ORIGINAL ARTICLE

HBV X protein targets hBubR1, which induces dysregulation of the mitotic checkpoint

S Kim[1], S-Y Park[1], H Yong[1], JK Famulski[2], S Chae[1], J-H Lee[1], C-M Kang[3], H Saya[4], GK Chan[2] and H Cho[1]

[1]Department of Biochemistry, Ajou University School of Medicine, Suwon, Korea; [2]Department of Oncology, University of Alberta, Edmonton, Alberta, Canada; [3]Korea Institute for Radiological and Medical Sciences, Seoul, Korea and [4]Division of Gene Regulation, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan

Accurate chromosomal segregation is monitored by the mitotic checkpoint, and an increased rate of chromosomal missegregation leads to chromosomal instability (CIN). Here, we demonstrate that the HBV X protein (HBx) binds BubR1, a component of the mitotic checkpoint complex and co-localizes with BubR1 at the kinetochores. HBx binding to BubR1 attenuates the association between BubR1 and CDC20, an activator of the anaphase-promoting complex/cyclosome (APC/C) and induces slippage of mitotic arrest in the presence of microtubule poisons. In addition, HBx binding to BubR1 results in the accumulation of lagging chromosomes and chromosome bridges. In contrast, a C-terminally truncated HBx mutant (HBx$^{1-100}$) fails to bind BubR1 and does not cause aberrant chromosomal segregation. This provides a novel mechanism for dysregulation of the mitotic checkpoint by a viral pathogen linking it to the accumulation of chromosomal instability in HBV-associated hepatocarcinogenesis.

Introduction

Chromosome instability (CIN) is directly related to aneuploidy, which is regarded as one of the hallmarks of cancer (Sen, 2000). CIN can be caused by an increased rate of chromosomal missegregation during mitosis (Gollin, 2004), and abnormal numbers of centrosomes are associated with multipolar spindles that cause chromosomal missegregation (Lingle et al., 2002). In addition, a defective mitotic checkpoint has also been recognized as a causative factor of CIN (Kops et al., 2004). The mitotic checkpoint monitors microtubule attachment at kinetochores during mitosis and prevents cells with unaligned chromosomes from proceeding to anaphase by inhibiting the anaphase-promoting complex/cyclosome (APC/C) (Fang et al., 1998; Fang, 2002). In mammalian cells, APC/C is regulated by the mitotic checkpoint complex (MCC), which is composed of BubR1, Bub3, Mad2 and CDC20 (Sudakin et al., 2001). Mouse embryonic fibroblasts (MEFs) derived from heterozygous BubR1$^{+/-}$, Mad2$^{+/-}$ or CENP-E$^{+/-}$ mice exhibit chromosome missegregation and aneuploidy (Michel et al., 2001; Dai et al., 2004; Weaver et al., 2007).

Hepatocellular carcinoma (HCC) is a leading cause of death worldwide. One of the primary etiological factors for the development of liver cancer is chronic infection with hepatitis B virus (HBV) and CIN plays an important role in liver carcinogenesis. Analysis of primary liver tumors identified two distinct groups based on CIN status, with one group showing high frequencies of allelic imbalance and chromosomal loss being closely associated with HBV infection (Laurent-Puig et al., 2001). This is likely associated, at least in part, with the observation that integration of the HBV genome causes rearrangement of cellular DNA (Henderson et al., 1988). In addition, our group and others have observed that the HBV X protein (HBx) viral oncoprotein is directly involved in hyperamplification of centrosomes, which leads to chromosome aberrations (Forgues et al., 2003; Yun et al., 2004; Fujii et al., 2006).

HBx is a multifunctional protein (Murakami, 2001), which deregulates cell cycle checkpoint controls (Madden and Slagle, 2001) potentially including the G$_2$/M checkpoint (Lee et al., 2002). Notably, HBx-expressing cells become multinucleated under microtubule-damaging conditions (Lee et al., 2002), raising the possibility of mitotic checkpoint defects.

Here, we demonstrate that (1) HBx interacts with the BubR1 mitotic checkpoint protein and interferes with the binding of BubR1 to CDC20 and (2) HBx binding to BubR1 triggers aberrant chromosomal segregation.

