HBxAP\(\alpha\)/Rsf-1-mediated HBx–hBubR1 interactions regulate the mitotic spindle checkpoint and chromosome instability

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Hepatitis B virus (HBV) X protein encoded by the HBV genome, is involved in the development of HBV-mediated liver cancer, whose frequency is highly correlated with chromosomal instability (CIN). We reported previously that HBx induces mitotic checkpoint dysfunction by targeting the human serine/threonine kinase BubR1 (hBubR1). However, the underlying mechanism remained unresolved. Here, we show that HBx protein-associated protein \(\alpha\) (HBxAP\(\alpha\))/Rsf-1 associates with hBubR1 and HBx in the chromatin fraction during mitosis. Depletion of HBxAP\(\alpha\)/Rsf-1 abolished the interaction between HBx and hBubR1, indicating that HBxAP\(\alpha\)/Rsf-1 mediates these interactions. Knockdown of HBxAP\(\alpha\)/Rsf-1 with small interfering RNA did not affect the recruitment of hBubR1 to kinetochores; however, it did significantly impair HBx targeting to kinetochores. A deletion mutant analysis revealed that two Kunitz domains of HBx, the Cdc20-binding domain of hBubR1 and full-length of HBxAP\(\alpha\)/Rsf-1 were essential for these interactions. Thus, binding of HBx to hBubR1, stabilized by HBxAP\(\alpha\)/Rsf-1, significantly attenuated hBubR1 binding to Cdc20 and consequently increased the rate of mitotic aberrations. Collectively, our data show that the HBx impairs hBubR1 function and induces CIN through HBxAP\(\alpha\)/Rsf-1, providing a novel mechanism for induction of genomic instability by a viral pathogen in hepatocarcinogenesis.

Introduction

Aneuploidy, an abnormal number of chromosomes generated by an increased rate of chromosomal missegregation due to chromosomal instability (CIN), is a very common characteristic of human solid tumors (1,2). It has been proposed that the mechanisms leading to CIN are associated with defects in the cohesion of sister chromatids, bipolar spindle formation and/or spindle attachment (3). In addition, dysfunction of the mitotic checkpoint has been recognized as a major cause of CIN (4). The mitotic checkpoint prevents sister chromatid separation until all chromosomes are properly attached to the mitotic spindle by inhibiting the activity of the E3 ubiquitin ligase APC/C (anaphase-promoting complex/cyclosome) (5,6). This checkpoint is composed of a number of proteins, including Mad1, Mad2, Bub1 and BubR1, which collectively inhibit the activity of APC/C by interacting with the APC/C coactivator protein Cdc20 (6,7). Mouse models in which mitotic checkpoint signaling is decreased show an increase in CIN (8–11). Thus, impaired activity of the mitotic checkpoint is an important contributing factor to CIN.

The hepatitis B virus (HBV) is a risk factor for the development of hepatocellular carcinoma (12,13). HBV-associated hepatocellular carcinoma exhibits a higher rate of CIN in the form of micronuclei and multinucleation than hepatocellular carcinoma caused by other risk factors (14–16). The HBV genome is 3.2kb in size and consists of four overlapping open reading frames encoding DNA polymerase, surface antigen, core protein and the regulatory X protein, HBV X protein (HBx). HBx has been shown to enhance HBV replication and contribute to HBV-induced hepatocarcinogenesis (17,18). It also disrupts cell-cycle control through binding to various proteins involved in cell-cycle regulation (19–26). Notably, HBx has been implicated in CIN, which is in turn involved in defects in mitotic processes (27). We and others have demonstrated that HBx induces dysregulation of mitotic checkpoint and centrosome dynamics, which leads to mitotic aberrations, including misaligned chromosomes in metaphase and lagging chromosomes in anaphase (27–29).

