Oncogene (2009), 1–11

www.nature.com/onc

ORIGINAL ARTICLE

NF-κB signaling mediates the induction of MTA1 by hepatitis B virus transactivator protein HBx

TM Bui-Nguyen[1,8], SB Pakala[1,8], RD Sirigiri[1], W Xia[2], M-C Hung[2,3], SK Sarin[4,5], V Kumar[6], BL Slagle[7] and R Kumar[1]

[1]*Department of Biochemistry and Molecular Biology, Institute of Coregulator Biology, The George Washington University, Washington, DC, USA;* [2]*Department of Molecular and Cellular Oncology, MD Anderson Cancer Center, Houston, TX, USA;* [3]*Center for Molecular Medicine, Graduate Institute of Cancer Biology, China Medical University and Hospital, Taichung, Taiwan;* [4]*Department of Gastroenterology, G.P. Pant Hospital, Institute of Liver and Biliary Sciences, New Delhi, India;* [5]*Department of Hepatology, Institute of Liver and Biliary Sciences, New Delhi, India;* [6]*Virology Laboratory, International Centre for Genetic Engineering and Biotechnology, New Delhi, India and* [7]*Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, USA*

Metastasis-associated protein 1 (MTA1), a master chromatin modifier, has been shown to regulate cancer progression and is widely upregulated in human cancer, including hepatitis B virus-associated hepatocellular carcinomas (HCCs). Here we provide evidence that hepatitis B virus transactivator protein HBx stimulates the expression of MTA1 but not of MTA2 or MTA3. The underlying mechanism of HBx stimulation of MTA1 involves HBx targeting of transcription factor nuclear factor (NF)-κB and the recruitment of HBx/p65 complex to the NF-κB consensus motif on the relaxed *MTA1* gene chromatin. We also discovered that MTA1 depletion in HBx-expressing cells severely impairs the ability of HBx to stimulate NF-κB signaling and the expression of target proinflammatory molecules. Furthermore, the presence of HBx in HBx-infected HCCs correlated well with increased MTA1 and NF-κB-p65. Collectively, these findings revealed a previously unrecognized integral role of MTA1 in HBx stimulation of NF-κB signaling and consequently, the expression of NF-κB targets gene products with functions in inflammation and tumorigenesis.
Oncogene advance online publication, 14 December 2009; doi:10.1038/onc.2009.404

Keywords: MTA1; hepatitis B virus; HBx; signaling; MTA coregulator; liver cancer

Introduction

Hepatocellular carcinoma (HCC) is the most common form of liver cancer in adult that accounted for 1 million deaths every year worldwide (Dvorchik *et al*., 2007). Of those, high morbidity and mortality rate are found in Asia and Africa. In the United States, according to the American Cancer Society, there will be 22 000 new cases of HCC, up to 19 000 of them fatal, during the year of 2008. Over the years, a large number of epidemiological studies suggest that more than 75% of HCC cases are associated with chronic hepatitis B virus (HBV) and/or hepatitis C virus (HVC). The infection of HBV increases the risk of a wide spectrum of clinical manifestations ranging from self-limited acute or fulminant hepatitis, asymptomatic infection or chronic hepatitis with progression to liver cirrhosis that can lead to HCC. Indeed, elevated serum HBV DNA level is a strong risk predictor of HCC (Chen *et al*., 2006; Lupberger and Hildt, 2007). The circular virion genome of HBV has four overlapping reading frames that are responsible for the generation of seven different hepatitis B proteins. Being encoded by the *X* gene, the smallest reading frame, HBx has been implicated in the regulation and transactivation of a variety of cellular genes in hepatocytes. HBx targets a gamut of cytoplasmic and nucleus gene products with diverse functions including modulating nuclear factor (NF)-κB signaling, regulating cell survival and producing inflammatory cytokines, contributing to HBV infection, replication, pathogenesis and putatively to carcinogenesis (Bouchard and Schneider, 2004).

Among the Rel/NF-κB family of proteins, the RelA (p65) and NF-κB1 (p50; p105) have crucial roles in immune response, cell survival and cellular transformation (Pahl, 1999; Li and Verma, 2002). A notable body of literature suggests that HBx interacts with all major forms of NF-κB (Zhang *et al*., 2006) and activates target promoters bearing NF-κB, cAMP-response element and AP-1 consensus motifs (Chirillo *et al*., 1996; Bergametti *et al*., 1999; Hoffmann and Baltimore, 2006); induces the degradation of p105 NF-κB1 and IκBα (Su and Schneider, 1996; Zhang *et al*., 2006) or sequesters IκBα to sustain NF-κB activation (Lucito and Schneider, 1992; Weil *et al*., 1999; Yun *et al*., 2002), where its activity is enhanced by VHL binding protein in a cooperative manner (Kim *et al*., 2008). Yet, the mechanism for HBx activation of NF-κB remains undefined.

Correspondence: Professor R Kumar, Department of Biochemistry and Molecular Biology, George Washington University, 2300, I street, Washington, DC 20037, USA.
E-mail: bcmrxk@gwumc.edu
[8]These authors contributed equally to this work.
Received 22 April 2009; revised 22 July 2009; accepted 9 September 2009

In addition to HBV, the process of liver carcinogenesis has been also shown to be intimately linked with the upregulation of metastasis-associated protein 1 (MTA1), a component of nucleosome remodeling histone deacetylase (NURD) complex (Yoo *et al*., 2008). The MTA family has emerged not only as a crucial modifier of chromatin remodeling but also as a potential prognostic indicator after hepatectomy for HCC (Hamatsu *et al*., 2003; Manavathi and Kumar, 2007). MTA1 overexpression has been shown to associate with carcinogenesis and angiogenesis in animal tumor model systems and in human cohort studies (Manavathi *et al*., 2007b) in addition to be consistently correlated with a higher tumor grade and increased tumor angiogenesis in gastrointestinal carcinoma tumors (Kidd *et al*., 2006) and in colorectal carcinoma as compared to the matched normal tissues (Giannini and Cavallini, 2005). In postoperative patients with HCC, MTA1 expression in HBV associated HCC is higher than that of HCV-associated HCC, although the median survival rate of the MTA1-positive patients with HCC has been shown to significantly shorter than those with MTA1-negative HCCs (Ryu *et al*., 2008). Although these studies suggest a strong positive correlation between the HBx and MTA1 in HCC (Yoo *et al*., 2008), the role of MTA1 in HBx regulation of cellular pathways remains poorly understood. Here we elucidated the molecular basis of MTA1 upregulation in HBx-transfected cells and unexpectedly discovered a mandatory role of MTA1 in HBx regulation of NF-κB signaling and consequently, the expression of NF-κB target genes that collectively have a vital role in HBV-mediated hepatocarcinogenesis.

Results

HBx induces MTA1 expression at the transcription level

To investigate the potential mechanistic basis between the HBx and MTA1, we first tested the effect of increasing amount of HBx on the levels of MTA1 expression in a human hepatocarcinoma cell line HepG2. We found that HBx expression in HepG2 is accompanied by increased expression of MTA1 (Figure 1a) but not of MTA2 or MTA3 (Figure 1b). Similarly, we also noticed a substantial upregulation of MTA1 in HepG2X, an HBx-stable transfectant of HepG2 as compared with vector-transfected cells (Ding *et al*., 2005) or in nonhepatic cell line human embryonic kidney (HEK) 293 (Figure 1c). Because HCC has been also linked with HCV (Ray and Ray, 2001), we examined whether HCV core protein also influences MTA1 expression. However, we found only albeit effect of HCV overexpression on the level of MTA1 in HepG2 cells (Figure 1d), suggesting that MTA1 is preferably induced by HBx and not by HCV core protein.

