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Chapter 6

The hepatitis B virus protein X counteracts the viral restriction factor talin-1

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Submitted for publication
Abstract

The hepatitis B virus (HBV) expresses one accessory protein called X (HBx) that transactivates HBV RNA transcription and is essential for viral replication in vivo. HBx interacts with the host protein DDB1, and may utilise the DDB1 associated ubiquitin ligation machinery to induce the proteasomal degradation of a viral restriction factor. To identify this HBV replication restricting factor, we co-immunoprecipitated HBx interacting proteins using a biologically inactive GFP-HBx fusion construct in the presence and absence of wildtype HBx. Thereby we identified 2376 HBx interacting proteins, including DDB1 and other previously described HBx interacting proteins. The amount of talin-1 (TLN1) that precipitated with GFP-HBx was markedly reduced in the presence of wildtype HBx. We show that TLN1 is indeed proteasomally degraded upon HBx expression. TLN1 links the cytoplasmatic tails of β-integrins to the actin cytoskeleton and is critically involved in the formation of focal adhesions. Disrupted focal adhesion kinetics have previously been associated with HBx expression. We show that TLN1 overexpression neutralizes the transcriptional transactivation by HBx but does not affect transcription in absence of HBx. Like HBx expression, shRNA mediated knockdown of TLN1 transactivated transcription. In HepG2 cells, TLN1 knockdown stimulated HBV replication and prevented HBx from further augmenting HBV replication. This indicates that HBx-mediated TLN1 degradation is essential and sufficient to stimulate HBV replication in these cells. Our data show that TLN1 can act as a viral restriction factor that suppresses HBV replication, and that the HBV accessory protein X relieves this restriction by inducing TLN1 degradation.

Importance

Chronic infection with HBV is a mayor health burden, affecting an estimated 240.000.000 people worldwide and causing more than 780.000 deaths per year. Current antiviral therapies are expensive and generally not curative because they fail to affect viral transcription in infected hepatocytes. The HBV accessory protein HBx is essential to maintain viral RNA transcription, and thereby drives the ongoing viral protein production. We show that HBx functions by inducing the degradation of TLN1, a restriction factor that suppresses HBV transcription. Knowledge of this mechanism may aid the development of antivirals that can counteract HBx function and curb the ongoing viral protein production during the treatment of chronic HBV infection.
Introduction

The hepatitis B virus (HBV) is a member of the hepadna virus family, and infects hepatocytes in the human liver. After entry, the core particle releases the partially double stranded HBV genome in the nucleus, where it is subsequently repaired by host enzymes to form the HBV covalently closed circular DNA (cccDNA). This circular DNA template of about 3,200 base pairs encodes the HBV structural core and surface genes, its polymerase, which reverse transcribes the viral pregenomic RNA into the partially double stranded DNA, and one accessory protein called X (HBx). HBx expression is essential to initiate and maintain HBV replication in vivo. The mechanism by which HBx supports HBV replication is unknown. HBx expression affects various processes such as apoptosis, the cell cycle, and DNA repair. In the absence of HBx, viral RNA transcription from the HBV cccDNA is epigenetically silenced. In most hepatoma derived cell lines, such as HepG2 cells, HBx expression stimulates HBV transcription but is not essential. HBx can also transactivate transcription from other circular DNA templates. The capacity to transactivate RNA transcription is related to the in vivo function of HBx and can be used to assess HBx functionality.

For its function in vivo HBx requires an interaction with the damaged DNA binding protein 1 (DDB1). DDB1 can form a complex with Cul4A and an E3 ring ubiquitin ligase that ubiquitinates substrate proteins, which are subsequently degraded by the proteasome. Various viral accessory proteins, such as simian immunodeficiency virus (SIV) vpx, human immunodeficiency virus (HIV) vpr, and paramyxovirus SV5-V protein are known to “hijack” this machinery by acting like a DDB1-Cul4A associated factor (DCAF) in order to specifically degrade a host protein that restricts viral replication.

Reasoning that HBx may also function by inducing the degradation of a host protein that restricts HBV replication, we generated an eCFP-HBx fusion protein that failed to transactivate transcription but still interacted with previously identified HBx binding partners. To identify proteins degraded by HBx, we immunoprecipitated proteins interacting with this eCFP-HBx fusion construct in the presence and absence of HBx expression. Subtractive massspectrometric analysis revealed a strong reduction in the amount of talin-1 (TLN1) precipitating with eCFP-HBx in the presence of wildtype HBx. Subsequent analysis showed that TLN1 was indeed degraded by HBx and that TLN1 suppressed HBV replication by interfering with HBV RNA transcription.