Hepatitis B virus X protein-associated protein (HBxAP) was identified as a protein that interacts with HBx and is involved in HBx-dependent activation of HBV transcription (30). There are three HBxAP variants: \(\alpha\), \(\beta\) and \(\gamma\) (31). These proteins contain a plant homeodomain-type zinc finger domain, which is found in many proteins that play roles in chromatin remodeling, transcriptional regulation and protein–protein interaction. Of these variants, HBxAP\(\beta\) protein is the most abundant (31). HBxAP\(\beta\), also known as Rsf-1, is one of the non-catalytic subunits of the human ISWI-containing factor, RSF (remodeling and spacing factor), which has been reported to participate in nucleosome assembly and chromatin remodeling with FACT (facilitates chromatin transcription) (32–34). A number of recent studies have shown that overexpression of HBxAP\(\beta\)/Rsf-1 promotes cell proliferation and tumor growth. Moreover, HBxAP\(\beta\)/Rsf-1 is overexpressed in various human cancers, suggesting a possible link between HBxAP\(\beta\)/Rsf-1 and tumorigenesis (35–38). In addition, the fact that HBxAP\(\beta\)/Rsf-1 is required for the stable assembly of histone H3 variant CENP-A (centromere protein A) into centromeric chromatin in mid-G1 suggests that Rsf-1 may play other roles in cell-cycle progression (39).

Recently, we observed that HBx deregulates mitotic checkpoint function by targeting human BubR1 (hBubR1) and thereby induces mitotic aberrations (27). Here, we investigated the mechanism underlying this action of HBx. We found that the chromatin-remodeling factor HBxAP\(\alpha\)/Rsf-1 is required for HBx recruitment to kinetochores and subsequent binding to hBubR1. Depletion of HBxAP\(\alpha\)/Rsf-1 increases hBubR1 binding to Cdc20 by abolishing the interaction between HBx and hBubR1 and results in decreased CIN. These results indicate that HBxAP\(\alpha\)/Rsf-1 plays a critical role in HBx-mediated CIN.

Materials and methods

Cell culture

HBx-transfected ChangX, HeLa and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and maintained in CO\(_2\) at 37°C. ChangX cells were originally established by stably cotransfecting pTelX and pUHD172-1 as described previously (40).

Reagent and antibodies

Antibodies against Myc, topoisomerase II\(\alpha\) and green fluorescent protein (GFP) antibodies were purchased from Santa Cruz Laboratory (Santa Cruz, CA). HBxAP\(\alpha\)/Rsf-1 antibody was from Abnova. BubR1, Aurora B and Bub3 antibodies were purchased from BD Biosciences (Franklin Lakes, NJ) and Flag antibody was from Sigma (St Louis, MO). \(\alpha\)-Tubulin antibody was from Oncogene (Cambridge, MA). V5 antibody was purchased from Invitrogen (Carlsbad, CA).

Abbreviations: APC/C, anaphase-promoting complex/cyclosome; CIN, chromosomal instability; GFP, green fluorescent protein; GST, glutathione-S-transferase; hBubR1, human BubR1; HBV, hepatitis B virus; HBx, HBV X protein; HBxAP, hepatitis B virus X protein-associated protein; siRNA, small interfering RNA.
Small interfering RNA, plasmid transfection

The small interfering RNAs (siRNAs 1 and 2) of HBxAPα/Rsf-1 and control were purchased from Invitrogen. The sequences were 5′-UCUUUGUC UCGACCAAUGGC-3′ for siRNA 1 and 5′-CCAUGUCAACAUU UCUU-3′ for siRNA 2. The siRNA 3 of HBxAPα/Rsf-1 was purchased from Ambion. The sequence was 5′-GGGAAUGUGCAAAACAUU-3′ for siRNA 3. The siRNAs were transfected using transfection reagent (Invitrogen) according to manufacturer’s instructions. To construct RNAi-resistant HBxAPα/Rsf-1, the nucleotide sequence CCTATGAGCTAATTCTT was changed to CCATCCTGAGCTAATTCTT. The mutations were verified by DNA sequencing. For generation of the full-length and various Myc-tagged deletion mutant HBx constructs, corresponding regions were PCR amplified using HBx (adv) as the template and subcloned into the pCMV-Myc. These plasmids were then transiently transfected into human liver carcinoma (HepG2) cells according to manufacturer’s instructions. Various pcDNA6-Rsf1-V5 constructs were kind gift from Dr Ie-Ming Shin (Johns Hopkins Medical Institutions). Various pEGFP-BubR1 constructs were kind gift from Dr Gordon Chan (University of Alberta, Canada).