The observed upregulation of MTA1 protein by HBx was at transcriptional level as HBx-mediated induction of MTA1 mRNA could be effectively blocked by the inclusion of actinomycin D, a DNA replication inhibitor (Figure 1e). To further validate these findings, we next tested the effect of HBx on a murine MTA1 promoter cloned in a TATA-less-pGL3-luciferase reporter system. We found that HBx expression in HepG2, HEK 293 (Figure 1f, supplementary Figure S1A and supplementary Figure S1B, respectively) and HepG2X cells (Figure 1g) was accompanied by stimulation of the MTA1 transcription. In brief, HBx induces MTA1 expression at the transcription level.

HBx uses NF-κB pathway to stimulate MTA1 transcription

The activation of NF-κB and resulting stimulation of the NF-κB transactivation activity confers cell-survival phenotype in HBx-expressing cells (Su and Schneider, 1996; Yun *et al*., 2002). Recent data from our laboratory suggest that MTA1 promoter contains five NF-κB consensus sites and that p65/RelA directly binds and stimulates MTA1 transcription (SB Pakala and R Kumar, unpublished findings, paper in review elsewhere). We examined the mechanism by which HBx stimulates MTA1 transcription with a particular focus on the NF-κB pathway. To examine the use of the NF-κB pathway by HBx to induce MTA1 expression, we showed that HBx-mediated stimulation of the MTA1 promoter activity in HepG2 cells could be effectively blocked by the inclusion of NF-κB-inhibitor parthenolide (Figure 2a). Because HBx uses p65 pathway to stimulate MTA1 transcription (above), there was no additional potentiating effect of coexpressing HBx and p65 on the MTA1 promoter activity (Figure 2b, supplementary Figure S1C). To define the molecular insights of HBx regulation of MTA1 chromatin, we next determined the region of the MTA1 promoter that is being targeted by HBx by performing a chromatin immunoprecipitation (ChIP)-based MTA1 promoter walk in HepG2-transiently expressing HBx. Recruitment of HBx or p65 to all four potential NF-κB consensus sites was analyzed. Flag-tagged antibody-based and p65 antibody-based ChIP analysis showed that HBx as well as p65 were recruited onto two regions of the MTA1 promoter (−3814 to −4152 and −2874 to −3207). Because HBx induces the expression of MTA1, we also examined whether acetylated histone H4, a marker of relaxed activated chromatin, is also recruited to the same sites in the MTA1 promoter in the HBx-expressing cells. Indeed, Acetylated H4 antibody-based ChIP analysis again showed that acetylated H4 is recruited to the regions of MTA1 promoter targeted by HBx (Figure 2c). Using sequential ChIPs, we further showed that HBx/p65 complex is recruited to the MTA1 promoter and such recruitment could be substantially reduced by NF-κB-inhibitor parthenolide (Figure 2d). In brief, these observations suggest that HBx/p65 binding to the MTA1 promoter leads to transcriptional stimulation of MTA1 chromatin. Because HBx is not known to bind directly to DNA, this interaction is presumed to be indirect.

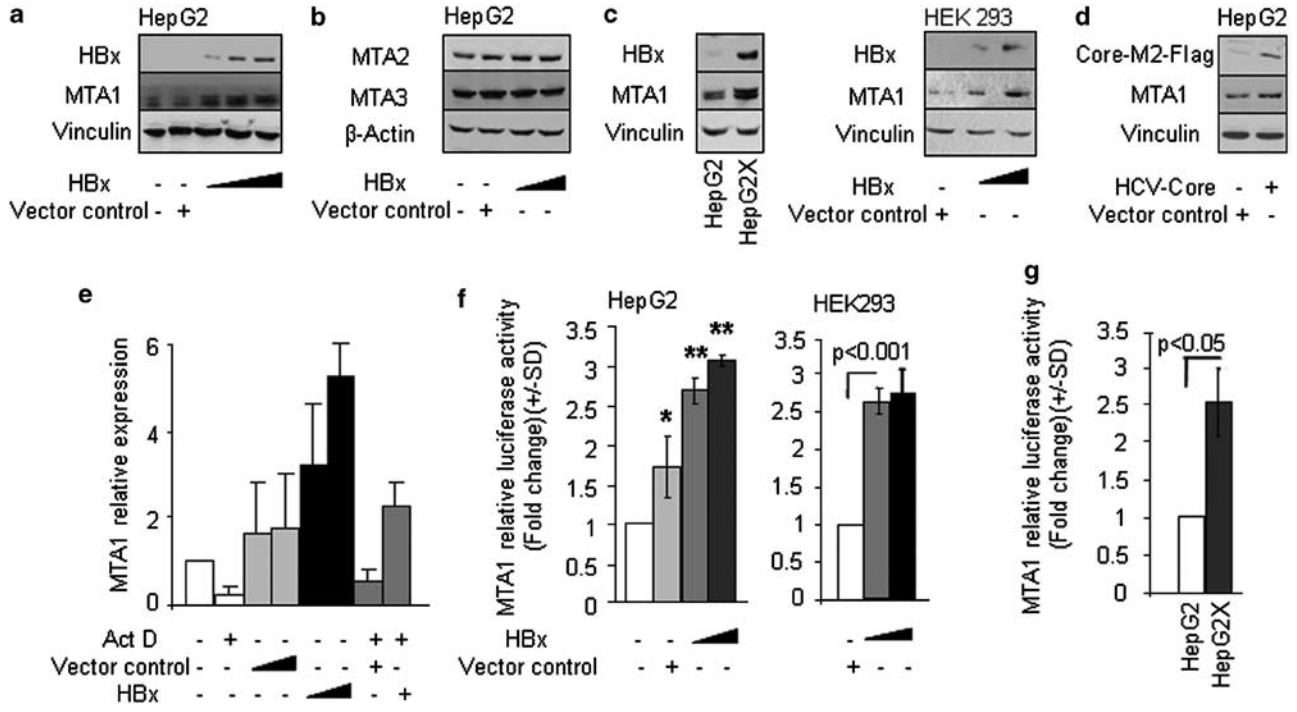

Figure 1 HBx induces metastasis-associated protein 1 (MTA1) expression at the transcriptional level. (**a**) Western blot analysis of HBx and MTA1 in HepG2 cells transfected with either control vector (250 ng per reaction) or HBx expression vector (50, 100 and 250 ng per reaction). (**b**) Western blot analysis of MTA2 and MTA3 proteins in HepG2 cells transfected with control vector (250 ng per reaction) or HBx expression vector (50 and 250 ng per reaction). (**c**) Western blot analysis of MTA1 protein in HepG2X, an HBx-stable transfectant of HepG2, and HEK 293 cells transfected with either control vector (250 ng per reaction) or HBx (50 and 250 ng per reaction). (**d**) Western blot analysis of MTA1 protein in HepG2 cells transfected with either control vector or HCV core protein (250 ng per reaction). (**e**) q–PCR analysis of MTA1 in HepG2 cells treated with actinomycin D (Act D) (5 μg/ml) after being transfected with either control vector or HBx (50–200 ng per reaction). Expression levels of MTA1 were normalized with β-actin. (**f**) MTA1 promoter activity in HepG2 cells and HEK 293 cells transfected with either control vector (250 ng per reaction) or HBx (100–250 ng per reaction; $^{*}P<0.05$,$^{**}P<0.001$). (**g**) MTA1 promoter activity in HepG2X.

Essential role of the YFKD motif in HBx regulation of MTA1 transcription

To define the minimal region of HBx required for stimulation of MTA1 transcription, we used a series of well-characterized truncated mutant of HBx. We found that deletions of the N-terminal regions (aa1–84) or the nonconserved region (aa141–154) had marginal effect on the HBx-mediated MTA1 transactivation, whereas deletions in the region between residue 58 and residue 140 of HBx resulted in significant decrease in the level of HBx-mediated stimulation of MTA1 transcription. Interestingly, there was a good correlation between the effects of HBx-deletion constructs on the MTA1 promoter activity and MTA1 protein expression in HepG2 cells (Figure 3a), providing clue about the biological significance of the region of HBx encompassing aa58–120. Because this region contains the binding motif for p65 (Vijay Kumar, personal communication) and because HBx stimulates MTA1 transcription through NF-κB pathway (this study), we wanted to define the minimal region on HBx that may impact MTA1 expression. We mutated the YFKD motif to FAEN in the full-length HBx protein (designated as mut-HBx) and examined the impact of mut-HBx on MTA1 promoter activity as well as on MTA1 upregulation. We found that as compared to the wild-type (WT) HBx, mut-HBx is unable to efficiently induce the MTA1 promoter activity as well as MTA1 protein in HepG2, NIH3T3 and murine embryonic fibroblasts (MEFs; Figure 3b, supplementary Figure S2A-B).