2

Results

HBx targets hBubR1

Some viral oncoproteins such as Tax and simian virus40 (SV40) T antigen interact with mitotic checkpoint components and these interactions contribute to aberrant mitotic phenotypes (Cotsiki et al., 2004; Liu et al., 2005). Similarly, HBx-induced multinucleated cells under microtubule-damaging conditions (Lee et al., 2002) could be potentially mediated through defects in the mitotic checkpoint. To examine this possibility, we first used a targeted yeast two-hybrid assay to detect binding between HBx and any of the MCC components. A full-length cDNA encoding HBx$^{1-154}$ was cloned into a D-galactose (gal)-inducible B42 activation domain vector (pJG4-5). We found that a fusion protein combining LexA and full-length hBubR1$^{1-1050}$ interacted with B42-HBx to yield colonies in leucine-free media and was positive on an X-Gal plate (Figure 1a).
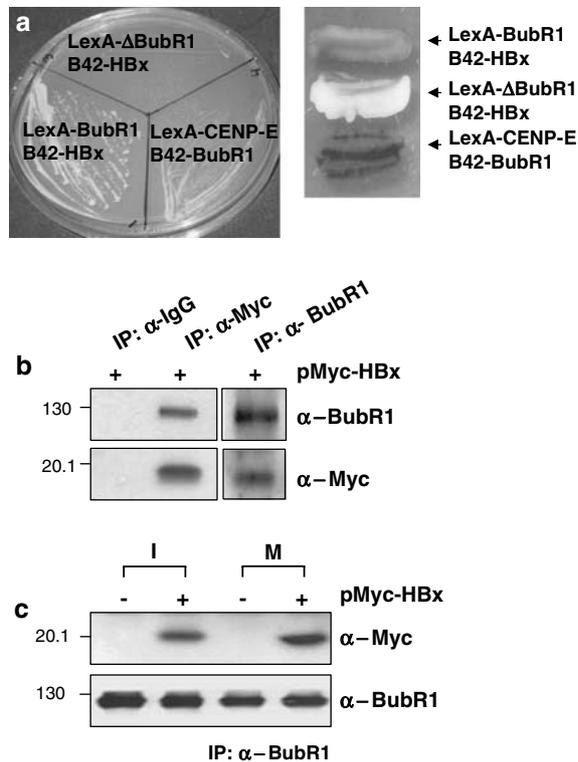
Figure 1 HBV X protein (HBx) interacts with BubR1. (**a**) In a yeast two-hybrid assay, yeast expressing LexA-BubR1$^{1-1050}$ and B42- HBx$^{1-154}$ resulted in growth in media lacking leucine (left) and blue colonies on the X-gal plate (right). Interaction between LexA-CENP-E and B42-BubR1 was used as a positive control. (**b**) The pMyc-HBx plasmid was transfected into HeLa cells. Cell lysates were immunoprecipitated (IP) with either goat anti-mouse-IgG, anti-Myc or anti-BubR1 antibody. The precipitates were analysed by western blotting (WB) with anti-BubR1 antibody (top) and with anti-Myc antibody (bottom). (**c**) The pMyc-HBx plasmid was transfected into HeLa cells. Interphase cell lysates were prepared at 48 h post transfection (I; interphase). Mitotic cell lysates were prepared after 12 h of nocodazole (100 ng ml^{-1}) challenge at 36 h post transfection (M, mitosis). The immunoprecipitates obtained with anti-BubR1 antibody were analysed by western blotting with anti-Myc antibodies (top) and anti-BubR1 antibodies (bottom).

However, neither the LexA-fused Cdc20, Mad2 nor Bub3 interacted with B42-HBx (Supplementary Figure 1), confirming the specific interaction of HBx with BubR1. We further found that yeast expressing an hBubR1 C-terminal truncation construct (LexA-hBubR1$^{1-467}$) and B42-HBx failed to grow on the selection media, suggesting that the N-terminal region of hBubR1 is not involved in HBx binding. Binding between a LexA-fused C-terminal fragment of CENP-E$^{1958-2662}$ and its known binding partner, B42-BubR1$^{408-1050}$, was used as a positive control (Chan et al., 1998; Figure 1a).

We confirmed the binding of HBx and BubR1 in mammalian cells. Since mammalian MCC components were originally identified in HeLa cells (Sudakin et al., 2001), we transfected HeLa cells with a pMyc-tagged HBx construct. Forty-eight hours post transfection, cell lysates were immunoprecipitated with either an anti-mouse IgG antibody or a mouse anti-Myc antibody, followed by western blotting using an anti-BubR1 antibody. Endogenous hBubR1 was present in immune complexes obtained with the anti-Myc antibody, which targeted the Myc-tagged HBx, but not with anti-mouse IgG (Figure 1b). A reciprocal immunoprecipitation experiment using an anti-BubR1 antibody also confirmed the interaction between HBx and hBubR1 (Figure 1b). To examine whether these interactions occur in a cell-cycle-dependent manner we next prepared cell lysates from both asynchronously growing cells (I: mainly interphase) and mitotic cells (M: obtained after nocodazole treatment for 12 h). Immunoprecipitation with the anti-BubR1 antibody revealed that HBx bound hBubR1 to similar degrees in interphase and mitotic phase cells (Figure 1c). Since BubR1 functions as a mitotic checkpoint protein, we analysed strictly mitotic cell lysates in the following experiments. These results are the first indication that BubR1 can be targeted by the HBx viral oncoprotein.