Immunoblotting and immunoprecipitation

Cells were trypsinized, washed once with phosphate-buffered saline and resuspended in buffer B [30 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40, 0.1% sodium dodecyl sulfate, 0.1% sodium deoxycholate, 5 mM ethylenediaminetetraacetic acid and 5 mM sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed according to manufacturer’s instructions. To construct recombinant HBxα/Rsf-1, the nucleotide sequence CCATCCTGAGCTAATTCTT was changed to CCATCCTGAGCTAATTCTT. The mutations were verified by DNA sequencing. For generation of the full-length and various Myc-tagged deletion mutant HBx constructs, corresponding regions were PCR amplified using HBx (adv) as the template and subcloned into the pCMV-Myc. These plasmids were then transiently transfected into human liver carcinoma (HepG2) cells according to manufacturer’s instructions. Various pcDNA6-Rsf1-V5 constructs were kind gift from Dr Ie-Ming Shin (Johns Hopkins Medical Institutions). Various pEGFP-BubR1 constructs were kind gift from Dr Gordon Chan (University of Alberta, Canada).

Results

HBxAPα/Rsf-1 mediates HBx–hBubR1 interactions in mitosis

We found previously that HBx interacts with the mitotic checkpoint components hBubR1 in intact cells and demonstrated that this interaction is enhanced in mitotic cells relative to interphase cells (27). However, the underlying mechanism remained unclear. To further explore this mechanism, we performed binding assays in intact cells (in vivo) and cell-free systems (in vitro). Interestingly, binding of Myc-tagged HBx and hBubR1 in vivo was increased in mitotic cells compared with interphase cells (Figure 1A). Consistent with mitosis-specific binding, treatment with nocodazole or paclitaxel, which arrest cells in prometaphase, increased this interaction (Figure 1B). Again, recombinant glutathione-S-transferase (GST)-tagged HBx bound weakly to hBubR1 purified from SF9 insect cells in vitro (Figure 1C), but Myc-tagged HBx bound strongly to hBubR1 in vivo (Figure 1A and B). These results suggest that the interaction of HBx with hBubR1 might involve other mediating proteins. We, therefore, focused on discovering hBubR1-interacting proteins using a yeast two-hybrid system (Y2H). These screens identified HBxAPα/Rsf-1, which is also known to interact with HBx (30) and plays a role in HBx-dependent activation of HBV transcription, as a hBubR1-interacting protein (data not shown). To test whether HBxAPα/Rsf-1 functions as a mediator to accelerate HBx and hBubR1 binding, we performed reciprocal immunoprecipitations using Myc-tagged HBx, endogenous HBxAPα/Rsf-1 and endogenous hBubR1. We found that HBxAPα/Rsf-1 bound both Myc-tagged HBx and hBubR1 in mitotic HeLa cells (Figure 1E). hBubR1 is normally localized at centromeric chromatin regions when spindle checkpoints are activated, suggesting potentially a functional implication among these proteins in the regulation of kinetochore function in mitosis. To test this possibility, we investigated whether these proteins were detected in the chromatin-bound fraction and whether HBxAPα/Rsf-1 interacted with both HBx and hBubR1 to function as an intermediate. After transfecting cells with Myc-tagged HBx, we isolated chromatin-bound fraction (P3) or soluble fraction (S2) from mitotic cells (prometaphase), generated by treatment with nocodazole (Figure 1D). Endogenous HBxAPα/Rsf-1 was only detected in chromatin-bound fraction (P3) (Figure 1F, Input panel). On the other hand, the majority of ectopic HBx (Myc-HBx) proteins were shown in chromatin-bound fraction (P3) but also detected in soluble fraction (S2). In addition, hBubR1 consistently appeared in both soluble and chromatin-bound fractions (Figure 1F, Input panel). Notably, HBxAPα/Rsf-1 and Myc-tagged HBx proteins were detected in hBubR1 immunoprecipitates from the chromatin-bound fraction, but not those from the soluble fraction (Figure 1F, IP panel). These data indicate that recruitment of HBxAPα/Rsf-1-associated HBx to the chromatin region, especially the kinetochore, may regulate the mitotic spindle checkpoint and/or chromosome segregation in coordination with hBubR1.