Although a large body of previous HBx studies have suggested both direct and indirect involvement of NF-κB to HBx transactivation, evidence showing direct interaction between HBx and NF-κB-p65 remains elusive. Hence, we next expressed the WT HBx and mut-HBx constructs as ^{35}S-labeled proteins and performed a glutathione *S*-transferase pull-down assay using the glutathione *S*-transferase-NF-κB-p65 fusion protein. Results in Figure 3c showed a direct interaction of the NF-κB-p65 with HBx but not with mut-HBx. These findings also confirmed that the YFKD motif has an essential role in the noted HBx-NF-κB-p65 interaction. To further validate these results and to establish the significance of the YFKD motif, we next examined the recruitment of the Flag-HBx/p65 complex to the native MTA1 chromatin by sequential ChIPs involving antibodies against the Flag and p65 in the first and second ChIPs, respectively. We found that the WT Flag-HBx but not Flag-mut-HBx was recruited onto the putative binding regions of the MTA1 promoter (−3814 to −4152 and −2874 to −3207; Figure 3d). To establish a direct interaction of the HBx protein to the NF-κB

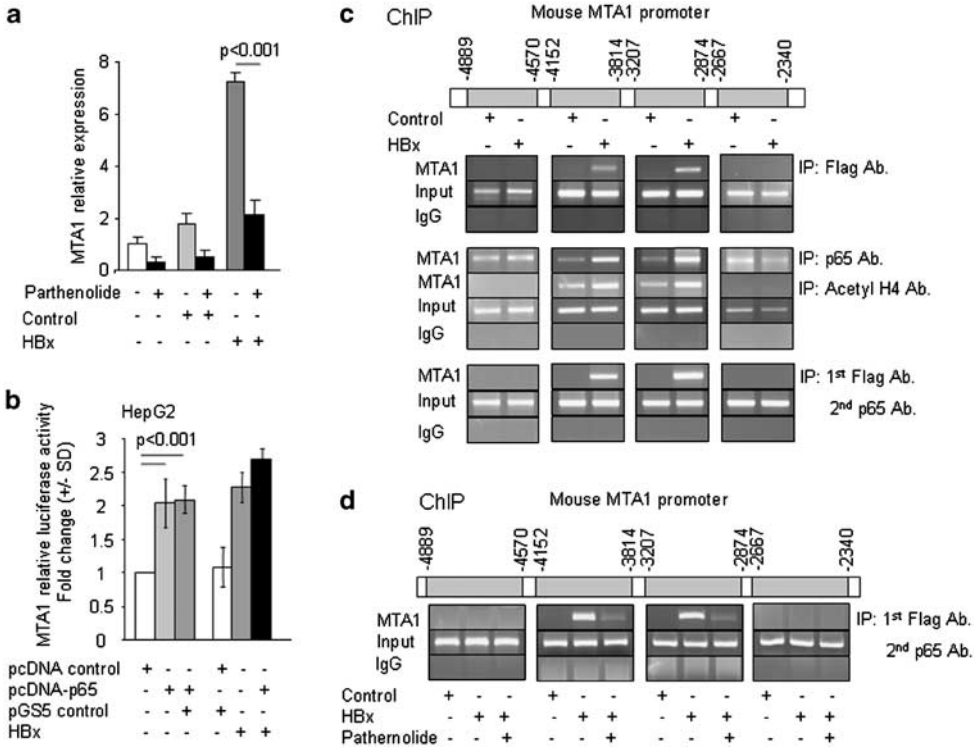

Figure 2 HBx activates metastasis-associated protein 1 (MTA1) transcription through nuclear factor (NF)-κB. (**a**) q–PCR analysis of MTA1 in HepG2 cells treated with parthenolide (5 μM) after being transfected with either control vector or HBx expression vector. Expression levels of MTA1 were normalized with β-actin. (**b**) Effect of HBx or NF-κB-p65 on MTA1 promoter activity in HepG2 cells. (**c**) Recruitment of HBx or NF-κB-p65 or acetyl H4 to *MTA1*-chromatin (−3814 to −4152 and −2874 to −3207) by chromatin immunoprecipitation (ChIP) assay in NIH3T3 cells using Flag-tagged antibody (Flag Ab), NF-κB-p65 antibody (p65 Ab) or acetyl H4 antibody (Acetyl H4 Ab) after co-transfecting with pCMV vector control or pCMV-HBx. Row 3, sequential ChIP assay showing the recruitment of HBx followed by NF-κB-p65 to *MTA1*-chromatin (−3814 to −4152 and −2874 to −3207). (**d**) Recruitment of HBx followed by NF-κB-p65 to *MTA1*-chromatin (−3814 to −4152 and −2874 to −3207) by sequential double ChIP assay in NIH3T3 cells treated with parthenolide after being transfected with either pCMV vector control or pCMV-HBx.

consensus motif in the MTA1 promoter DNA and the significance of the YFKD motif, we next performed electrophoretic gel mobility shift assay (EMSA). To this end, we subjected the nuclear extracts from the HepG2 cells transfected with HBx or mut-HBx to EMSA analysis using a 300-bp DNA fragment containing a single NF-κB consensus motif in the MTA1 promoter. We found that extracts from the HBx, but not from mut-HBx, expressing cells resulted in the formation of a distinct protein/DNA complex (Figure 3e, compare lane 5 with lane 2), which could be competed by the cold DNA probe (lane 8). Interestingly, the observed HBx protein/DNA complex could be effectively supershifted by anti-p65 antibodies but not by IgG (compare lane 6 with lane 8). These results suggested that HBx presumably uses NF-κB-p65 to interact with the MTA1 DNA as mut-HBx, which cannot interact with p65, was also unable to bind to MTA1 DNA fragment in the EMSA (lanes 9–14).

MTA1 is required for HBx transactivation function

Recent studies suggest that MTA1 is not only a target of p65 but also a component of NF-κB signaling network (SB Pakala, TM Bui-Nguyen and R Kumar, unpublished findings). To understand the impact of endogenous MTA1 on HBx transactivation activity, we examined the ability of HBx to induce MTA1-luc activity in the WT MEF and MEF-MTA1-KO MEFs. Unexpectedly, we found that HBx was unable to induce MTA1-luc activity in MTA1-deficient MEFs whereas it did so in the WT MEFs (Figure 4a). To independently validate these results, we next showed that selective siRNA-mediated knockdown of MTA1 in HepG2 cells also impaired both the basal and HBx-induced MTA1 promoter activity (Figure 4b). Taken together, these results revealed an integrated role of MTA1 in the transactivation activity of HBx.