Materials and Methods

Cell culture and transfection

HEK 293 and HEK 293T cells were maintained in Dulbecco’s Modified Eagle Medium without HEPES (DMEM) supplemented with 10% heat inactivated fetal calf serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) (Gibco Pen Strep). HepG2 cells were maintained in William’s Medium E w/o L-Gln (LONZA, Basel, Switzerland), supplemented with 10% v/v inactivated fetal calf serum, 2 mM L-glutamin (LONZA, Basel, Switzerland), penicillin (100 U/mL), streptomycin (100 µg/mL) and 5 uM...
Dexamethasone (Sigma Aldrich). HEK 293T and HepG2 cells were maintained in a humidified 10% CO2 incubator at 37°C and HEK 293 cells were maintained in a humidified 5% CO2 incubator at 37°C. We used HEK 293 cells in the experiments in which transactivation of HBx was studied to prevent episomal replication of the vectors by the large T antigen present in HEK 293T cells. For subsequent Western blotting experiments we used HEK 293T cells because of their higher transfection efficiency. Twenty-four hours before transfection the cells were plated into 6- or 96-well culture plates. The calcium phosphate method was used for transfection. Briefly, plasmid DNA was diluted in 42 mM HEPES pH 7.2 and 2.5 M CaCl2 was added to a final concentration of 0.15 M CaCl2. The DNA mixture was added to an equal volume of 2x HEPES buffered saline (HBS)(275 mM NaCl, 10 mM KCl, 1.4 mM Na2HPO4, 42 mM HEPES pH 7.2) and after a 15 minute incubation at room temperature the mixture was added to the cells. Cells were incubated overnight in a humidified 3% CO2 incubator at 37°C and subsequently the medium was replaced. Luciferase activity was determined 48 hours after transfection.

Expression vectors and lentiviral transduction

The pHSV-HBx vector expressing HSV-tagged HBx was previously generated and described. This vector was used to generate pHSV-HBx.R96E using targeted mutagenesis (QuickChange II XL Site-Directed Mutagenesis Kit, Bio connect (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer’s instructions. The primer pairs used were HBx.R96E-Fwd (5’-CCCAAGGTCTTTACATAAGGAGACTTCGAGTCCCAACG-3’) and HBx.R96E-Rev (5’-GCTGGGAGTCCAAGAGTCTTATGTAAAGACCTTGGG-3’). As a vector control, the empty pcDNA 3.1 A(-)(Invitrogen, Carlsbad, CA, USA) was used. For the construction of plasmids expressing the fusion protein eCFP-HBx-HSV, HBx was cloned by PCR from the pHSV-HBx construct using primers containing an EcoR1 restriction site in the forward primer and a Kpn1 restriction site in the reverse primer. The PCR product was cloned in-frame with eCFP in pcDNA 3.1A (-) vectors. The HBV replication assay was previously described in detail. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pBlue3LTR-luc from Dr. Rienk Jeeninga and Dr. Ben Berkhour. The PDE-Luc constructs, expressing luciferase under control of the human PDE8A promoters was previously described. To generate the hEF1α-Luc construct, expressing luciferase under control of the human elongation factor 1 promoter, the luciferase gene from the LTR-Luc construct was amplified using the primers FW_Luciferase_BamHI (5’-GATAGGATCCATGGAGGAGCAGCAGA-3’) and REV-Luciferase-Kpn1 (5’-CTATGGTACCTTACACGGCGATCTTCC-3’). The amplification product was digested with BamHI and Kpn1 and ligated after the hEF1α promoter in a lentiviral vector digested with the same enzymes. The TG-Luc construct bearing a luciferase reporter under control of the thyroglobulin promoter was a kind gift of Dr. A. Ilgun. The R9 vector, which contains a 1.2 x overlength HBV DNA genome (subtype adw) in a pGEM 7zf+ backbone was kindly provided by Dr. Baumert. The GFP-TLN1 construct (Addgene plasmid 26724) was derived from Addgene and was previously described. The TLN2 expressing construct was a kind gift of Dr. Iwamoto. The primers to
generate the luciferase reporters under control of the different HBV promoters were adapted from Du et al.\textsuperscript{36} to make them suitable to the HBV adw subtype. Using these primers, the different promoter and Enhancer I sequences were amplified by PCR from the R9 vector. The primers used to amplify the HBV promoters and the HBV Enhancer I sequence were: Corepromoter-F: 5'-CCCGAGCCTCCAAGGTCTTAATAAG-3'; Corepromoter-R: 5'-CCCAAGCTTGGAGCTGGAACAGT-3'; S1promoter-F: 5'-CCCGAGCTTGCTCTTACTTTTGAAG-3'; S1promoter-R: 5'-CCCAAGCTTATAAGAATACCCAGCC-3'; S2promoter-F: 5'-CCCAAGCTTTTGCTCACCATA-3'; S2promoter-R: 5'-CCCGAGCTCTGGAGACCTTTTGT-3'; Xpromoter-F: 5'-CCCGAGCTCTGGAGACCTTTTGT-3'; Xpromoter-R: 5'-CCCAAGCTTGGAACAGTATATT-3'; HBVEnhancer1-F: 5'-GOGGTTACCGTATAAGCTAAACA-3'; HBVEnhancer1-R: 5'-CCCGAGCTCTGGAGACCTTTTGT-3'. The PCR products containing the HBV promoters were cut with SacI and HindIII and ligated in front of the luciferase reporter of the pGL3Basic vector (Promega). The constructs that also contained the HBV Enhancer I sequence were generated from these constructs by cloning of HBV Enhancer I containing PCR product cut with KpnI and SacI in the respective vectors cut with the same enzymes. All constructs were validated by sequencing the BigDye Terminator v1.1 Cycle Sequencing kit (ABI Prism, Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol on a 3730xl DNA analyser (Applied Biosystems). Sequencing conditions were 5 min at 94 °C, 45 cycles of 15 s at 94 °C, 10 s at 50 °C, 2 min at 60 °C, and a 10 min extension at 60 °C. The shRNAs targeting TLN1 (#1: TRCN0000123104, #2: TRCN0000123105 and #3: TRC0000123107) and the control shRNA (SHC001) in a pLKO.1 backbone were derived from the MISSION\textsuperscript{TM} TRC-Hs 1.0 library (Sigma-Aldrich St. Louis, MO, USA). shRNA #1 and control shRNA were also used to generate the VSV-G-pseudotyped lentiviruses by cotransfection of 1 µg of these constructs together with 0.67 µg pCMV-VSV-G, 0.47 µg pREV and 1.22 µg pMDL per well in a 6 well plate. 24 hours after the medium change the supernatant was harvested and filtered. HepG2's were transduced in 6 well plates for 24 hours. Subsequently the medium was changed and two days later the cells were reseeded for transfection experiments.