HBx co-localizes with BubR1 at the kinetochores

Activation of the mitotic checkpoint may require the proper assembly of mitotic checkpoint components at the kinetochores (Cleveland et al., 2003). Since HBx binds BubR1, we hypothesized that HBx might localize to the kinetochores during mitosis. To test this notion, we utilized HBx-expressing ChangX cells (Yun et al., 2004) previously developed in our lab and SNU368 cells that contain the HBV genome and express HBx protein(Kim et al., 2007). Specific expression of HBx protein in ChangX and SNU368 cells but not in Huh7 and Chang cells were shown (Figure 2c). We first confirmed that BubR1 in SNU368 cells co-localized at the kinetochores of the condensed chromosomes with the kinetochore-specific marker, CREST (Moroi et al., 1980; data not shown). Notably, a portion of the HBx protein co-localized with hBubR1 at the kinetochores in SNU368 cells as well as in ChangX cells (Figure 2a). Magnification of the kinetochore staining (Figure 2a, inset) clearly indicated co-localization of HBx and hBubR1 at the kinetochores. We next examined whether
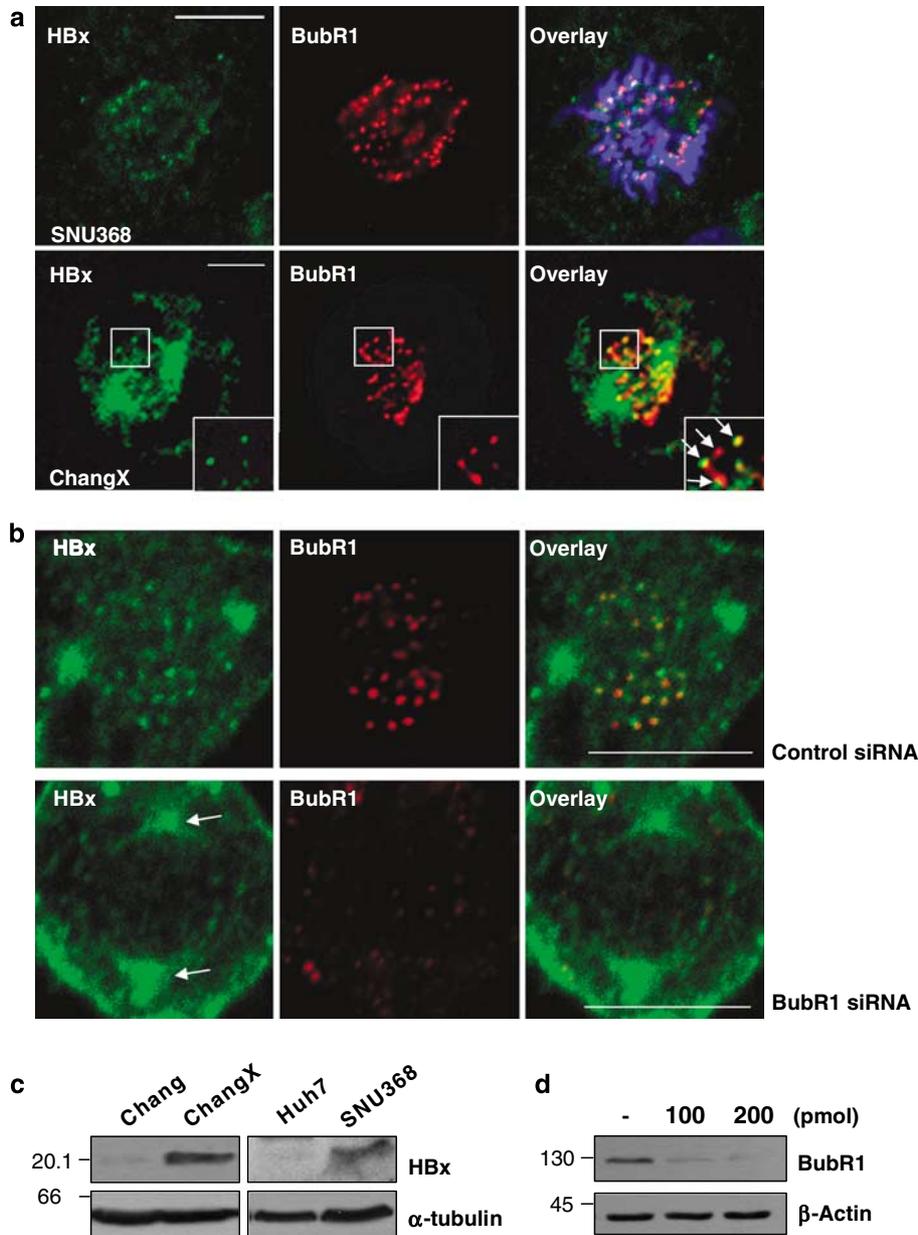
Figure 2 HBV X protein (HBx) co-localizes with BubR1 at the kinetochores. (**a**) Asynchronously growing ChangX and SNU368 cells were fixed with a mixture of methanol/acetone and immunofluorescence staining was carried out using rabbit anti-HBx (FITC) and mouse anti-BubR1 (Cy3) antibodies with DAPI staining (blue). Fluorescence images of mitotic cells were captured by confocal microscopy. The inset image depicts the magnified region of interest. Arrows in the inset indicate kinetochore co-localization. Scale bar = 10 μm. (**b**) ChangX cells were transfected with siRNA duplexes targeting the hBubR1 sequence using Lipofectamine 2000 and immunofluorescence staining was carried out. Arrows indicate the spindle poles. (**c**) HBx expression was determined by western blotting. (**d**) Expression levels of BubR1 were determined 48 h after transfection of siRNA duplex targeting hBubR1. Molecular weight markers (kDa) are indicated at the left side of the panels of western blot analysis.