HBxAPα/Rsf-1 is required for targeting HBx to kinetochores

The localization of hBubR1 to kinetochores is critical for mitotic checkpoint activation, whereas HBx localization to the kinetochore promotes abnormal chromosome segregation (27,41). To determine whether HBxAPα/Rsf-1 affects chromosome alignment and causes a defective mitotic checkpoint, we knocked down HBxAPα/Rsf-1 expression in HeLa cells using small interfering RNA (siRNA). We confirmed that HBxAPα/Rsf-1 levels were reduced in cells transfected with targeting siRNAs compared with cells transfected with control siRNA (Figure 2A). HBxAPα/Rsf-1-knockdown cells were then transfected with Myc-tagged HBx, and mitotic lysates were obtained after treatment with nocodazole. Knockdown of HBxAPα/Rsf-1 significantly reduced the binding of HBx to hBubR1 compared with controls (Figure 2B). To further confirm that HBxAPα/Rsf-1 is...
Fig. 1. The interaction between HBx and hBubR1 in vitro and in vivo. (A) Cells were subjected to attached (interphasic cells; I) and unattached (mitotic cells; M) cells. (B) HeLa cells were transfected with Myc-tagged HBx (Myc-HBx, +) or control (−). After 36 h transfection, cells were treated with nocodazole (100 ng/ml) or paclitaxel (100 nM) for 12 h. Cell lysates were immunoprecipitated with anti-BubR1 antibody and then analyzed by immunoblotting with the indicated antibodies. (C) GST pull down assay in vitro. GST–HBx fusion protein overexpressed in Escherichia coli and purified with glutathione. Human BubR1 (hBubR1) protein was overexpressed in Sf9 insect cells and purified with Ni-NTA bead. The interaction between GST–HBx and BubR1 was analyzed to immunoblotting with the indicated antibodies. (D) Illustration of experiment for cellular fractionation analysis. (E and F) HeLa cells were transfected with Myc-HBx (+) or control (−). After 36 h transfection, cells were treated with nocodazole (100 ng/ml) for 12 h and then mitotic cells were collected by mitotic shake-off. (E) Cell lysates were immunoprecipitated with anti-HBxAPα/Rsf-1 antibody and then analyzed by immunoblotting with the indicated antibodies. (F) Mitotic cells were fractionated into the soluble (S2) and insoluble chromatin-bound (P3) fractions. The immunoprecipitates obtained with anti-BubR1 antibody were analyzed by immunoblotting with the indicated antibodies. Topoisomerase (Topo) IIα and Aurora B, and GAPDH were used as the controls for chromatin-bound and soluble fractions, respectively.
necessary for the interaction of HBx with hBubR1, we ectopically expressed HBxAPα/Rsf-1 in HBxAPα/Rsf-1-depleted cells using a vector encoding a His-tagged HBxAPα/Rsf-1 DNA (HBxAPα/Rsf-1-His-R) resistant to HBxAPα/Rsf-1 siRNA. Immunoprecipitation analyses revealed that binding of HBx to hBubR1 was restored by exogenously expressing HBxAPα/Rsf-1 protein (Figure 2C). These results suggest that HBxAPα/Rsf-1 may arbitrate the function of the HBx–hBubR1 complex through mitotic checkpoint inactivation.