**Luciferase assay**

Luciferase activity was measured 48 hours after transfection by adding 25 µl substrate (0.83 mM ATP, 0.83 mM D-luciferin (Duchefa, Haarlem, The Netherlands), 18.7 mM MgCl\textsubscript{2}, 0.78 µM Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}, 38.9 mM Tris pH 7.8, 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100, and 2.6 µM dithiothreitol) directly to the culture medium. Luminescence was measured for 1 s per well using a luminometer (Berthold, Bad Wildbad, Germany). All experiments were performed in triplicate. The amount of DNA transfected per well was kept constant by cotransfection of our vector control.
qPCR and quantification of HBV DNA in core particles

To assess the effect of HBx and the different shRNA constructs on PDE8A and TLN1 mRNA, HEK 293T cells were transfected in a 6 well plate with 200 ng per well of a vector control or pHSV-HBx. Forty-eight hours after transfection, total RNA was purified using TriPure Isolation Reagent (Roche, Basel, Switzerland) according to the manufacturer’s instructions. cDNA was prepared using random hexamers (Invitrogen) and 200 U M-MLV reverse transcriptase (Promega, Madison, WI, USA) in the presence of 20 U RNAse inhibitor (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Subsequently, 2 µl of cDNA was used in a quantitative PCR using the SYBR Green I Master (Roche) and a LightCycler® 480 system (Roche). PDE8A cDNA was quantified relative to β-actin cDNA using the primers TLN1-F: 5'- CTGGTGCAGAGCTGCAAGGC-3’ and TLN1-R: 5’ CACCTAACCAGCTGATGC3’ for TLN1, PDE8A-1 F: 5’-CGTTTTATACGTATGCAAATCCT-3’ and PDE8A-1 R: 5’-GCTTTGACGCTGCAAGGC-3’ for PDE8A and BA-F: 5’-GGCCCAGTCTCTCCCCAAGTCCAC-3’ and BA-R: 5’-GGTAAGCCCTGGCTGCCTCCACC-3’ for β-actin. The following program was used for qPCR: 10 min 95ºC, followed by 50 cycles of 10 sec 95ºC, 20 sec 59ºC, 30 sec 72ºC with a single acquisition during the 72ºC step. The quantification of HBV DNA in core particles was previously described in detail. Briefly, HBV replicating HepG2 cells were lysed in isosmotic lysis buffer. After removal of cell nuclei and debris, lysates were treated with DNase to remove non-encapsidated DNA before HBV DNA was quantified.