BubR1 is required to tether HBx at kinetochores by knockdown of BubR1 expression. The hBubR1 siRNA duplex significantly reduced BubR1 expression in ChangX cells (Figure 2d) and we found that the kinetochore localization of HBx was diminished in cells lacking kinetochore hBubR1 (Figure 2b, lower panel). In contrast, centrosomal localization of HBx (Fujii et al., 2006) appeared not to be affected by BubR1 siRNA (Figure 2b, arrows). Localization of CDC20 to kinetochores appeared to be stable in cells depleted of BubR1 (data not shown). Thus, the interaction between HBx and BubR1 may contribute to the kinetochore localization of HBx.

HBx reduces the association between hBubR1 and CDC20

We next examined the HBx-binding region of BubR1. N-terminally truncated hBubR1$^{408-1050}$ (BubR1ΔN, harboring the CDC20 binding site and the kinase domain)

4

and C-terminally truncated $hBubR1^{1-685}$ (BubR1ΔC, lacking the kinase domain) were generated as fusion proteins with GFP (Figure 3a; Chan et al., 1999) and co-transfected with the pMyc-HBx vector. Cell lysates were immunoprecipitated with an anti-GFP antibody, and the presence of HBx in the immunocomplexes was determined. Both BubR1 deletion mutants retained the ability to bind HBx; pGFP-BubR1ΔN showed some

decrease in HBx binding, whereas pGFP-BubR1ΔC retained nearly all of its HBx-binding activity (Figure 3b). Both deletion mutants shared amino acids 408–685 of hBubR1; this region contains the CDC20 binding site (Fang, 2002), suggesting that HBx and CDC20 share a common binding region within hBubR1 (Figure 3a).