We next tested which domain of HBxAPα/Rsf-1 is important for the interaction between HBx and hBubR1. HEK293T cells were cotransfected with Myc-tagged HBx and wild-type (WT) or mutant constructs (R1 and R2) of HBxAPα/Rsf-1 (Figure 2D), and cell lysates were immunoprecipitated and analyzed with an anti-V5 antibody. We found that only WT HBxAPα/Rsf-1 interacted with HBx and hBubR1 protein, whereas mutants containing the plant homeodomain-type zinc finger domain actually interfered with this association (Figure 2E). From these results, we conclude that full-length HBxAPα/Rsf-1 is necessary for the mitosis-specific relationship among HBx, HBxAPα/Rsf-1 and hBubR1.

Given that HBx interacts with chromatin-bound hBubR1 in mitosis, we next examined whether HBxAPα/Rsf-1 is involved in the recruitment of hBubR1 and/or HBx to the kinetochore. HBx-expressing cells were transfected with siRNA against HBxAPα/Rsf-1 and immunostained with an anti-BubR1 or anti-HBx antibody, and an anti-centromere antibody (kinetochore marker). siRNA-mediated depletion of HBxAPα/Rsf-1 did not influence the localization of hBubR1 to the kinetochore, but did dramatically disrupt kinetochore localization of HBx (Figure 3A and B). To confirm again these results, we assessed changes in the level of mitotic chromatin-bound HBx and hBubR1 proteins induced by HBxAPα/Rsf-1 knockdown. Chromatin-bound hBubR1 was maintained in HBxAPα/Rsf-1-depleted mitotic cells, but chromatin-bound HBx was significantly reduced, indicating that HBxAPα/Rsf-1 is required for targeting HBx to the kinetochore in mitosis (Figure 3C).

Kunitz domains of HBx interplay with Cdc20-binding domain of hBubR1 upon mitotic checkpoint activation

We demonstrated previously that HBx binding to hBubR1 attenuates the association of hBubR1 with Cdc20 and showed that this interaction induces slippage of mitotic arrest by activating the mitotic spindle (27). To determine which domains are required for this functional interaction, we generated a series of HBx deletion mutants (Figure 4A). HBx consists of 154 amino acids and contains a negative regulatory domain (amino acids 1–50), a transactivation domain (amino acids 51–154) and two Kunitz domains (amino acids 61–76 and 133–145) (42). The Kunitz domain is a serine protease type zinc finger domain that is essential for transcriptional activation of HBx (43). HEK293T cells were transfected with Myc-tagged HBx wild-type (HBxW T) or deletion mutants (H1, HBx(1–133); H2, HBx(1–78); H3, HBx(79–154); H4, HBx(51–154); H5, HBx(50–100) and interactions with BubR1 were analyzed by immunoprecipitation using an anti-BubR1 antibody. HBxWT as well as H4 mutant, retaining the two Kunitz domains, strongly bound hBubR1, whereas HBx deleted of one Kunitz domain (H1, H2, H3 and H5) showed profoundly reduced hBubR1 binding (Figure 4B). It is of note that the H2 construct was very unstable and we failed to get a significant amount of expression level (Input) even though we transfected more DNA into cells. We thus concluded that both Kunitz domains of HBx are necessary for the interaction with hBubR1. Next, to determine which domain of hBubR1 associates with HBx, we constructed a series of EGFP–hBubR1 mutants (WT, 1–1050 amino acids; B1, 1–467 amino acids; B2, 408–904 amino acids; B3, Δ476–687 amino acids) (Figure 4C). HeLa cells were cotransfected with Myc-tagged HBx and WT or mutant EGFP–hBubR1 fusion protein constructs, and then cell lysates were immunoprecipitated with an anti-EGFP antibody. Interestingly, the interaction with HBx was preserved in WT hBubR1 and the B2 mutant retaining the Cdc20-binding region, whereas mutants B1 and B3 lacking the Cdc20-binding domain of hBubR1 failed to bind HBx (Figure 4D). These results suggest that HBx and Cdc20 might share or compete with a common binding site(s) within hBubR1.