An essential role of MTA1 in HBx stimulation of NF-κB signaling

As MTA1-depletion compromises HBx transactivation activity, we next examined whether HBx activation of NF-κB requires MTA1. To examine the above hypothesis, we examined the effect of MTA1-siRNA on the ability of HBx to induced NF-κB signaling pathway in HepG2 cells. As expected from the previous studies, HBx expression was accompanied by increased levels of phosphorylated IκBα and phosphorylated p65 as well as MTA1 expression. However, comparable levels of HBx in HepG2 cells were unable to induce p65/RelA activation in the absence of MTA1 (Figure 5a). Consistent with these results, the levels of HBx-mediated stimulation of the NF-κB DNA-binding activity was

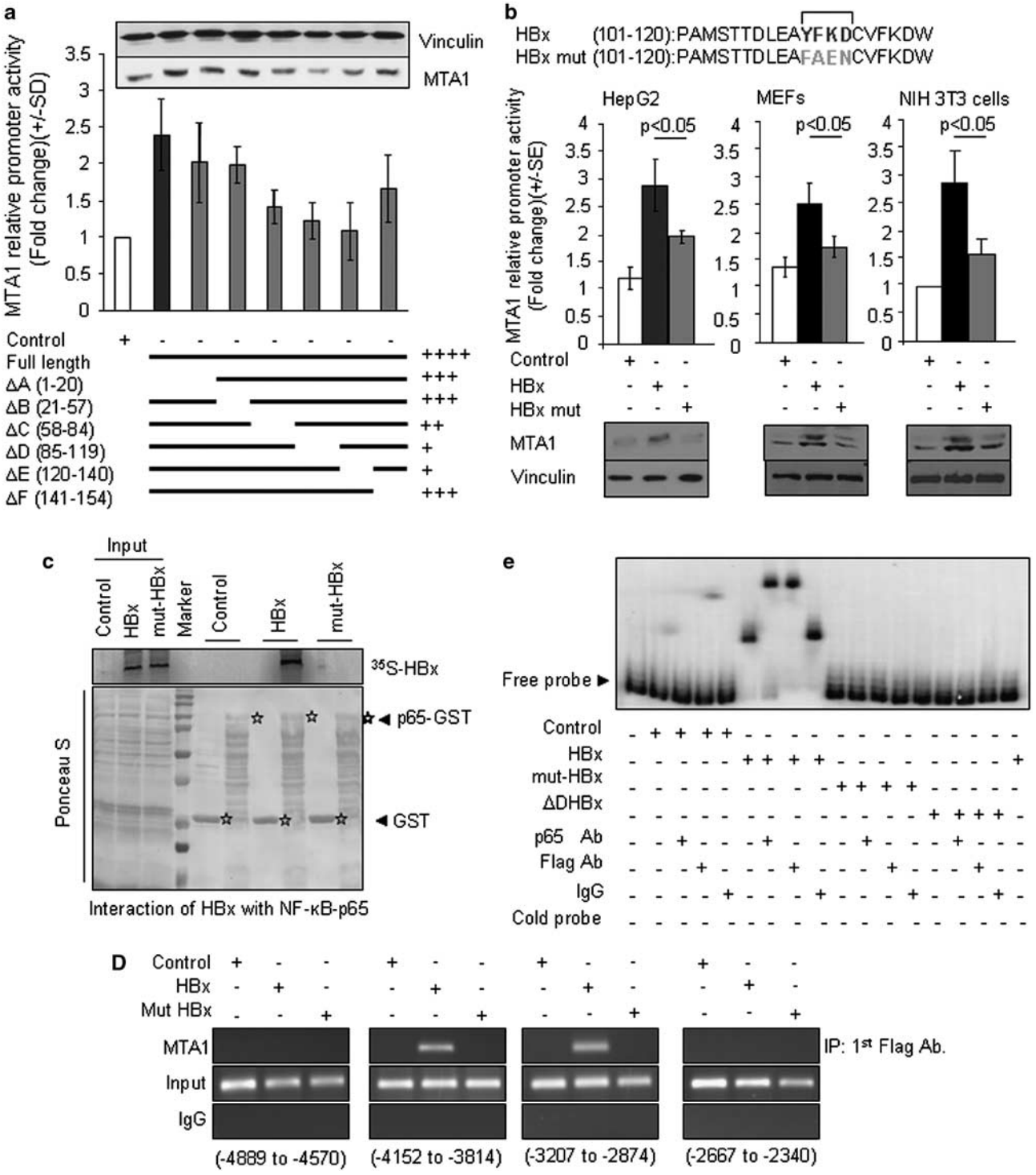

Figure 3 Role of YFKD motif of HBX in regulation of metastasis-associated protein 1 (MTA1) transcription. (**a**) MTA1 promoter activity in HepG2 cells transfected with either control vector or HBx expression vector or HBx-deletion constructs. Inset, western blot analysis of MTA1 protein in HepG2 cells after transfecting with vector or HBx or HBx-deletion constructs. (**b**) MTA1 promoter activity in HepG2 or NIH3T3 or murine embryonic fibroblast (MEF) cells after being transfected with either control vector or HBx expression vector or mut-HBx (YFKD was mutated to FAEN) expression vector. Lower panels are western blot analysis of MTA1 protein in HepG2 or NIH3T3 or MEF cells after being transfected with either control vector or HBx expression vector or mut-HBx expression vector. (**c**) Glutathione *S*-transferase pull-down assays with ^{35}S-labeled *in vitro*-translated HBx and mut-HBx and glutathione *S*-transferase-NF-κB-p65 protein. (**d**) Recruitment of HBx to *MTA1*-chromatin (−3814 to −4152 and −2874 to −3207) by chromatin immunoprecipitation (ChIP) assay in the NIH3T3 cells. (**e**) Electrophoretic gel mobility shift assay (EMSA) analysis of NF-κB-p65 binding to the mouse MTA1 promoter using PCR product encompassing functional NF-κB consensus sequence in transfected cells and controls. Nuclear extract of HepG2 cell transfected with either Flag-tagged HBx, or Flag-tagged mut-HBx or Flag-tagged ΔD-deletion HBx (2000 ng per lane), probe control (0.3 ng per lane), NF-κB-p65 antibody (Ab), anti-Flag antibody and IgG control (1000 ng per lane), cold probe (15 ng per lane) were used.

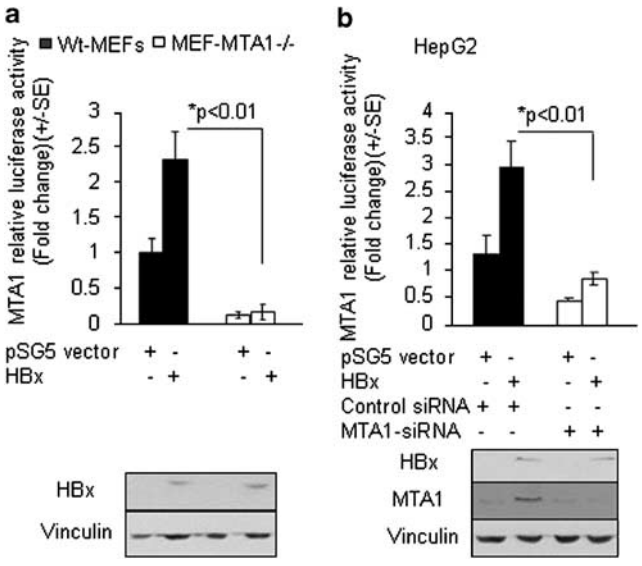

Figure 4 Metastasis-associated protein 1 (MTA1) is required for HBx transactivation function. (**a**) MTA1 promoter activity in wild-type (WT) and MTA1$^{-/-}$ murine embryonic fibroblasts (MEFs) after being transfected with either control vector or HBx. (**b**) MTA1-promoter activity in HepG2 cells with or without MTA1 knockdown by siRNA after being transfected with either control vector or HBx.

substantially reduced in MTA1-deficient MEFs as compared to WT MEFs (data not shown). We next determined whether the noticed defect in NF-κB signaling pathway by HBx under condition of MTA1 deficiency is also accompanied by a defective NF-κB-target gene expression. To explore this notion, we examined the effect of MTA1-siRNA on the ability of HBx to induce transcription from the NF-κB-luc reporter in HepG2 and MEFs. We found that MTA1 downregulation impairs the ability of HBx to induce NF-κB-luc activity (Figures 5b-c) and to induce the formation of the protein/DNA sequence harboring NF-κB consensus motif complex (Figure 5d). Accordingly, MTA1 depletion in HepG2 by siRNA (Figure 5e, inset) also impairs the ability of HBx to induce the expression of NF-κB target genes such as *COX2* and *TNF-α* (Figure 5e). Because previous studies have shown a strong positive correlation of COX2 upregulation with HBx expression and in HBV-associated chronic liver diseases (Cheng *et al*., 2004), these studies suggest that HBx-dependent stimulation of NF-κB signaling and resulting inflammatory targets such as *COX2* and *TNF-α* are at least, in part, are dependent on the presence of MTA1. Together, these findings imply that HBx-induced inflammation, which overtime could contribute to transformation, may be dependent on the cellular status of MTA1. Together, these findings suggest that MTA1 has an essential mechanistic role during HBx-mediated stimulation of NF-κB signaling and resulting expression of target genes and functions.