HBx mainly consists of two domains, a regulatory domain (1–50) and a transactivation domain (51–154;
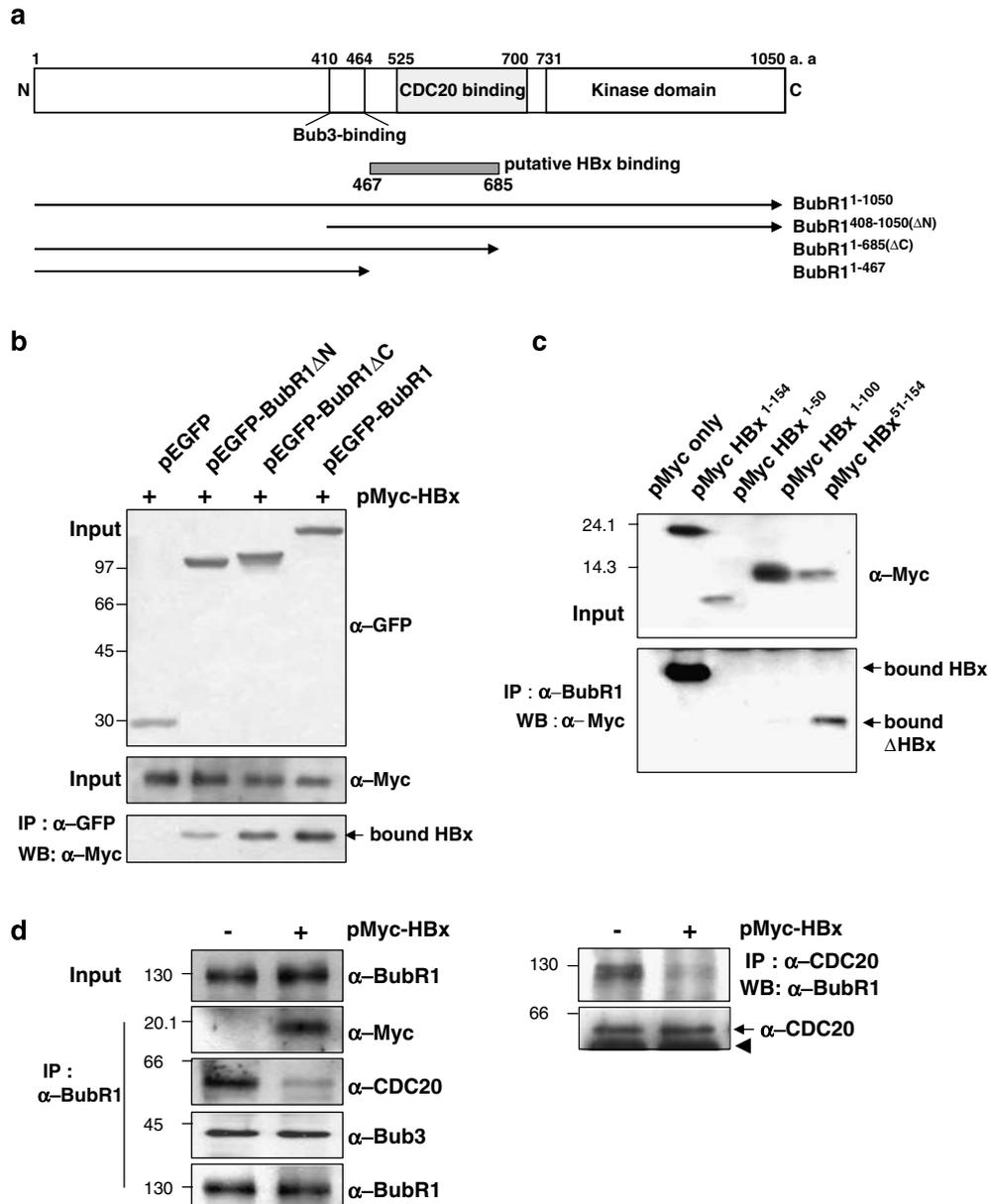
Figure 3 HBV X protein (HBx) inhibits the binding between BubR1 and CDC20. (**a**) A schematic representation of hBubR1, its constructs and its functional domains. (**b**) The full-length and deletion constructs of pEGFP-BubR1 (BubR1ΔN; $hBubR1^{408-1050}$, BubR1ΔC; $hBubR1^{1-685}$), pEGFP (vector control) and pMyc-HBx were co-transfected into HeLa cells and expression levels were shown (input). Cell lysates were immunoprecipitated with anti-GFP antibody and the presence of HBx in the resulting pellets was determined (bottom). (**c**) Full-length and deletion HBx constructs were transfected into HeLa cells and analysed for expression (input). Cell lysates were immunoprecipitated with anti-BubR1 antibody and the presence of HBx in the pellets was determined (bottom). (**d**) HeLa cells were transfected for 36 h and followed by nocodazole treatment for 12 h. The cell lysates were divided and immunoprecipitated with either anti-BubR1 antibody (left) or anti-CDC20 antibody (right). The immunoprecipitates were resolved and analysed by immunoblotting with the indicated antibodies. Molecular weight markers are indicated in each panel. Arrowhead indicates the heavy chain of immunoglobulin G (IgG).

Lin *et al.*, 1997). We found that the HBx$^{1-50}$ and HBx$^{1-100}$ fragments did not bind to hBubR1 (Figure 3c). In contrast, a stable interaction between hBubR1 and HBx$^{1-154}$ was detected, and the HBx$^{51-154}$ fragment was found to retain comparable BubR1 binding ability relative to the amount of expressed protein (Figure 3c).

Since HBx and CDC20 seem to share a binding region within hBubR1, we investigated whether HBx competes with CDC20 for binding to hBubR1. HeLa cells are used because it has a robust mitotic checkpoint. First, we observed that BubR1 efficiently recruits CDC20 in HeLa cells treated with nocodazole (Supplementary Figure 2). In the BubR1 immunoprecipitates, we found that the interaction between BubR1 and CDC20 was significantly reduced when HBx was present (Figure 3d). Densitometric analysis revealed that BubR1 binding to CDC20 in the presence of HBx was reduced to 27% of the control value. Three other independent experiments consistently showed reduced binding (27–52% of the control value) between BubR1 and CDC20 in the presence of HBx. Reciprocal immunoprecipitations using an anti-CDC20 antibody also showed that the CDC20–BubR1 interaction was reduced in the presence of HBx (Figure 3d, right panel). However, HBx did not affect the interaction between BubR1 and Bub3 (Figure 3d, left panel). Together, these results demonstrate that HBx interferes with BubR1 binding to CDC20 in the MCC of mammalian cells, suggesting that the binding of HBx to BubR1 may disturb mitotic checkpoint function.