Western blotting

For Western blotting, proteins were immunoprecipitated proteins or cells were treated as indicated and subsequently dissolved in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with Complete® EDTA free protease inhibitor (Roche). Immunoprecipitated proteins or treated cell lysates were loaded on a 10% Bis-Tris gel (NuPAGE 10% Bis–Tris precast gel) together with the Odyssey Protein Weight Marker (LI-COR, Lincoln, NE, USA) and separated by electrophoresis using MES SDS running buffer (Invitrogen). Subsequently, gel separated proteins were blotted on a nitrocellulose membrane (Protran, Schleicher & Schuell, Dassel, Germany; 2 hours 150V) using NuPAGE transfer buffer. Blots were stained overnight at 4°C in PBS (Gibco) supplemented with 0.1 % Tween 20 (Merck) and 5% BSA (United states biochemical corp., Cleveland Ohio). The following antibodies were used to visualize proteins: mouse monoclonal anti DDB1 (1:1000; BD transduction laboratories); SC-1616 anti-β-actin antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA); Mouse monoclonal anti-non-muscle Myosin IIA antibody (1:1000; Abcam ab55456); mouse monoclonal anti talin-1 (1:200, Abcam [93E12] ab104913); Rabbit monoclonal anti talin-2 antibody (1:10,000, Abcam ab108967). IRDye 800CW conjugated goat anti-mouse IgG or 680LT conjugated donkey anti rabbit (1:15,000; 926–32210, LI-COR, Lincoln, NE, USA) were used as secondary antibodies to visualize expression using the Odyssey infrared image system (LI-COR).
**Immunoprecipitation and mass spectrometry**

HEK 293 cells were seeded in 6 well plates and transfected with 1 µg of eCFP-HBx expressing vector per well, and cotransfected with 500 ng of empty vector or HSV-HBx expressing vector. 48 hours after transfection cells from three 6 well plates per condition were harvested by trypsin digestion, the cells were pelleted at 400 g for 10 minutes in 50 ml PBS. The cells were lysed in one ml of lysis buffer L (20 mM Heps, 0.5 M NaCl, 1 mM EDTA, 0.25% Triton X-100, 1 mM EGTA supplemented with Complete® EDTA free protease inhibitor (Roche). Immunoprecipitations were performed using a polyclonal antibody against GFP (1 µl per IP, AB290, BIO connect) and 25 µl ProtG beads (Thermo scientific). After an overnight incubation at 4ºC rotating, the beads were washed 4 times with IP buffer (25 mM Tris, 150 mM NaCl, pH 7.2) supplemented with protease inhibitor (Roche). Immunoprecipitated proteins were dissolved in 1x sample buffer (NuPAGE LDS loading buffer, Life Technologies) supplemented with 0.1 M DTT, incubated for 5 min at 95ºC, separated on a 4%-12% NuPAGE Novex Bis-Tris gel (Life Technologies) and visualized using Coomassie Brilliant Blue (CBB) staining (Imperial Protein Stain, Thermo Scientific). Each gel lane was cut in eight slices and proteins were in-gel digested with trypsin (37). Peptides were loaded onto Empore-C18 StageTips38, eluted with 80% ACN, 0.5% acetic acid and analysed by mass spectrometry.

**Mass spectrometry data acquisition**

Digested peptides were loaded onto a C18 pre-column (Acclaim Pepmap100, 75 µm × 2 cm, C18 3 µm 100 Å, Thermo Scientific) and separated by a C18 analytical column (Acclaim Pepmap RSLC, 75 µm × 15 cm, C18 2 µm 100 Å, Thermo Scientific) coupled online to a linear trap quadrupole (LTQ)-Orbitrap XL-ETD (Thermo Scientific) via a nanoelectrospray ion source (Nanospray Flex Ion Source, Thermo Scientific) with a spray voltage of 2.0 kV. Peptides were loaded for 10 min at 4% mobile phase (80% ACN, 0.5% acetic acid) and eluted by increasing the mobile phase from 4-30% (10-110 min) and 30-60% (110-135 min), followed by a 5 minute wash to 95% and a 10 min regeneration at 4%. Full scan MS spectra were acquired in the Orbitrap analyser with a resolution of 60,000 at m/z 400, and a target value of 1,000,000 charges. The 5 most intense precursor ions in the full scan with a charge state of 2+ or higher were selected for collision-induced dissociation (CID) using an isolation width of 2 Da, a 30% normalized collision energy and an activation time of 30 ms. CID spectra were acquired in the linear ion trap. All data were acquired with Xcalibur software.