Levels of MTA1 and p65 in HBV-infected HCC

To evaluate the significance of noted HBx regulation of MTA1 and in turn, a mechanistic role of MTA1 in HBx-induced NF-κB pathway in human HCC, we examined the expression characteristics of HBx, MTA1 and p65 in HCC specimens and matched nontumor tissue from the same patient. Western blot analysis of 22 matched sets of specimens indicated that there were 9 HCC-HBx-positive specimens out of 44 analyzed here, whereas MTA1 and p65 were upregulated in 20 and 19 samples, respectively, and consistently upregulated in HCC as compared to the matching nontumor tissues (Figure 6a). Overall, there were 7 out of 22 HCC samples wherein HBx presence correlated with increased MTA1 and p65. Interestingly, high levels of MTA1 expression were often ensued by high levels of NF-κB-p65 expression in both tumor and non-HCC samples. To further support these observations, we performed immunohistochemical analysis of 39 additional samples in microtissue array format (Figure 6b). HCC (25) and non-HCC (14) samples were examined for the expression of HBx, MTA1 and p65 using immunohistochemical staining. There was a significant correlation between HBx expression and the detected levels of MTA1 ($P=0.0001$) in a mixed population of HCC and non-HCC samples. Strong correlation between the intensity of MTA1 and p65, however, was only found in HCC samples ($P<0.0001$) but not in non-HCC samples ($P=0.46$) (Figure 6c). Altogether, our data suggest that high expression levels of HBx are accompanied by increased expression of MTA1 and p65RelA and that MTA1 has a pivotal role in HBx transactivation in HCC.

Discussion

The expression of HBx, a multifunctional HBV viral transactivator, targets a variety of cellular proteins, including NF-κB with putative functions ranging from transcription, inflammation, survival and cancerous phenotypes. However, at the moment, cellular targets of HBx have not been exploited to develop strategies to inhibit or slowdown the process of HBV-mediated HCC carcinogenesis. Overexpression of MTA1 is known to be associated with the degree of intrahepatic invasion and metastasis in patients with HCC (Hamatsu *et al*., 2003; Ryu *et al*., 2008). While this study was in process, Yoo *et al*. showed that HBx induces MTA1 but that study did not address possible mechanistic insights (Moon *et al*., 2004). Here we delineated the molecular mechanism underlying the induction of MTA1 by HBx and showed the integral role of MTA1 during HBx-mediated activation of NF-κB signaling and its targets. We further established that although both HBV and HCV have been linked with HCC, MTA1 is targeted by HBV-HBx protein and not by HCV core protein. We further showed that HBx selectively induces MTA1 but not MTA2 and MTA3. Ongoing cloning of a murine MTA1 promoter allowed us to provide a detailed mechanistic insight of HBx regulation of MTA1 transcription. We showed that HBx targets p65 to physically interact with the MTA1 promoter containing a functional p65 motif, and such interactions lead to a

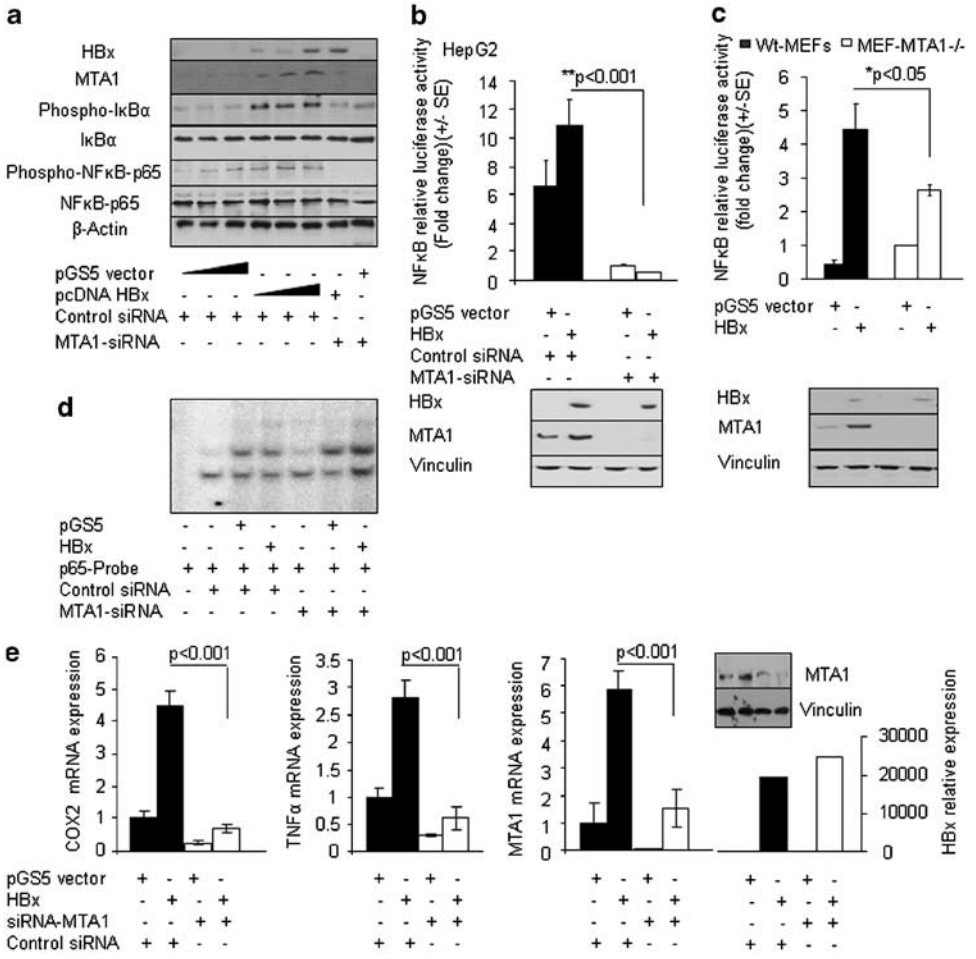

Figure 5 Metastasis-associated protein 1 (MTA1) is needed for HBx stimulated nuclear factor (NF)-κB signaling. (**a**) Effect of selective knockdown of MTA1 on the activation status of the NF-κB signaling components in HepG2 cells by western blot analysis after being transfected with either control vector or HBx (250 ng per reaction in a six-well plate). HepG2 cells transfected with increased amount of control vector and HBx (50, 250 and 600 ng per reaction) were used as controls. (**b**) NF-κB-promoter activity in HepG2 cells with or without MTA1 knockdown by siRNA-MTA1 after being transfected with either vector or HBx. Lower panel is the control western blot analysis for the aforementioned experiments. Vinculin was used as a control. (**c**) NF-κB-promoter activity in murine embryonic fibroblast (MEF) cells after being transfected with either vector or HBx. Lower panel is the control western blot analysis for the aforementioned experiments. Vinculin was used as a control. (**d**) Nucleus extracts from HepG2 cells transfected with either vector control or HBx expression vector after MTA1 knockdown by siRNA-MTA1 were subjected to electrophoretic gel mobility shift assay (EMSA) analysis using an NF-κB-consensus sequence. Extracts from wild-type HepG2 transiently transfected with HBx were used as controls. (**e**) q–PCR analysis of COX2, TNF-α, MTA1 and HBx mRNAs in HepG2 cells with or without MTA1 knockdown by siRNA-MTA1 after being transfected with control vector or vector-expressing HBx. Control siRNA was used in indicated experiments. Expression levels of COX2, TNF-α, MTA1 and HBx were normalized with β-actin. Inset, western blot analysis for MTA1 in HepG2 cells after being co-transfected with siRNA-MTA1 and HBx or control vector. Vinculin was used as a control.

productive MTA1 transcription in HBx-expressing cells. Indeed, we showed that HBx uses the *YFKD* motif to interact with the p65 subunit and consequently, the HBx/p65 complex interacts with the MTA1 promoter element through the NF-κB response elements. Nevertheless, the inability of both ΔC-HBx (HBx with intact aa101–120) and ΔE-HBx (HBx with intact aa101–120) to stimulate the p65 luc-promoter activity (supplementary Figure S1D) suggests that individual domains of HBx may not mimic the activity conferred by the intact HBx.