Compromised mitotic checkpoint in cells expressing HBx protein

A compromised mitotic checkpoint led to mitotic slippage in BubR1$^{+/-}$ MEFs expressing low BubR1 level (Dai *et al.*, 2004). Similarly, the reduced binding of hBubR1 to CDC20 in the presence of HBx might also induce defective mitotic checkpoint function. In fact, we found that HBx-expressing ChangX cells escaped from mitotic arrest after nocodazole treatment (Figure 4a). At 18 h, ∼5% of ChangX cells escaped from mitotic arrest (Supplementary Figures 3Ab and B), whereas a significant portion of them (25–30%) also underwent cell death (Supplementary Figures 3Ac, Ad and B) as determined by aceto-orcein staining. At 24 h, 75% of Chang cells remained arrested at mitosis whereas the mitotic index in ChangX cells was reduced to 20% even in the presence of nocodazole (Supplementary Figure 3B). Mitotic slippage during metaphase to anaphase transition can be further characterized by the APC/C-dependent cyclin B and securin degradation. Since prolonged treatment of nocodazole sensitized ChangX cells to death, we next synchronized cells by the double thymidine block (DTB) method and added nocodazole to cells at G_2/M phase to shorten the exposure to nocodazole. Immunoblot revealed that levels of cyclin B and securin in Chang cells were maintained until 10 h whereas those in ChangX cells were already reduced after 4–6 h under nocodazole challenge (Figure 4b). We also confirmed early degradation of securin in cells

transiently transfected with HBx (Figure 4c). Thus, these results demonstrated that HBx expression induced mitotic slippage in cells under microtubule damaging conditions.

Binding of HBx to hBubR1 increases the rate of chromosome instability

Since perturbation of the mitotic checkpoint in cells can increase the rate of chromosomal missegregation during mitotic progression (Cleveland *et al.*, 2003), we evaluated the frequency of aberrant chromosomal segregation in HBx-expressing cells by aceto-orcein staining. In these experiments, we have only included the aberrant chromosomal segregation derived from bipolar mitotic spindle poles and excluded those driven from multiple centrosomes (Forgues *et al.*, 2003; Yun *et al.*, 2004). The number of cells with unaligned chromosomes at metaphase (Figure 5a, arrows) and chromosome bridges at anaphase/telophase (Figure 5b, arrows) were significantly increased in cells expressing HBx$^{1-154}$ and HBx$^{51-154}$. Less than 5% of control metaphase cells had unaligned chromosomes, while the percentage seen in metaphase HBx$^{1-154}$- or HBx$^{51-154}$-expressing cells was elevated at least threefold (*$P<0.05$, **$P<0.005$, Student's *t*-test). Similarly, mitotic bridges were seen in ∼10% of control cells, whereas those rates were significantly higher in cells transfected with HBx$^{1-154}$ and HBx$^{51-154}$ (Figure 5b). In contrast, expression of hBubR1 binding-deficient HBx$^{1-50}$ or HBx$^{1-100}$ did not alter the occurrence of aberrant chromosome segregations. Taken together, these results indicate that binding of HBx to BubR1 significantly increases aberrant chromosome segregation.

Discussion

We have demonstrated for the first time that BubR1, a key component of the MCC, is a target of the HBx viral oncoprotein. Importantly, the HBx–BubR1 interaction was found to disrupt CDC20 binding in the MCC and HBx-expressing cells underwent faulty mitotic progression. Thus, the interaction between HBx and BubR1 provides a novel mechanism that may account, at least in part, for increased chromosomal instability during HBV-mediated carcinogenesis.

Our findings add to the list of viral proteins that are known to interact with mitotic checkpoint proteins. SV40T antigen interaction with Bub1 promoted endoreduplication under nocodazole treatment (Cotsiki *et al.*, 2004). Tax was reported to bind Mad1 (Jin *et al.*, 1998) and recently to the CDC20-associated APC/C (Liu *et al.*, 2005), prematurely activating the APC/C. However, the mechanisms of action through which these viral oncoproteins affect the mitotic checkpoint and their functional consequences are poorly characterized.