**Mass spectrometry data analysis**

The RAW MS files were analysed using the MaxQuant computational platform, version 1.3.6.039 and Proteome Discoverer 1.4 software (Thermo Scientific). For Maxquant analysis, proteins and peptides were identified using the Andromeda search engine by querying the human Uniprot database (release-2012 01, 81,213 entries)40 enabling the ‘match between run’
option. To search for precursor and fragment ions, an initial maximal mass deviation of 20 ppm and 0.5 Da, respectively, was required. Trypsin with full enzyme specificity and only peptides with a minimum length of 7 amino acids were selected. A maximum of two missed cleavages was allowed. Carbamidomethylation (Cys) was set as fixed modification, while Oxidation (Met) and N-acetylation as variable modifications. For protein and peptide identification, we required a maximum false discovery rate (FDR) of 1%. Reverse and contaminant hits were eliminated from the output files. For Proteome Discoverer analysis, peptides were identified using the SEQUEST HT search algorithm by querying the human Uniprot database uniprot-organism-9609-AND-keyword-kw-0181.fasta. In the SEQUEST HT search a precursor mass tolerance of 10 ppm was allowed. Fragment mass tolerance was 0.6 Da. Trypsin with full enzyme specificity and only peptides with a minimum length of 7 amino acids were selected. A maximum of two missed cleavages was allowed. Carbamidomethylation (Cys) was set as fixed modification, while Oxidation (Met) and N-acetylation as variable modifications. Peptide spectral matches (PSM) were validated using percolator based on q-values at a 1% FDR. With Proteome Discoverer, peptide identifications were grouped into proteins according to the law of parsimony and filtered to 1% FDR.

Software and statistics

Data from luciferase experiments and qPCR analysis were analysed using GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA, USA). Significance of differences in luciferase activity and mRNA expression were determined by two-sided T testing.

Results

HBx transactivates transcription by inducing the degradation of a host protein

Although HBx expression is essential for in vivo HBV replication, the virus can replicate in HepG2 cells in the absence of HBx. We induced HBV replication in HepG2 cells by transfection with the R9ΔHBx vector, which contains a 1.2 x overlength head-to-tail HBV genome lacking functional HBx expression. The absence of HBx resulted in lower viral replication as compared to wild type R9 HBV. HBV replication could be restored by cotransfection of a construct expressing HBx, showing that HBx protein expression augments viral replication in this in vitro model (Fig. 1A). It has been described that HBx can act as a broad transactivator in vitro, and that the capacity to transactivate is related to its in vivo function. To investigate under which conditions HBx can transactivate transcription in HEK 293 cells, we transfected HEK 293 cells with luciferase reporters. Cotransfection of an HBx expressing construct markedly increased the transcriptional activity of luciferase reporters under control of the endogenous HBV promoters (Fig. 1B) and several unrelated promoters (Fig. 1C). Transactivation by HBx did not depend on the promoters’ basal activity (S1A Fig.) or the presence of HBV enhancer 1 (S1B Fig.), and maximum transactivation was already reached by the lowest HBx concentration tested (S1C-E Figs.). HBx was able to interact with
DDB1 in HEK 293 cells (Fig. 1D). The well described HBx.R96E mutant, which does not interact with DDB1, failed to transactivate luciferase reporters in HEK 293 cells (Fig. 1b S1B-E Figs.). HBx also failed to transactivate promoters in the presence of proteasome inhibitor MG132, indicating that proteasomal degradation is required for transactivation by HBx (Fig. 1E). While HBx efficiently transactivated a phosphodiesterase 8A (PDE8A) promoter driven luciferase reporter (Fig. 1C), PDE8A mRNA transcription from the chromosomal promoter was not affected by HBx (Fig. 1F). These findings indicate that the transcriptional transactivation by HBx in HEK 293 cells involves the proteasomal degradation of a host protein.

Figure 1. HBx transactivates transcription by inducing proteasomal degradation of a host protein. (A) HBV replication was initiated in HepG2 cells by transfection of a construct containing a 1.2 fold overlength HBV genome that initiates the full HBV replication cycle. The replication of a mutant lacking HBx was impaired but could be rescued by cotransfection of a construct that expresses HBx. (B,C) Activity of luciferase reporter constructs, cotransfected with plasmids expressing HBx or the HBx.R96E mutant that does not interact with DDB1, into HEK 293 cells 48 hours after transfection. The luciferase reporters were under control of the hepatitis B core (c), X (x), S1 (S1) and S2 (S2) promoters, or the HIV-1 LTR (LTR), the human elongation factor 1α (hEFG1α), the phosphodiesterase 8A (PDE8A), or thyroglobulin (TG) promoters. (D) eCFP-HBx was expressed in HEK 293-T cells and lysates were immunoprecipitated with anti GFP. A Western blot of SDS-page separated proteins was stained with anti-DDB1, showing that in these cells HBx interacts with DDB1. (E) Activity of the HBV core promoter 48 hours after cotransfection with HBx in the absence and presence of proteasome inhibitor MG132, showing that transactivation by HBx critically depends on proteasomal degradation. (F) mRNA production from the chromosomal PDE8A promoter in the absence and presence of HBx reveals that transactivation by HBx is specific for extrachromosomal templates. *: P<.05, **: P<.01, ***: P<0.001
HBx induces the degradation of talin-1

