBXIP occurs regardless of p53 status.

The mechanisms by which HBx dysregulates centrosome dynamics remain to be defined. Biochemical analysis of centrosomes in HBx-expressing cells showed evidence of altered assembly, as determined by the mobility of centrosomal complexes in sucrose gradients, and by altered modifications of pericentrin and centrin-2 proteins. Similarly, RNAi-mediated depletion of cellular HBXIP altered centrosome assembly and induced modifications of centrosomal proteins. Thus, HBXIP appears to be required for proper centrosome assembly or stability, with viral HBx dysregulating centrosome structure. The effects of viral HBx on centrosome structure are absent in cells expressing HBx mutants that fail to bind HBXIP, demonstrating dependence on interaction with cellular HBXIP for the disruption of normal centrosome structure. The structural defects in centrosomes observed following depletion of HBXIP or treatment with viral HBx could arise either through production of centrosome intermediates that fail to mature during duplication or where centrosomes fail to symmetrically split to form the bipolar spindle (28).

It has been shown that proteolyzed pericentrins interfere with normal spindle formation and centrosome assembly during mitosis, leading to prometaphase arrest (23–25). Interestingly, pericentrin from control HeLa cells was present mostly as an ~250-kDa species, whereas a smaller ~150-kDa form of pericentrin predominated in cells expressing either viral HBx or treated with RNAi to delete HBXIP. Thus, the centrosome defects observed in cells expressing viral HBx and in cells with HBXIP deficiency may be secondary to aberrant regulation of Pericentrin processing. In this regard, pericentrins are essential factors for centrosome formation that bind to γ-tubulin and anchor γ-tubulin-containing ring complexes to centrosomes (29), thus presumably explaining why their aberrant processing causes dysregulation of centrosome dynamics in dividing cells. We also noticed that whereas centrin-2 normally is present as a pair of 20- and 16-kDa isoforms, only a single 20-kDa form of centrin-2 was found in HBXIP-deficient cells and in cells expressing viral HBx. The origin of these different forms of centrin-2 is currently unknown, but theoretically they could...
arise by either proteolytic processing or expression from alternatively spliced mRNAs (30). Their functions are also undefined to date.

Although HBXIP and HBx associate with centrosomes, it is unlikely that HBXIP is a core component of these organelles for two reasons. First, our prior immunolocalization and fluorescent protein tagging experiments showed that HBXIP is targeted to spindle microtubules of dividing cells (9). Thus, HBXIP may be connected to centrosomes via interactions with tubulin or tubulin-associated proteins, but is not a core component of these organelles. Second, recent RNAi experiments showed that silencing of the expression of 14 of 15 centrosome core components caused G1 arrest, which is p53-dependent (31). In contrast, inhibiting HBXIP expression with RNAi in p53-defective HeLa cells causes G2 arrest, which is p53-independent (9). Also, dysregulating HBXIP by expression of viral HBx produces tripolar and multipolar spindles, regardless of p53 status and without preventing mitosis from occurring, at least in the majority of cells examined.

HBXIP shares in common with several other proteins the ability to control both centrosome splitting to form bipolar spindles and cytokinesis to split dividing cells during telophase. The list of proteins with this dual function minimally includes Eg5, \( \gamma \)-tubulin, dynamin 2, dynein, dynactin, KIFC5A, and NudC (32–36). Interestingly, centrosomes have been implicated in cytokinesis, based on experiments where it was possible to remove or disrupt centrosomes (37, 38). We observed that although most HBx-expressing cells failed to complete mitosis, those cells that managed to segregate chromosomes became arrested upon attempting cytokinesis. In contrast, mutants of viral HBx that failed to bind HBXIP executed cytokinesis normally. Thus, viral HBx dysregulates both the bipolar-spindle formation and cytokinesis functions of cellular HBXIP. Defects in either of these processes contribute to genetic instability, and thus could have a tumor-promoting effect.

Viral oncoproteins often have multiple cellular targets and mechanisms of action (39). Through interactions with vari-

FIGURE 8. Subcellular fractionation analysis of centrosome targeting by WT and mutant HBx proteins. A, HeLa cells were transfected with pchA-HBx WT or mutants (AAAA, ADDD). After 24 h, cells were synchronized with nocodazole (0.1 \( \mu \)g/ml) for another 24 h and lysed, and their nuclei were removed by centrifugation. The supernatant was analyzed (50 \( \mu \)g of total protein per lane), by SDS-PAGE/immunoblotting using anti-HA (top) and anti-\( \alpha \)-tubulin (bottom) antibodies. B and C, HeLa cells were transfected with plasmid encoding HA-HBx WT or mutants (AAAA, ADDD). After 24 h, cells were synchronized with nocodazole (0.1 \( \mu \)g/ml) for another 24 h, and centrosome-containing fractions were prepared and analyzed by sucrose gradient centrifugation, followed by SDS-PAGE/immunoblot analysis of the sucrose fractions using antibodies recognizing HBx protein (B) or \( \alpha \)-tubulin (C). Molecular weight markers are shown in kilodaltons. CNTL, control.

FIGURE 9. HBx but not HBXIP nonbinding HBx mutants cause centrosomal protein modifications. A and B, HeLa cells were transfected with plasmids encoding WT, AAAA, or ADDD HBx proteins. After 24 h, cells were synchronized with nocodazole (0.1 \( \mu \)g/ml) for another 24 h, and centrosome-containing fractions were prepared and analyzed by sucrose density gradient centrifugation. Fractions were analyzed by SDS-PAGE/immunoblotting using anti-pericentrin (A) or anti-centrin-2 (B) rabbit polyclonal antibodies. Arrowheads indicate the \(-22\) and \(-16\)-kDa isoforms of centrin-2. C and D, HeLa cells were transfected with control pc-HA vector (C) or HBXIP-targeting (D) RNAi reagents. Cells were prepared as described above, and fractions were analyzed using antibodies recognizing HBXIP, pericentrin, or centrin-2.

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