The HBx regulatory protein has been established by many laboratories to activate NF-κB activities in transfected cells, and this is presumed to have a role in HCC (Su and Schneider, 1996; Kim *et al*., 2008). As shown in Figure 5a, we did observe a transient induction of IκBα phosphorylation, which is a direct indicator of functional upstream signaling feeding into the IκBα-p65 linearity. For reasons not fully understood at the moment, we did not observe any downregulation of IκBα protein (Figure 5a, 4th panel) as was the case in Chirillo *et al*. (1996). Several possibilities may be attributable to this discrepancy such as the use of the HeLa cells by Chirillo *et al*. (1996) as opposed to the relevant use of HepG2 cells in our study, possible HBx interaction with papilloma virus in the HeLa cells, leading to the observed effects reported by Chirillo *et al*. (1996). Although it is clear that MTA1 is essential for HBx transactivation of NF-κB, the manner by which MTA1 could feed into HBx/NF-κB pathway remains to be an important question for future investigation.

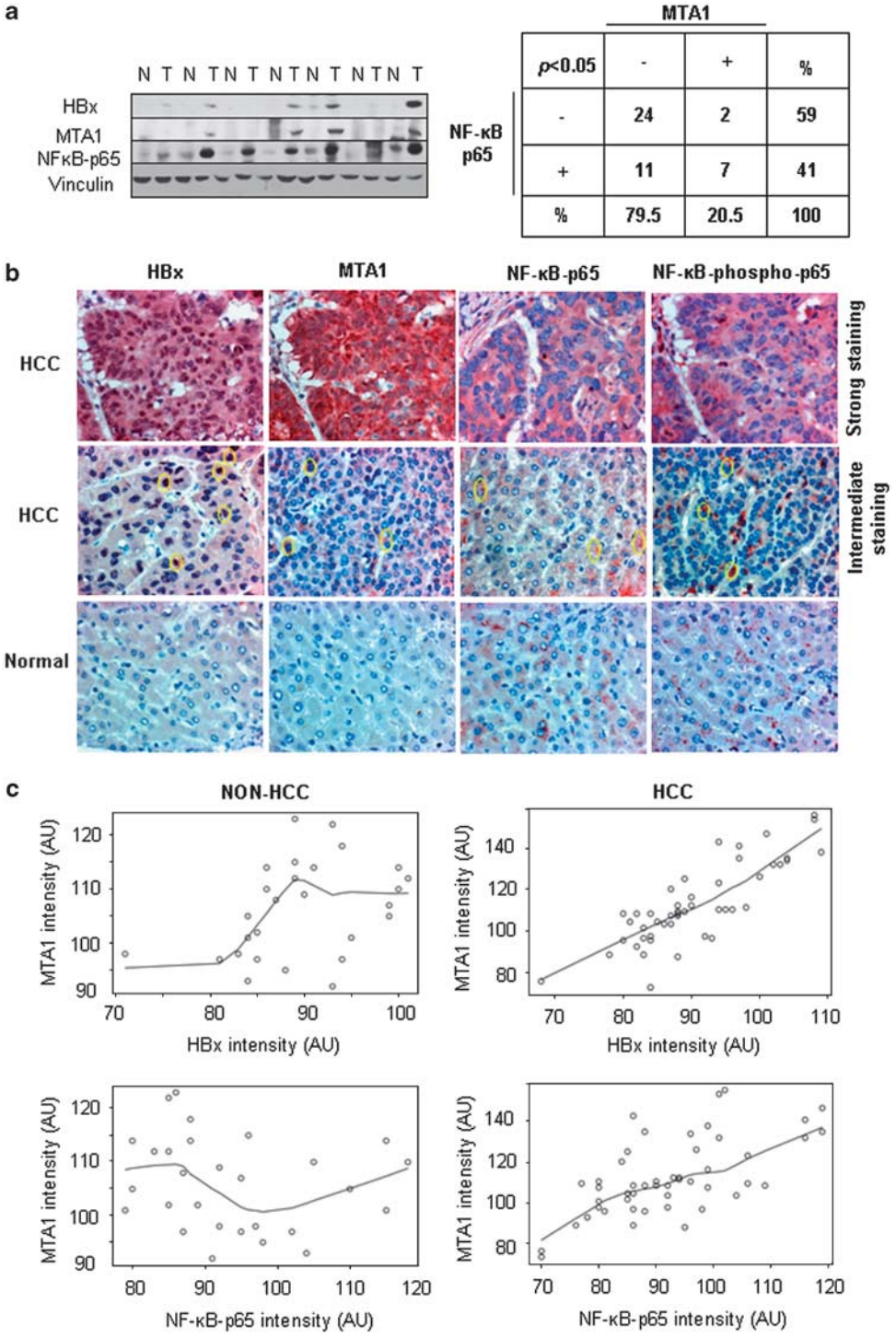

Figure 6 Prevalence of HBx, metastasis-associated protein 1 (MTA1) and nuclear factor (NF)-κB-p65 in hepatocellular carcinoma (HCC). (**a**) Western blot analysis for MTA1, NF-κB-p65 and HBx proteins in human HCC specimens and matched nontumor tissue from the same patient ($n = 44$). Statistical analysis of the data was performed using Fisher's Exact test. (**b**) Representative images of immunohistochemical analysis for HBx, MTA1, NF-κB-p65 and NF-κB-phospho-p65 in HCC and non-HCC samples in microtissue array. Circular marks denoted positive staining regions. (**c**) A multiple linear mixed model for the MTA1 intensity (arbitrary unit, AU) with those of HBx or NF-κB-p65 and the interaction between these markers and disease group fitted as covariates was assembled. Positive association between MTA1 and HBx and MTA1 and NF-κB-p65 intensity was observed in the HCC patients but not in non-HCC patients. Particularly, in HBx-positive HCC patients, there was a significant positive association between NF-κB-p65 and MTA1 intensity ($P < 0.0001$).

Because MTA1 now affects the stability of proteins (Li *et al*., 2009), it is possible that HBx-induced MTA1 in turn, stabilizes specific component or components of proximal NF-κB signaling. If so, such changes may translate into an amplified NF-κB signaling. Furthermore, because MTA1 is a chromatin modifier, it is

conceivable that MTA1 may affect the net transcription of the components of NF-κB pathway, and consequently, affect the signaling.