To identify the host protein that is degraded upon interaction with HBx, we employed immunoprecipitation using an HBx - enhanced Cyan Fluorescent Protein fusion protein (eCFP-HBx). We noticed that while this eCFP-HBx construct could still co-precipitate the HBx interacting protein DDB1(Fig. 1D), it failed to transactivate transcription in HEK 293 cells (data not shown). As eCFP is large as compared to HBx, we speculate that the eCFP-HBx fusion protein would still interact with the protein targeted for degradation, but that steric hindrance of the GFP protein may prevent the formation of the HBx-DDB1-Cul4 complex required to induce its proteasomal degradation. To identify the host protein that is specifically degraded by wild type HBx, eCFP-HBx was expressed in HEK 293 cells in the presence and absence of wild type HBx. Cells were lysed and eCFP-HBx co-precipitating proteins were separated by SDS-page and visualized by colloid blue staining, but we did not observe an eCFP-HBx interacting protein that was specifically degraded in the HEK 293 cells expressing wild type HBx (Fig. 2A). Next, we analysed all proteins that precipitated with eCFP-HBx using mass spectrometry. Peptides from 2376 (MaxQuant analysis, S1 Table) and 2885 (Proteome Discoverer analysis, S2 Table) proteins were identified. From the identified proteins heat shock 70 kDa protein 1A/1B (HSP1A/HSP1B), which was previously described to interact with HBx[1], was the protein most abundantly co-precipitated with eCFP-HBx in both conditions (S2A Fig.), showing that eCFP-HBx indeed has not lost the capacity to interact with previously described HBx interacting proteins. We observed that for several proteins the number of identified peptides was enriched in the immunoprecipitation in the absence of wild type HBx (Fig. 2B and S2B Fig.). This suggested that these proteins are possible targets for HBx-DDB1 mediated degradation. The three proteins that were most enriched in the absence of wild type HBx and for which more than 10 unique peptides were detected, were selected for further analysis: myosin heavy chain 9 (MYH9), talin-1 (TLN1) and elongation factor Tu, mitochondrial (TUFM).
Figure 2. HBx induces proteasomal degradation of talin-1. (A) HEK 293 cells were transfected with eCFP-HBx (left), or eCFP-HBx + wild type HBx and lysed 48 hours post transfection. eCFP-HBx was immune precipitated and interacting proteins were separated by SDS-page and visualized using colloidal blue staining. (B) Scatter plots of the ratio of the number of unique peptides in the immunoprecipitation in the absence/presence of HBx as a function of the number of unique peptides in the immunoprecipitation in the presence of HBx, based on MaxQuant analysis of mass spectrometry data. (C) Western blot analysis of lysates of HEK 293T cells stained with antibodies against the indicated proteins. HBx expression resulted in degradation of TLN1 but did not affect MYH9 or TUFM expression. (D) HBx expression induced TLN1 degradation in a dose-dependent manner. (E) HEK 293-T cells were transfected with constructs expressing either HBx or a short hairpin RNA (shRNA) against TLN1. While HBx expression did not affect TLN1 mRNA production, expression of the shRNA against TLN1 resulted in mRNA degradation. (F) Western blot analysis of lysates of HEK 293T cells stained with antibodies against the indicated proteins. HBx-mediated degradation could be prevented by adding proteasome inhibitor MG132. The HBx.R96E mutant did not affect TLN1 levels.

We observed that wild type HBx expression in HEK 293T cells did not induce the degradation of MYH9 or TUFM, indicating that wild type HBx efficiently competed with eCFP-HBx for binding to MYH9 and TUFM, but that wild type HBx did not induce their proteasomal degradation (Fig. 2C). When we expressed HBx in HEK 293T cells, we observed a marked reduction in TLN1 protein levels (Fig. 2C). Further research indicated that HBx induced TLN1 degradation in a dose-dependent manner (Fig. 2D), without affecting the TLN1 mRNA expression levels (Fig. 2E). Expression of the HBx.R96E mutant did not affect TLN1 levels and addition of proteasome inhibitor MG132 prevented HBx-mediated TLN1 degradation (Fig. 2F), confirming that HBx needs to interact with DDB1 to induce proteasomal degradation of TLN1. In addition, the levels of talin-2 (TLN2), which is highly homologous to TLN1 (74% amino acid sequence identity), were not affected by HBx expression (S3A Fig.).