A defect in mitotic checkpoint function leads to increased CIN during mitotic progression (44). To test whether interfering with the association between HBx and hBubR1 contributes to a defective mitotic phenotype, we stained HeLa cells transfected with Myc-tagged WT or mutant HBx with aceto-orcein and counted the frequency of aberrant chromosome segregations. As expected, chromosome bridges at anaphase/telophase were substantially increased in cells transfected with WT HBx or H4 mutant containing the hBubR1-interacting Kunitz domains (40–50%), whereas mutants lacking one of these domains (H1, H2, H3 and H5) did not produce abnormal phenotypes (10–15%) (Figure 4E). Collectively, these data strongly suggest that HBx interferes with mitotic checkpoint function via association with hBubR1.

HBxAPα/Rsf-1 increases the frequency of abnormal mitotic events by disrupting hBubR1–Cdc20 interactions

It has been shown previously that HBx directly interacts in vitro and in vivo with an HBxAPα/Rsf-1 region that includes the plant homeodomain-type zinc finger domain and that HBxAPα/Rsf-1 regulates the transcriptional activity of HBV in an HBx-dependent manner (30). The mitotic checkpoint component BubR1 has been shown to bind directly to Cdc20, thereby inhibiting the metaphase-to-anaphase transition through APC/C-mediated ubiquitination of cyclin B and securin (6). We thus examined whether aberrant chromosome transmission due to a defective mitotic checkpoint causes the HBxAPα/Rsf-1-mediated interaction of HBx with hBubR1, thereby disrupting the association of hBubR1 and Cdc20. Cells were transfected with different amounts of Myc-tagged HBx and treated with nocodazole to activate the mitotic checkpoint. Cell lysates were immunoprecipitated with an anti-BubR1 antibody. As shown in Figure 5A, the interaction between hBubR1 and Cdc20 was dramatically disrupted in Myc-tagged HBx in a concentration-dependent manner. These results suggest that inhibition of hBubR1–Cdc20 association by exogenously expressed HBx attenuates mitotic checkpoint activation. Because this HBx–hBubR1 association is dependent on HBxAPα/Rsf-1 protein (Figure 2B and C), we determined whether HBxAPα/Rsf-1 was capable of regulating hBubR1 binding to Cdc20 in Myc-tagged HBx-expressing cells by knocking down HBxAPα/Rsf-1 in these cells using siRNA. The interaction between hBubR1 and Cdc20 was significantly reduced by exogenously expressed Myc-tagged HBx in cells transfected with control siRNA, whereas this interaction was restored in cells depleted of HBxAPα/Rsf-1 (Figure 5B). Finally, we asked whether depletion of HBxAPα/Rsf-1 attenuated mitotic aberrations induced by inhibiting interactions within the HBx–hBubR1–Cdc20 complex. The frequency of defective mitotic events (e.g., chromosome bridges and lagging chromosomes) in HBxAPα/Rsf-1-depleted cells (30%) was decreased compared with that in cells expressing only Myc-tagged HBx (55%) (Figure 5C). Taken together, these data suggest that dysfunction of the mitotic checkpoint is a bona fide enhancer of CIN through mitotic checkpoint blockade caused by competition between strong HBxAPα/Rsf-1-mediated HBx–hBubR1 interactions and weak hBubR1–Cdc20 interactions.