Both inflammation and MTA1 have been linked to cancer, particularly to HCC. Chronic inflammation in liver, depending on the magnitude of NF-κB activity, promotes hepatocarcinogenesis whereas MTA1 overexpression is known to associate with tumor growth and metastasis (Toh *et al*., 1995; Hamatsu *et al*., 2003; Bagheri-Yarmand *et al*., 2004; Giannini and Cavallini, 2005; Maeda *et al*., 2005; Jang *et al*., 2006; Kidd *et al*., 2006; Manavathi and Kumar, 2007; Manavathi *et al*., 2007b). Nevertheless, the role of the nucleosome remodeling histone deacetylase complex in NF-κB signaling remains unknown until our recent discovery. Our recent study showed that MTA1, the founding member of the nucleosome remodeling histone deacetylase family of coregulators, is a part of NF-κB pathway and regulates host inflammatory response. Here we took this observation further and established regulatory contribution of MTA1/NF-κB cross talk in the transactivation activity of HBx. Results from ChIP studies suggested that HBx may use p65 as a coactivator and that HBx/p65 recruitment to the MTA1 promoter is correlated with the recruitment of H4-specific acetyltransferase complexes. The overexpression of p65 can also mimic the effect of HBx-mediated stimulation of the MTA1 transcription. However NF-κB, which is known to regulate the transcription of many acute-phase proteins (Pahl, 1999), requires other transcription factor and coregulator such as SRC-3 (Anderson and Kedersha, 2007) for its maximum activity. In this context, it is worth mentioning that the removal of MTA1 had a profound impairment of HBx transactivation and expression of the NF-κB's targets, for example COX2 and TNF-α. These findings suggest an integral role of MTA1 in the previously shown regulation of NF-κB activity by HBx and support the notion that MTA1 is required efficient transactivation of HBx in hepatocytes. Although HBx has been shown to influence a large number of molecules in the cell lines, only a few have been shown to be involved in the physiologically relevant human specimens as well. In this context, this study has used both the model systems and points to the fact that the newly described HBx/MTA1/NF-κB pathway may be physiologically relevant in the pathogenesis of HCC. Because TNF-α is a target as well as activator of NF-κB, the status of MTA1 in HBx-expressing cells is likely to have an important effect on carcinogenesis process. These findings suggest that MTA1 may not only represent a novel HCC prognostic indicator (Hamatsu *et al*., 2003), but also can be a therapeutic target.

Materials and methods

Materials

Antibodies against IκBα (sc-371), NF-κB p65 (sc-372), phospho-NF-κB p65 (sc-33020) and NF-κB p65 (286-H) X (sc-7151 X) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). COX2 antibody (4842) was from Cell Signaling (Beverly, MA, USA). NF-κB p65 was from Abcam (Cambridge, MA, USA). Flag-M2 monoclonal antibody (F3165), β-actin and vinculin monoclonal antibodies, normal mouse IgG, rabbit IgG were from Sigma Chemical Co. (St Louis, MO, USA). MTA1 polyclonal antibody (1805) was from Bethyl Laboratories Inc. (Montgomery, TX, USA). HBx antibody (MAB8419) was from Millipore/Chemicon (Danvers, MA, USA). Rabbit polyclonal anti-HBx was used as previously described (Lee *et al*., 1995). Plasmids pSI-HBx and pSI-GFP plasmids were cloned as described (Keasler *et al*., 2007). pCMVXF and pCMV-Core are kind gifts from Dr A Siddiqui, University of Colorado Health Sciences, Denver, CO. pSG5-HBx full-length and pSG5-HBx mutant constructs are from Dr V Kumar, International Centre for Genetic Engineering and Biotechnology, India. Cells used were obtained from the American Type Culture Collection. HepG2-X is a gift from Dr Mien-Chie Hung, MD Anderson Cancer Center, Houston, TX. All cell types were cultured as described previously or otherwise stated (Bergametti *et al*., 1999; Manavathi *et al*., 2007a).

Cells and tissue

HepG2 liver cells, HEK 293 cells and NIH3T3 cells were obtained from the American Type Culture Collection. HepG2X is a gift from Dr Mien-Chie Hung, Molecular and Cellular Oncology, MD Anderson Cancer Center. All cell types were cultured as described previously or otherwise stated (Bergametti *et al*., 1999; Manavathi *et al*., 2007a). Human HCC and nontumor tissues were obtained from the Qidong Liver Cancer Institute in Shanhair, People's Republic of China, as described in Slagle *et al*. 1991. There was evidence of HBV infection (circulating antibody to HBV surface antigen, antibody to HBV core antigen or HBV DNA integrated into tumor DNA) for all patients. Samples were collected according to institutional IRB approvals. Tissue microarray sections (LVC961) were obtained from Pantomics Inc. (Richmond, CA, USA).

Quantiative RT–PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and first-strand cDNA synthesis was carried out with SuperScript II reverse transcriptase (Invitrogen) using 2 μg of total RNA and poly (dT) primer.

ChIP and reporter assays

HepG2 cells were transiently transfected with HBx expression vector before ChIP analysis and reporter assays. ChIP assays were carried out by using Flag antibody, NF-κB, MTA1 and H4 acetylation antibodies as described previously (Gururaj *et al*., 2006; Manavathi *et al*., 2007a). The primers used for ChIP are listed in Supplementary Tables 1. MTA1-luciferase and NF-κB-luciferase assay were performed according to the manufacturer's instructions (Promega, Madison, WI, USA).

siRNA transfection

siRNA against MTA1 and negative control siRNA were purchased from Dharmacon (Chicago, IL, USA). Cells were seeded at 40% density 24 h before transfection in six-well plates. Transfection was performed using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Cells were subsequently subjected to further studies after 36 h of transfection. Western blot analyses were performed as described previously (Manavathi *et al*., 2007a).

EMSA
Nuclear extracts were prepared using a Nonidet P-40 lysis method (Schreiber *et al*., 1989). EMSA for DNA binding was performed using the annealed and [γ-^{32}P] ATP end-labeled NF-κB Consensus Oligonucleotide (Promega) or MTA1 in a 20 μl reaction mixture for 15 min at 20 °C. The reactions were then terminated and run on a nondenaturing 5% polyacrylamide gel and imaged by autoradiography. Supershift complex was detected by the addition of 1 μg of antibodies.

Immunohistochemical analysis
The immunoperoxidase staining method used in these studies was a modification of the avidin–biotin complex technique as described previously. The modifications from the standard method were incorporated to ensure high sensitivity and specificity. Tissue microarray sections (LVC961; Pantomics Inc.) were deparaffinized, dehydrated and subjected to antigen retrieval using PT Module (Thermo Fisher Scientific, Fremont, CA, USA) for 30 min at room temperature. The endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide for 10 min and the slides were then treated with 10% normal horse or goat serum for 30 min. Incubation with primary antibodies was performed at 4 °C overnight. Following washes with phosphate-buffered saline, the slides were incubated with biotinylated secondary antibodies and incubated with avidin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Detection was performed with the 0.125% aminoethylcarbazole chromogen substrate solution (Sigma Chemical Co.). In the study for the correlation of NF-κB expression, a polyclonal antibody (Abcam) was used to detect NF-κB in 25 hepatitis cancer (HCC) tumor specimens and 14 non-HCC cases. Concentrations of the antibodies used were as follows: NF-κB, 0.200 mg/ml (diluted 1:800), a polyclonal antibody of p-NF-κB p65 (Ser 536, SC-33020) from Santa Cruz Biotechnology, 0.2 mg/ml (diluted 1:100), a polyclonal antibody of MTA1 (IHC-00026) (diluted 1:200, from Bethyl Laboratories Inc.) and monoclonal antibody of HBx, 1 mg/ml (diluted 1:50; Chemicon).

Imaging analysis
To ensure absolute objectivity of the immunohistochemical studies, we used in these experiments the ACIS III Automated Cellular Imaging System (DAKO) to analyze tissues scoring and quantification (nuclear or membrane and cytoplasm applications based on percent and intensity). The percentage of positive tumor cells was used for statistical analysis.

Statistical analysis and reproducibility
The results are given as the mean ± standard error. Statistical analysis of the data was performed by using Student's *t*-test, Fisher's Exact test or otherwise described.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Aleem Siddiqui for pCMVXF and pCMV-Core constructs, Ranjit Banerjee for the HVC core plasmid, Amanda J Hodgson for technical assistance and Xuemei Wang for statistical analysis. The work was supported by NIH Grant CA98823 (RK). Work in Dr Betty L Slagle laboratory is supported by the NIH Grant 95388.

References

Anderson P, Kedersha N. (2007). On again, off again: the SRC-3 transcriptional coactivator moonlights as a translational corepressor. *Mol Cell* **25**: 796–797.