Talin-1 suppresses HBV replication by interfering with RNA transcription

Next, we analysed whether TLN1 is indeed the host protein restricting the transcription from our luciferase reporters in HEK 293 cells. To reduce TLN1 protein levels, HEK 293 cells were transfected with plasmids expressing shRNAs targeting TLN1. shRNA expression induced a strong reduction of TLN1 mRNA (Fig. 2E) and protein levels (Fig. 3A) 48 hours post transfection. Like HBx expression, shRNA mediated reduction of TLN1 levels markedly increased transcription from luciferase reporters under control of the HBV core- (Fig. 3B), HBV X- and human PDE8A promoters (S3B and C Figs.). Conversely, overexpression of a GFP-TLN1 fusion construct prevented HBx mediated transactivation of luciferase reporters under control of the HBV core (Fig. 3C), and human PDE8A promoters (S3D Fig.) in a dose dependent manner. This effect was highly specific for HBx expression, as GFP-TLN1 overexpression did not affect transcription from these reporters in the absence of HBx. In contrast, overexpression of TLN2 did not affect HBx-mediated transactivation of a luciferase reporter under control of the HBV core promoter (Fig. 3D). To assess the effect of TLN1 on the full HBV replication cycle, we generated a lentiviral vector to express TLN1 shRNA #1.
HepG2 cells were transduced with this lentiviral vector, resulting in a decrease in TLN1 mRNA (S3E Fig.). Upon induction of HBV replication by transfection with the R9ΔHBx vector, the TLN1 knockdown cells produced five-fold more HBV DNA in core particles (Fig. 3E). Replication of HBV in the presence of functional HBx also increased in the TLN1 knockdown cell-line, indicating that the low level of HBx produced by the wild type virus accounts for restricted TLN1 degradation, enough to support HBV replication. In TLN1 knockdown cells, HBx was unable to augment HBV replication, showing that TLN1 degradation is essential and sufficient for the function of HBx.
Discussion

Expression of the HBV accessory protein X is required for in vivo HBV replication. HBx interacts with DDB1, a host protein often “hijacked” by viral accessory proteins to induce the degradation of a host protein that interferes with viral replication. We observed that in HEK 293 cells, HBx-mediated transcriptional transactivation required interaction with DDB1 and proteasomal degradation. By applying subtractive mass spectrometry, we identified TLN1 as an HBx interacting protein that is degraded in the presence of HBx in these cells. Disruption of TLN1 levels by shRNA-mediated knockdown or by HBx expression enhanced transcription driven by viral and non-viral promoters. HBx-transactivated transcription could be restored to basal levels by overexpression of (GFP-)TLN1. In the absence of HBx, transcription was not affected by similar levels of TLN1 overexpression. In HepG2 cells, TLN1 knockdown stimulated HBV replication and prevented HBx from further augmenting viral replication. These data demonstrate that HBx-mediated TLN1 degradation is a crucial factor in the regulation of HBV RNA transcription (Fig. 3F).

Figure 3. Talin-1 interferes with HBV replication by suppressing RNA transcription. (A) Western blot of lysates of HEK 293-T cells 48 hours after transfection with plasmids expressing shRNAs against TLN1, showing that all shRNAs against TLN1 reduce TLN1 protein level. (B) HEK 293 cells were transfected with a luciferase reporter under control of the HBV core promoter, and cotransfected with the plasmids expressing shRNAs against TLN1, showing that TLN1 knockdown efficiently transactivates transcription. (C) Cotransfection of a vector expressing a biologically active GFP-TLN1 fusion protein with a luciferase reporter under control of the HBV Core promoter prevented transactivation by HBx in a dose dependent manner. (D) Activity of a luciferase reporter under control of the HBV Core promoter 48 hours after cotransfection with a construct expressing TLN2. TLN2 overexpression did not affect transactivation by HBx. (E) HepG2 cells were transduced with a lentivirus expressing the shRNA against TLN1. HBV replication was initiated by transfection of the 1.2x overlenght HBV containing R9 vector or its mutant (R9ΔHBx) lacking HBx expression. HBV DNA in core particles in the cytoplasm of the cells was analyzed 7 days post transfection. (F) Schematic representation of HBx function. *: P<.05, **: P<.01, ***: P<0.001

HBx induces TLN1 degradation

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TLN1 links the cytoplasmic domain of β integrins to the actin cytoskeleton and is involved in the formation of focal adhesions. Like HBx, TLN1 has been involved in a broad range of processes such as cell viability and apoptosis, proliferation, migration, signal transduction and transcription. In the cytoplasm, TLN1 forms a compact, autoinhibiting heterodimer that shields the active domains. Upon activation and monomerisation, TLN1 links the cytoplasmic domain of β integrins to the actin cytoskeleton, a fundamental process in the formation of focal adhesions. Notably, it has been observed that HBx expression disrupts the formation of focal adhesions and modulates the activity of the associated focal adhesion kinase.