Discussion

Chronic infection with HBV is one of the etiological factors causing liver cancer. We demonstrated previously that hBubR1 is a target of the viral oncoprotein HBx and showed that these interactions increase CIN (27). In the current report, we have provided evidence that HBxAPα/Rsf-1 mediates the interplay between HBx and hBubR1. Specifically, we demonstrated that HBxAPα/Rsf-1 not only stimulates the recruitment and localization of HBx to kinetochores (Figure 3B), it also serves as a mediator that coordinates the interaction between HBx and hBubR1 at kinetochores (Figures 2 and 3). Our results indicate that a mitotic checkpoint block caused by solidification of HBxAPα/Rsf-1-mediated HBx–hBubR1 coupling and attenuated hBubR1–Cdc20 binding increases CIN.

It is generally accepted that a dysfunction in the mitotic checkpoint is among the causes of CIN (3,4). The mitotic checkpoint protein BubR1 directly binds to Cdc20 to activate the spindle checkpoint and...
prevent APC/C activation (6,7). Here, we demonstrated that the two Kunitz domains of HBx and the Cdc20-binding domain of hBubR1 were crucial for their association during mitosis (Figure 4) and further showed that HBxAPα/Rsf-1 not only mediated these interactions, it might stabilize them. During mitosis, hBubR1 mainly functions at the kinetochores region. It is likely that kinetochore localization of HBx was stabilized by binding to HBxAPα/Rsf-1, which provides more chance to interact with hBubR1 at the kinetochores region. As shown in Figure 3B and C, in the absence of HBxAPα/Rsf-1, HBx binding to kinetochores was significantly suppressed, demonstrating that HBxAPα/Rsf-1 stabilizes the HBx/hBubR1 interaction at the kinetochores region. Thus, the Cdc20-binding domain of hBubR1 could be occupied by HBx in HBV-infected liver cells, thereby increasing free Cdc20. In keeping with this, downregulation of HBxAPα/Rsf-1

Fig. 2. Full-length of HBxAPα/Rsf-1 is critical for the HBx–hBubR1 interaction. (A) At 48 h after each siRNA transfection, HBxAPα/Rsf-1 was analyzed by immunoblotting. (B) HeLa cells were cotransfected with control siRNA (−) or HBxAPα/Rsf-1 siRNA (+) and Myc-HBx. After 36 h transfection, cells were treated with nocodazole (100 ng/ml) for 12 h. The cell lysates were immunoprecipitated with anti-BubR1 antibody and then analyzed by immunoblotting with the indicated antibodies. (C) HeLa cells were cotransfected with control siRNA or HBxAPα/Rsf-1 siRNA and Myc-HBx. After 12 h transfection, cells were transfected with HBxAPα/Rsf-1-His-R and continually incubated for 24 h. Cells were treated with nocodazole (100 ng/ml) for 12 h during 36 h post-transfection. Cell lysates were immunoprecipitated with anti-BubR1 antibody and then analyzed by immunoblotting with the indicated antibodies. (D) Schematic diagram of HBxAPα/Rsf-1 function domains. (E) HEK293T cells were cotransfected with WT or mutant constructs (R1 and R2) of HBxAPα/Rsf-1-V5 and Myc-HBx. At 36 h after transfection, cells were treated with nocodazole (100 ng/ml) for 12 h. Cell lysates were immunoprecipitated with anti-V5 antibody and then analyzed by immunoblotting with the indicated antibodies.
Fig. 3. HBxAPα/Rsf-1 is required for targeting HBx to kinetochore. (A and B) At 48 h after siRNA transfection, ChangX cells were fixed with 4% paraformaldehyde. Cells were stained with anti-BubR1 (green) and anti-centromere antibody (red) antibodies or anti-HBx (green) and anti-centromere antibody (red) antibodies. 4′,6-Diamidino-2-phenylindole was used for staining of nucleus (blue). Fluorescence images of mitotic cells were captured by confocal microscopy. Scale bar is 5 μm. BubR1 or HBx signal intensity at kinetochores was quantified from >100 kinetochores in 10 cells. (C) ChangX cells transfected with control (−) or HBxAPα/Rsf-1 (+) siRNA for 36 h and treated with nocodazole (100 ng/ml) for 12 h. Mitotic cells were fractionated into the soluble (S2) and insoluble chromatin-bound (P3) fractions. The amount of HBx and HBxAPα/Rsf-1 in each fraction was analyzed by immunoblotting with the indicated antibodies. Topoisomerase (Topo) IIα or GAPDH was used as the controls for chromatin-bound and soluble fractions, respectively (left panel). The relative ratio of chromatin-bound HBx to Topo IIα was determined by densitometric analysis (right panel). Data are means ± SD from three independent experiments. *P < 0.05, **P < 0.01 versus control by Student’s t-test.
diminished HBx–hBubR1 interactions; moreover, the mitotic checkpoint recovery obtained through hBubR1–Cdc20 binding diminished abnormal chromosome segregations. Taken together, our findings indicate that an HBx–HBxAPα/Rsf-1–hBubR1 protein network at the kinetochore is associated with a failed mitotic checkpoint and generation of CIN. However, HBxAPα/Rsf-1 is known to be involved in the maintenance of the epigenetic marker, CENP-A (39); thus, whether HBxAPα/Rsf-1 and HBx further regulate any other centromere/kinetochore functions remains an open question.