We have shown that binding of HBx to BubR1 disrupts mitotic checkpoint function. The HBx interaction domain of BubR1 (amino acids 408–685) overlaps with the CDC20 binding domain of BubR1 (Figure 3a
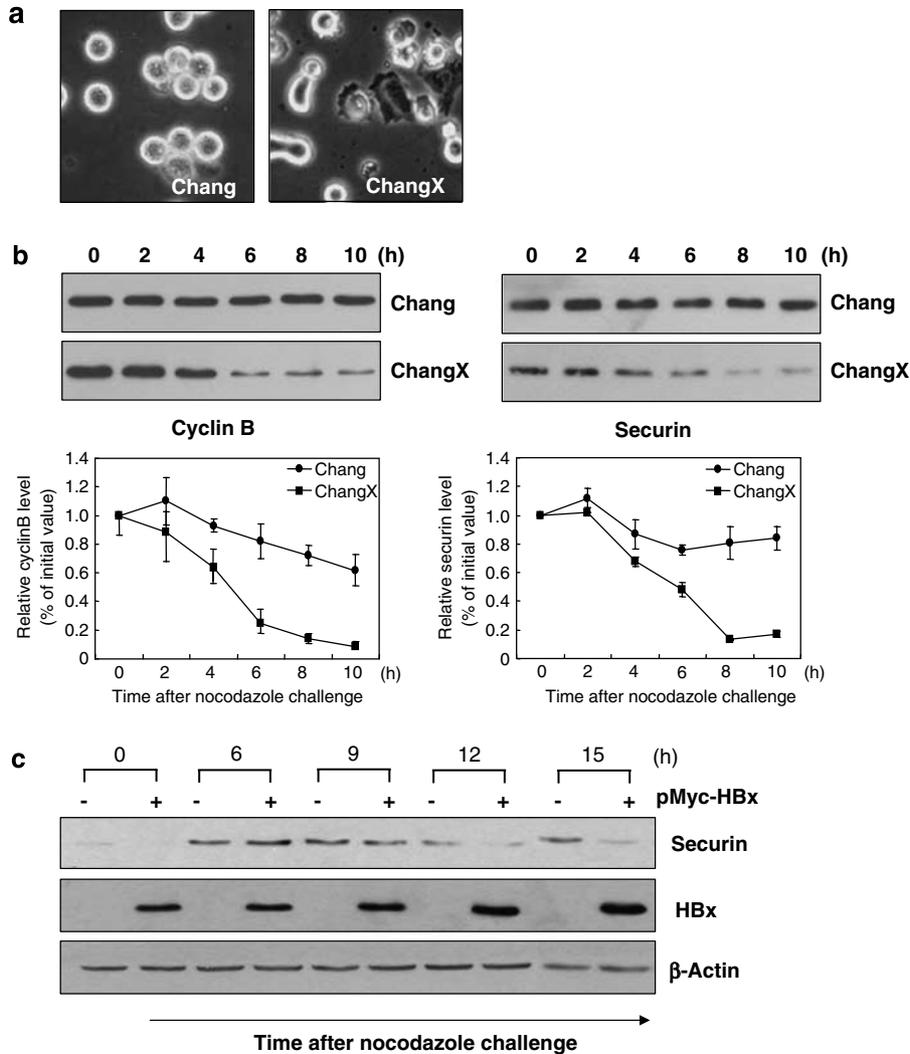
Figure 4 Compromised mitotic checkpoint in cells expressing HBV X protein (HBx). (**a**) A representative phase-contrast image of Chang and ChangX cells 14 h after nocodazole treatment. (**b**) ChangX cells were synchronized by the double thymidine block (DTB) method and nocodazole was added 10 h after DTB release. Expression profiles of cyclin B and securin in Chang and ChangX cells are shown and analysed by densitometry. (**c**) Chang cells transfected with pMyc-HBx were treated with nocodazole. Expression profiles of securin, HBx and β-actin were analysed by immunoblotting.

and b; Fang, 2002). Both Mad2 and BubR1 have been shown to directly bind CDC20 and inhibit the activity of the APC/C in vitro (Fang et al., 1998; Fang, 2002) with BubR1 functioning as a much more potent inhibitor of CDC20-associated APC/C activity (Sudakin et al., 2001; Tang et al., 2001). Thus, the binding of HBx to BubR1 may interfere with the ability of BubR1 to bind CDC20 and its inhibitory activity toward the CDC20-associated APC/C. The potential importance of the HBx–BubR1 interaction is highlighted by the observation that cells expressing HBx cannot properly arrest in mitosis under microtubule damaging condition (Figure 4). *BubR1* null mouse embryos failed to survive beyond day 8.5 (Wang et al., 2004) but BubR1 MEF$^{+/-}$ expressing 10–25% of normal BubR1 levels showed enhanced mitotic slippage (Baker et al., 2004; Dai et al., 2004). In our system, HBx expression may mimic the situation of low BubR1 level in cells and lack the complete inhibition of CDC20,

thereby allowing mitotic slippage and an accumulation of aberrant chromosomes. Thus, our findings provide new insights into viral pathogen-induced mitotic dysregulation.