Bagheri-Yarmand R, Talukder AH, Wang RA, Vadlamudi RK, Kumar R. (2004). Metastasis-associated protein 1 deregulation causes inappropriate mammary gland development and tumorigenesis. *Development* **131**: 3469–3479.

Bergametti F, Prigent S, Luber B, Benoit A, Tiollais P, Sarasin A *et al*. (1999). The proapoptotic effect of hepatitis B virus HBx protein correlates with its transactivation activity in stably transfected cell lines. *Oncogene* **18**: 2860–2871.

Bouchard MJ, Schneider RJ. (2004). The enigmatic X gene of hepatitis B virus. *J Virol* **78**: 12725–12734.

Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN *et al*. (2006). Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* **295**: 65–73.

Cheng AS, Chan HL, Leung WK, To KF, Go MY, Chan JY *et al*. (2004). Expression of HBx and COX-2 in chronic hepatitis B, cirrhosis and hepatocellular carcinoma: implication of HBx in upregulation of COX-2. *Mod Pathol* **17**: 1169–1179.

Chirillo P, Falco M, Puri PL, Artini M, Balsano C, Levrero M *et al*. (1996). Hepatitis B virus pX activates NF-kappa B-dependent transcription through a Raf-independent pathway. *J Virol* **70**: 641–646.

Ding Q, Xia W, Liu JC, Yang JY, Lee DF, Xia J *et al*. (2005). Erk associates with and primes GSK-3beta for its inactivation resulting in upregulation of beta-catenin. *Mol Cell* **19**: 159–170.

Dvorchik I, Demetris AJ, Geller DA, Carr BI, Fontes P, Finkelstein SD *et al*. (2007). Prognostic models in hepatocellular carcinoma (HCC) and statistical methodologies behind them. *Curr Pharm Des* **13**: 1527–1532.

Giannini R, Cavallini A. (2005). Expression analysis of a subset of coregulators and three nuclear receptors in human colorectal carcinoma. *Anticancer Res* **25**: 4287–4292.

Gururaj AE, Singh RR, Rayala SK, Holm C, den Hollander P, Zhang H *et al*. (2006). MTA1, a transcriptional activator of breast cancer amplified sequence 3. *Proc Natl Acad Sci USA* **103**: 6670–6675.

Hamatsu T, Rikimaru T, Yamashita Y, Aishima S, Tanaka S, Shirabe K *et al*. (2003). The role of MTA1 gene expression in human hepatocellular carcinoma. *Oncol Rep* **10**: 599–604.

Hoffmann A, Baltimore D. (2006). Circuitry of nuclear factor kappaB signaling. *Immunol Rev* **210**: 171–186.

Jang KS, Paik SS, Chung H, Oh YH, Kong G. (2006). MTA1 overexpression correlates significantly with tumor grade and angiogenesis in human breast cancers. *Cancer Sci* **97**: 374–379.

Keasler VV, Hodgson AJ, Madden CR, Slagle BL. (2007). Enhancement of hepatitis B virus replication by the regulatory X protein *in vitro* and *in vivo*. *J Virol* **81**: 2656–2662.

Kidd M, Modlin IM, Mane SM, Camp RL, Eick G, Latich I. (2006). The role of genetic markers—NAP1L1, MAGE-D2, and MTA1—in defining small-intestinal carcinoid neoplasia. *Ann Surg Oncol* **13**: 253–262.

Kim SY, Kim JC, Kim JK, Kim HJ, Lee HM, Choi MS *et al*. (2008). Hepatitis B virus X protein enhances NFkappaB activity through cooperating with VBP1. *BMB Rep* **41**: 158–163.

Lee T-h, Elledge SJ, Butel JS. (1995). Hepatitis B virus X protein interacts with a probable cellular DNA repair protein. *J Virol* **69**: 1107–1114.

Li Q, Verma IM. (2002). Identification of 14-3-3sigma mutation causing cutaneous abnormality in repeated-epilation mutant mouse. *Nat Rev Immunol* **2**: 725–734.

Li Da-Q, Ohshiro K, Reddy SDN, Pakala SB, Lee MH, Zhang Y *et al*. (2009). E3 ubiquitin ligase COP1 regulates the stability and functions of MTA1. *Proc Natl Acad Sci USA* **116**: 17493–17498.

Lucito R, Schneider RJ. (1992). Hepatitis B virus X protein activates transcription factor NF-kappa B without a requirement for protein kinase C. *J Virol* **66**: 983–991.

Lupberger J, Hildt E. (2007). A novel system for efficient gene transfer into primary human hepatocytes via cell-permeable hepatitis B virus-like particle. *World J Gastroenterol* **13**: 74–81.

Maeda S, Kamata H, Luo JL, Leffert H, Karin M. (2005). IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* **121**: 977–990.

Manavathi B, Kumar R. (2007). Metastasis tumor antigens, an emerging family of multifaceted master coregulators. *J Biol Chem* **282**: 1529–1533.

Manavathi B, Peng S, Rayala SK, Talukder AH, Wang MH, Wang RA *et al*. (2007a). Repression of Six3 by a corepressor regulates rhodopsin expression. *Proc Natl Acad Sci USA* **104**: 13128–13133.

Manavathi B, Singh K, Kumar R. (2007b). MTA family of coregulators in nuclear receptor biology and pathology. *Nucl Recept Signal* **5**: e010.

Moon EJ, Jeong CH, Jeong JW, Kim KR, Yu DY, Murakami S *et al*. (2004). Hepatitis B virus X protein induces angiogenesis by stabilizing hypoxia-inducible factor-1alpha. *FASEB J* **18**: 382–384.

Pahl HL. (1999). Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* **18**: 6853–6866.

Ray RB, Ray R. (2001). Hepatitis C virus core protein: intriguing properties and functional relevance. *FEMS Microbiol Lett* **202**: 149–156.

Ryu SH, Chung YH, Lee H, Kim JA, Shin HD, Min HJ *et al*. (2008). Metastatic tumor antigen 1 is closely associated with frequent postoperative recurrence and poor survival in patients with hepatocellular carcinoma. *Hepatology* **47**: 929–936.

Schreiber E, Matthias P, Muller MM, Schaffner W. (1989). Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* **17**: 6419.

Slagle BL, Zhou Y-Z, Butel JS. (1991). Hepatitis B virus integration event in human chromosome 17p near the p53 gene identifies the region of the chromosome commonly deleted in virus-positive hepatocellular carcinomas. *Cancer Res* **51**: 49–54.

Su F, Schneider RJ. (1996). Hepatitis B virus HBx protein activates transcription factor NF-kappaB by acting on multiple cytoplasmic inhibitors of rel-related proteins. *J Virol* **70**: 4558–4566.

Toh Y, Pencil SD, Nicolson GL. (1995). Analysis of the complete sequence of the novel metastasis-associated candidate gene, mta1, differentially expressed in mammary adenocarcinoma and breast cancer cell lines. *Gene* **159**: 97–104.

Weil R, Sirma H, Giannini C, Kremsdorf D, Bessia C, Dargemont C *et al*. (1999). Direct association and nuclear import of the Hepatitis B virus X protein with the NF-B inhibitor IB. *Mol Cell Biol* **19**: 6345–6354.

Yoo YG, Na TY, Seo HW, Seong JK, Park CK, Shin YK *et al*. (2008). Hepatitis B virus X protein induces the expression of MTA1 and HDAC1, which enhances hypoxia signaling in hepatocellular carcinoma cells. *Oncogene* **27**: 3405–3413.

Yun C, Um HR, Jin YH, Wang JH, Lee MO, Park S *et al*. (2002). NF-kappaB activation by hepatitis B virus X (HBx) protein shifts the cellular fate toward survival. *Cancer Lett* **184**: 97–104.

Zhang X, Zhang H, Ye L. (2006). Effects of hepatitis B virus X protein on the development of liver cancer. *J Lab Clin Med* **147**: 58–66.

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