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The HBx protein of hepatitis B virus confers resistance against nucleolar stress and anti-cancer drug-induced p53 expression

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ABSTRACT

The nucleolus is a stress sensor associated with cell cycle progression and a viral target. However, the role of the nucleolus during hepatitis B virus infection has not been studied. Here we show that under nucleolar stress, the HBx oncoprotein down-regulates p53 and p21^{waf1} levels by disrupting the interaction between ribosomal protein L11 and MDM2. Further, HBx inhibited Act D-mediated down-regulation of proliferative factors such as c-Myc and cyclin E and revived RNA pol I-dependent transcription under these conditions. Importantly, HBx also countered the action of anticancer drug Paclitaxel suggesting its possible role in drug resistance. Thus, HBx not only can facilitate cell proliferation under stress conditions but can confer resistance against anticancer drugs.

Structured summary of protein interactions:
RPL11 physically interacts with **HBx** and **MDM2** by anti bait coimmunoprecipitation (View interaction)
MDM2 physically interacts with **HBx** by anti bait coimmunoprecipitation (View interaction)
p53 physically interacts with **HBx** by anti bait coimmunoprecipitation (View interaction)

1. Introduction

Nucleolus is a non-membranous highly organised sub nuclear structure that is the seat of ribosomal RNA (rRNA) transcription and hub of ribosome biogenesis [1]. More recently, nucleolus is being linked to stress sensing and control of cell cycle progression [2,3]. Activation of p53–MDM2 regulatory loop by ribosomal proteins (RPs) plays an important role in mediating nucleolar stress and imposes cell cycle arrest and apoptosis. Nucleolar/ribosomal stress induced by diverse means such as overexpression or knock down of nucleolar proteins, treatment with actinomycin D (Act D) or anisomysin, serum starvation and contact inhibition [2–4] are reported to induce sequestration of MDM2 and inhibition of its ubiquitin ligase activity against p53 [1,5]. Importantly, RPs such as RPL11, RPL5 and RPS27a also help in maintaining a balance between rRNA transcription and ribosome biogenesis. Conditions that halt rRNA transcription also lead to imbalance in rRNA/RP ratio. The free pool of RP leaks out of nucleoli and inhibit MDM2 activity leading to stabilisation of p53 and stalling of cell proliferation [5].

Nucleolus is a potent target of viral infections [4,6]. All DNA, RNA as well as retro-viruses have evolved mechanisms to overcome the nucleolar stress response that seems to give survival advantage to the infected cells by averting clearance by the host immune system [6]. Further, this allows viruses to complete their life cycle [7]. The HBx protein of hepatitis B virus (HBV) has been linked with hepatocyte's ability to overcome various stress and facilitate cell cycle progression [8]. However, the exact mechanism employed by HBx to counter stress response still remains elusive. Today drug resistance in cancer therapy remains a major public health problem [9]. In this regard, constantly evolving nature of viruses owing to their error prone polymerase often makes drug therapy a failure due to emergence of viral strains. This is further complicated by the presence of viral structural or regulatory proteins that can confer resistance to both anti-viral and anti-cancer drugs which makes the removal of affected cells difficult [10,11]. Problems of drug resistance in the HBV-related tumours have been reported [12] but the molecular mechanism behind it still remains unexplained. Here, we show that viral HBx can inhibit p53 activation mechanisms in cells under nucleolar stress or during treatment with anti-cancer agents. Further, we expedite the role of HBx in conferring drug resistance to HBV infected cells.

Abbreviations: Act D, actinomycin D; ARPP, acidic ribosomal phosphoprotein P0; CHX, cyclohexamide; DMSO, dimethyl sulphoxide; HBV, hepatitis B virus; HBx, X protein of HBV; IHH, immortalised human hepatocytes; IP, immunoprecipitation; RPs, ribosomal proteins; RT-qPCR, real time quantitative PCR; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; WB, Western blotting

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2. Materials and methods

2.1. Cell culture and transfection

The immortalised human hepatocytes (IHH) were kindly provided by F. Danniel, while HepG2.2.15 cells were gift from Dr. S. Kamili. HepG2 (HB-8065) were procured from ATCC, USA. Huh7 and other cells were maintained and transfected as described earlier [13].

2.2. Plasmids chemicals and antibodies

HBx expression vector was described earlier [13]. Flag tagged RPL11 construct was a kind gift from K. Vousden [14]. All antibodies were procured from Santa Cruz Biotechnologies (USA). Act D, Paclitaxel and Doxorubicin were from Sigma Aldrich (USA). Drugs were added 24 h post transfection at a concentration of 5 nM for next 24 h.

2.3. RNA isolation and RT-qPCR

Total RNA isolation, reverse transcription and RT-qPCR have been described previously [15]. Acidic ribosomal phosphoprotein P0 (ARPP) or GAPDH were used as internal control for pol I and Pol II transcripts respectively. Analysis was performed with the ΔΔCt method [15]. Primer list is given in Supplementary Table S1.

2.4. Western blot and immunoprecipitation

Cells were lysed in lysis buffer (Cell Signaling Technology, MA) and immunoprecipitated or western blotted as described earlier [13]. The protein band intensities were determined by densitometry using NIH ImageJ software.