Interestingly, the HBxAPα/Rsf-1 gene is overexpressed in a variety of cancers (36,38) and is highly correlated with tumor aggressiveness and poor prognosis (37,45,46), suggesting that HBxAPα/Rsf-1 is
Mitotic dysfunction through HBxAPα/Rsf-1

HBxAPα/Rsf-1 is a potential oncogenic protein. Notably, Shih and colleagues recently demonstrated that acute expression of Rsf-1 induces DNA double-strand breaks, activating an ATM/p53-dependent pathway, whereas chronic induction of Rsf-1 leads to accumulation of chromosomal aberrations (47). In fact, we also observed that HBxAPα/Rsf-1 is highly overexpressed in association with accumulation of DNA double-strand breaks in HBV-related cancers (S. Cho et al., in preparation). This suggests that HBxAPα/Rsf-1 promotes CIN in HBV-related liver cancers through at least two different pathways: deregulation of mitotic BubR1 function versus accumulation of DNA double-strand breaks.

HBx also localizes to the centrosome through HBx-interacting protein (HBxIP), a centrosomal protein that regulates centrosome duplication during mitosis (28). This interaction leads to dysregulation of centrosome dynamics, affecting genomic instability (28,48). We observed that HBx binding to hBubR1 was enhanced in HBxIP-depleted mitotic cells compared with HBxAPα/Rsf-1-depleted cells (Supplementary Figure 1, available at Carcinogenesis Online). Therefore, it is interesting to speculate that the HBx-interacting proteins, HBxIP and HBxAPα/Rsf-1, cross talk to regulate centrosome/kinetochore functions in liver carcinogenesis.

In summary, we have demonstrated critical roles of HBxAPα/Rsf-1 in regulating the link between HBx and hBubR1 in mitotic disorders. One possible scenario is that, in the presence of HBx, mitotic checkpoint activation would be an accidental outcome of HBx–HBxAPα/Rsf-1-mediated disturbance of the collaboration among checkpoint/kinetochore proteins, and HBxAPα/Rsf-1 may principally function as an accelerating oncoprotein, serving to intercept the active mitotic checkpoint at kinetochore/centromere regions. Our findings provide important insights into HBxAPα/Rsf-1 function and shed light on the mechanisms that control mitotic checkpoint abortion and CIN in hepatocarcinogenesis.

Supplementary material
Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/
Funding
National Research Foundation of Korea grants funded by the Korea government (MEST) [No. 2012-0009203 (SRC)].

Acknowledgements
We thank the members of H.Cho laboratory for helpful comments and discussions.

Conflict of Interest Statement: None declared.

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Received December 1, 2012; revised March 9, 2013; accepted March 24, 2013