Materials and methods

Plasmids and antibodies

The cDNA expression vectors encoding HBx mutants were subcloned into pCMV-Myc. hBubR1 cDNA was subcloned into the Gateway pENTR vector (Invitrogen, Carlsbad, CA, USA) and subsequently transferred into the pWSGFP-GW destination vector using LR clonase recombination reaction (Chan et al, 1998). Rabbit anti-HBx antibody was made against a synthetic HBx peptide corresponding to residues 144–154 (Lab Frontier, Seoul, Korea). Antibodies for Myc and CDC20 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antibodies for BubR1 and Bub3
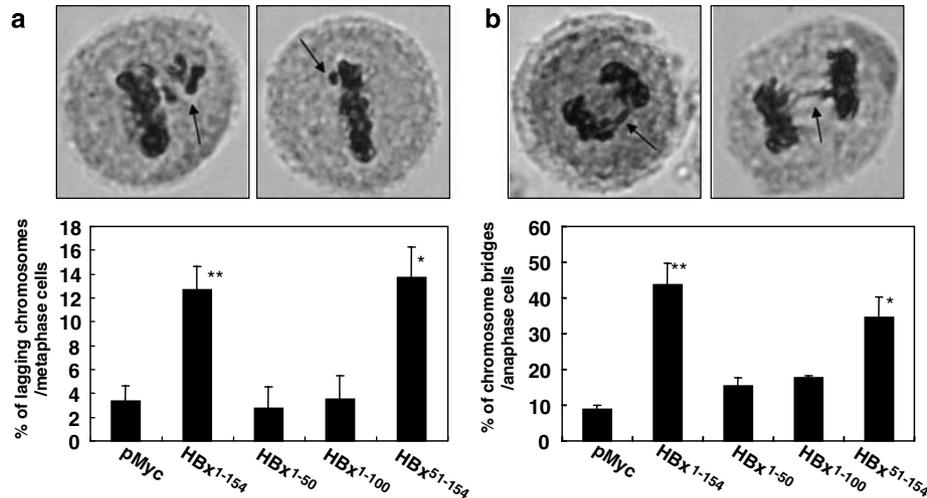
Figure 5 HBV X protein (HBx) results in the accumulation of chromosomal instability. At 48 h post transfection of different forms of pMyc-HBx, Chang cells were stained with aceto-orcein and the numbers of unaligned chromosomes (**a**) and mitotic anaphase bridges (**b**) were quantified. Three to six independent experiments were performed in which ∼100 mitotic cells were counted each time. *$P<0.05$, **$P<0.005$ by Student's *t*-test.

were from BD Transduction Laboratories (San Jose, CA, USA). Antibodies for securin and cyclin B were from ZYMED (San Francisco, CA, USA) and Santa Cruz, respectively.

Cell culture and synchronization
HBx-transfected ChangX cells (Yun *et al.*, 2004; Kim *et al.*, 2007) and Chang, HeLa and SNU 368 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies Co., Grand Island, NY, USA) in a humidified CO_2 incubator. SNU 368 cells were purchased from the Korean Cell Line Bank (Seoul, Korea).

Yeast two-hybrid assay
Yeast strains as well as yeast expression vectors pEG202, pJG4-5 and pSH18-34 were provided by Dr E Golemis (Fox Chase Cancer Center, PA, USA). The hBubR1, hBub3, hMad2 and CDC20 cDNAs were fused into pEG202 containing the LexA DNA binding domain, and HBx cDNA was fused into pJG4-5 harboring the D-galactose (gal)-inducible B42 activation domain. The positive interaction was determined by both β-galactosidase activity and leucine-dependent growth.

Transfection, immunoprecipitation and western blotting
Cells were transfected either using the calcium phosphate method (ProFection, Promega, Madison, WI, USA) or using the polyethylenimine (PEI; Polysciences, Warrington, PA, USA). For siRNA transfection, siRNA duplexes targeting the hBubR1 sequence 5′-AACAATACTCTTCAGCAGCAG-3′ were synthesized and tranfected into ChangX cells using lipofectamine 2000 (Invitrogen). Whole-cell lysates were prepared by extracting cells with radioimmunoprecipitation (RIPA) buffer and incubated with various primary antibodies for 8–16 h at 4 °C and the immune complexes were precipitated

using protein G-Sepharose and analysed by western blotting. The transfection efficiency using the pEGFP control construct was determined as 70–80% with both calcium phosphate method and PEI method (data not shown).

Microscopy analysis
Cells were seeded onto coverslips, fixed with methanol:acetone (1:1) solution, and permeabilized with PBS containing 0.075% TritonX-100 (Yun *et al.*, 2004). Fixed cells were pre-incubated in blocking solution, followed by incubation with primary antibodies at 4 °C and probed with fluorescein-conjugated antibody. Images were captured under confocal microscopy (LSM510, Zeiss, Jena, Germany). For the analysis of chromosome aberration, the nuclei were stained with 2% aceto-orcein solution 48 h post transfection with different HBx deletion mutants and numbers of mitotic cells with aberrant chromosomes were determined.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).