2.5. Determination of protein half-life

The Cells grown in 60 mm plates and treated with DMSO or 5 nM Act D for 16 h followed by treatment with protein translation inhibitor, cycloheximide (CHX) (30 μg/ml) in the continued presence of DMSO or Act D. Cell extracts were analysed by Western blotting.

2.6. Statistical analysis

The statistical significance of results was calculated with Student's *t*-test. A *P*-value of <0.05 was considered to be significant.

3. Results

3.1. HBx inhibits down-regulation of c-myc mRNA levels under nucleolar stress

Ribosomal proteins (RP) are crucial regulators of nucleolar stress induced p53 activation. Nucleolar stress leads to leakage of RP from nucleolus where they activate important checkpoints. RPL11 interacts and inhibits the function of c-Myc, transcription factor during rRNA transcription [16]. RPL11 can also destabilise *c-myc* mRNA by recruiting ago2-miRNA complexes to *c-myc* 3′ UTR [17]. Therefore, we checked *c-myc* mRNA levels in cells under HBx microenvironment. IHH cells treated with Act D were checked for *c-myc* mRNA levels by RT-qPCR. Act D treatment led to a significant reduction ($P < 0.05$) in the transcript levels as compared to control. However, the repressive effect of Act D on c-*myc* transcripts was abrogated in the presence of HBx (Fig. 1A) which would otherwise elicit apoptotic response and translate nucleolar stress into cell cycle arrest. HBx alone only marginally increased *c-myc* mRNA levels. To evaluate the RPL11 in these conditions, we overexpressed RPL11 in the absence or presence of Act D. As expected, this led to a significant reduction in the levels of *c-myc* transcripts like the control cells treated with Act D (Supplementary Fig. S1). We also checked the c-Myc protein levels in HepG2.2.15 cells that constitutively expresses HBx gene. c-Myc levels remained unaltered in HepG2.2.15 cells upon Act D treatment but declined in HepG2 cells (Fig. 1B). Thus, the presence of HBx seems to confer resistance to nucleolar stress.

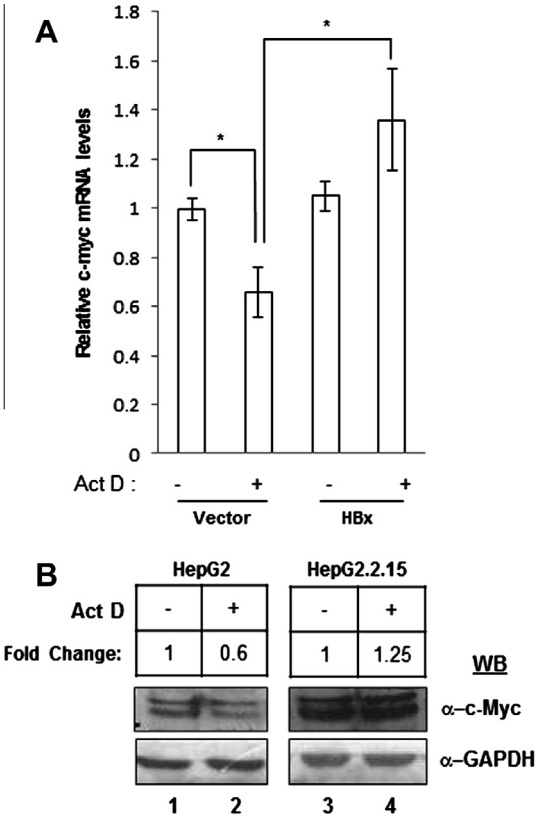

Fig. 1. Levels of *c-myc* under nucleolar stress. (A) IHH cells transfected either with control or HBx expression vectors were treated with DMSO or 5 nM Act D and analysed for *c-myc* mRNA levels by RT-qPCR. The Ct values were normalised against GAPDH. (B) HepG2 or HepG2.2.15 cells were either treated with DMSO (lanes 1 and 3) or 5 nM Act D (lanes 2 and 4) followed by WB for c-Myc. *Statistically significant at $P < 0.05$.

3.2. HBx interferes with stabilization of p53 under nucleolar stress

To understand the mechanism of HBx-dependent nucleolar stress response, we checked levels of key cell cycle regulator protein p53. The Act D-treated HepG2.2.15 cells showed low levels of p53 while HepG2 cells exhibited p53 accumulation (Fig. 2A). Under these conditions, MDM2 levels were higher in HepG2.2.15. Since, the post-translational modifications of MDM2 is well known to regulate its intracellular localisation, stability and activity [18], we next checked the phosphorylation status of MDM2. Decreased levels of pMDM2 (Thr-218) in HepG2.2.15 cells as compared to HepG2 cells suggested a sustained interaction with p53 in HepG2.2.15. Since, MDM2 is also a transcriptional target of p53, its accumulation under nucleolar stress was indicative of a post-transcriptional regulatory mechanism operational in HepG2.2.15 cells. Next we compared the stability of p53 and MDM2 in these cells. There was a marked increase in stability of