Several findings suggest that TLN1 can affect transcription, albeit the mechanism has not been defined in detail. In drosophila, TLN1 strongly suppresses the DE-cadherin and shotgun promoters. Interestingly, it was suggested in a comment that “Because talin has not been detected in the nucleus, it may affect transcription by sequestering a transcription factor in the cytoplasm.” In line with this suggestion, interactions at the cell membrane seem fundamentally involved in transcriptional regulation and can have a profound effect on transcriptional regulation. The involvement of TLN1 in HBV replication suggests that the lack of these interactions in vitro may contribute to the large difference between the effects of HBx expression on HBV replication in vitro and in vivo.

Chronic infection with HBV is the major etiological agent associated with hepatocellular carcinoma (HCC), and various lines of evidence suggest that HBx is involved in oncogenic transformation. In HCC, HBx is frequently expressed in chromosomally integrated HBV DNA fragments, and transgenic mice expressing HBx in their livers spontaneously develop HCC. TLN1 is frequently overexpressed in various types of cancer and serum TLN1 levels have been associated with HCC progression. TLN1 expression correlates with HCC dedifferentiation. Notably, HBV replication is inversely correlated to the hepatocyte differentiation state. Possibly, TLN1 mediated suppression of HBV replication involves integrin signaling, as genetic polymorphisms in the integrin αv gene are associated with HBV chronicity and HCC.

TLN1 has previously been implicated to play a role in the replication of other viruses. TLN1 suppresses the replication of retroviruses, although the inhibition is not related to viral transcription. The human cytomegalovirus protein pUL135 was shown to interact with TLN1 and to disrupt the interaction between infected cells and the extracellular matrix. In leukocytes, TLN1 is critically involved in the initiation of adaptive immune responses by activating integrins following cytokine or T cell receptor stimulation. Here, we identified TLN1 as a viral restriction factor that suppresses HBV replication by interfering with viral RNA transcription. HBx relieves this restriction by inducing TLN1 degradation. Knowledge of this mechanism may aid the development of antivirals that interfere with HBx and suppress HBV RNA transcription. Such antivirals may complement existing treatments by suppressing the ongoing viral antigen production in chronically infected patients.
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HCC

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Differentiation state

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References


Supplementary Materials

Figure S1. Transcriptional transactivation by HBx. (A) Basal activity of luciferase reporters under control of the HBV promoters in vectors in the absence and presence of the HBV Enhancer I 48 hours after transfection in HEK 293 cells. (B) Activity of the different HBV promoters in presence Enhancer I 48 hours after cotransfection with HBx or HBx.R96E expressing constructs. (C-E) Activity of the luciferase reporters under control of the HIV-1 LTR (LTR), the human elongation factor 1α (hEF1α) or the phosphodiesterase 8A (PDE8A) promoters respectively 48 hours after cotransfection with HBx or HBx R96E expressing constructs in HEK 293 cells. **: P<.01, ***: P<0.001

Figure S2. Peptide spectral matches of HBx interacting proteins. (A,B) The number of peptide spectral matches identified in the immunoprecipitation in the absence of wild type HBx is plotted versus the number of peptide spectral matches in the immunoprecipitation in the presence of wild type HBx based on Proteome Discoverer analysis of mass spectrometry data.
Figure S3. Talin-1 suppresses transcription and is specifically degraded in the presence of HBx (A) Western blot analysis of TLN2, showing TLN2 levels were not affected by HBx expression. (B,C) HEK 293 cells were transfected with luciferase reporters under control of the HBV X- and human PDE8A promoters, and cotransfected with the plasmids expressing shRNAs against TLN1, showing that TLN1 knockdown efficiently transactivates transcription. (D) Cotransfection of a vector expressing a biologically active GFP-TLN1 fusion protein with a luciferase reporter under control of the HBV X promoter prevented transactivation by HBx in a dose dependent manner. (E) TLN1 mRNA in HepG2 cells 48 hours after transduction with the lentivirus expressing shRNA against TLN1.

Supplementary Table legends

Table S1. Protein groups identified in the immunoprecipitation experiments based on mass spectrometry data analyzed by MaxQuant.

Table S2. Protein groups identified in the immunoprecipitation experiments based on mass spectrometry data analyzed by Proteome Discoverer.