Title: Expression of Pituitary Tumor Transforming Gene 1 (PTTG1)/Securin in hepatitis B virus-associated liver diseases: Evidence for a Hepatitis B virus X protein-mediated inhibition of PTTG1 ubiquitination and degradation.

Authors: Francisca Molina-Jiménez[1,2], Ignacio Benedicto[1,2], Miki Murata[3], Samuel Martín-Vílchez[2,4], Toshihito Seki[3], José Antonio Pintor-Toro[5], María Tortolero[6], Ricardo Moreno-Otero[2,4], Kazuichi Okazaki[3], Kazuhiko Koike[7], José Luis Barbero[8], Koichi Matsuzaki[3], Pedro L. Majano[1,2*], Manuel López-Cabrera[1,2,9*]
*These authors contributed equally to this work.
E-mail: fmolina.hlpr@salud.madrid.org, ibenedicto.hlpr@salud.madrid.org, muratami@takii.kmu.ac.jp, smvilchez@gmail.com, sekit@takii.kmu.ac.jp, jose.pintor@cabimer.es, torto@us.es, rmoreno.hlpr@salud.madrid.org, okazaki@takii.kmu.ac.jp, kkoike-tky@umin.ac.jp, jlbarbero@cib.csic.es, matsuzak@takii.kmu.ac.jp, pmajano.hlpr@salud.madrid.org, mlopez.hlpr@salud.madrid.org.

Affiliations: 1. Unidad de Biología Molecular, Hospital Universitario de la Princesa, Madrid, Spain. **2.** Centro de Investigación Biomédica en Red de enfermedades hepáticas y digestivas (CIBER-ehd), Instituto de Salud Carlos III (ISCIII), Madrid, Spain. **3.** Department of Gastroenterology and Hepatology, Kansai Medical University, Osaka, Japan. **4.** Unidad de Hepatología, Hospital Universitario de la Princesa, Madrid, Spain. **5.** Centro Andaluz de Biología Molecular y Medicina Regenerativa (CABIMER) Sevilla, Spain. **6.** Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain. **7.** Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan **8.** Departamento de Biología Celular y del Desarrollo, Centro de Investigaciones Biológicas (CIB), Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain. **9.** Centro de Biología Molecular Severo Ochoa (CBMSO), CSIC-UAM, Madrid, Spain.

Key words: PTTG1/Securin, hepatitis B virus X protein, hepatocarcinoma, ubiquitination, protooncogene, Skp1–Cul1–F-box (SCF) ubiquitin ligase complex.

Abbreviations: APC/C, anaphase-promoting complex/cyclosome; **CFP**, cyan fluorescent protein; **Dox,** doxycycline; **GFP**, green fluorescent protein; **HBV**, Hepatitis B virus; **HBx**, Hepatitis B virus X protein; **HCC**, hepatocellular carcinoma; **OA**, okadaic acid; **PP2A,** protein phosphatase 2A; **PTTG1**, pituitary tumor transforming gene 1 (or securin); **SCF**, Skp1–Cul1–F-box ubiquitin ligase complex; **SKP**, S-phase kinase-associated protein

Corresponding author: Manuel López Cabrera. Unidad de Biología Molecular, Hospital Universitario de La Princesa**.** C/ Diego de León 62, 28006 Madrid, Spain. Phone: 34-91-5202467; Fax: 34-91-3093911**.** e-mail: mlopez.hlpr@salud.madrid.org

Financial Support : This work was supported in part by grants : 1) CIBER-ehd [Funded by ISCIII] to Dr. R. Moreno-Otero, Dr. M. López-Cabrera, and Dr. P. Majano 2) SAF2007-61201 [Ministerio de Educación y Ciencia] to Dr. M. López-Cabrera, 3) CP03/0020 [ISCIII] and 4) SAF2007-60677 [Ministerio de Educación y Ciencia] to Dr. P. Majano. F. Molina-Jimenez was financially supported by ISCIII and Fundación para la Investigación Biomédica (FIB) del Hospital Universitario de la Princesa and I. Benedicto and S. Martín-Vílchez by CIBER-ehd.

ABSTRACT

Chronic infection with hepatitis B virus (HBV) is strongly associated with hepatocellular carcinoma (HCC), and the viral HBx protein plays a crucial role in the pathogenesis of liver tumors. Since the protooncogene Pituitary Tumor Transforming Gene 1 (PTTG1) is overexpressed in HCC herein we investigated the regulation of this protein by HBx. We analyzed PTTG1 expression levels in liver biopsies from patients chronically infected with HBV, presenting different disease stages, and from HBx transgenic mice. PTTG1 was undetectable in biopsies from chronic hepatitis B patients or from normal mouse livers. In contrast, hyperplastic livers from transgenic mice and biopsies from patients with cirrhosis, presented PTTG1 expression which was found mainly in HBx-expressing hepatocytes. PTTG1 staining was further increased in HCC specimens. Experiments *in vitro* showed that HBx induced a marked accumulation of PTTG1 protein without affecting its mRNA levels. HBx expression promoted the inhibition of PTTG1 ubiquitination which in turn impaired its degradation via proteasome. Glutathione S-transferase pull-down and co-immunoprecipitation experiments demonstrated that the interaction between PTTG1 and the Skp1–Cul1–F-box (SCF) ubiquitin ligase complex was partially disrupted possibly by a mechanism involving protein-protein interactions of HBx with PTTG1 and/or SCF. Furthermore, confocal analysis showed that HBx co-localized with PTTG1 and Cul1. We propose that HBx promotes an abnormal accumulation of PTTG1 which may provide new insights into the molecular mechanisms of HBV-related pathogenesis of progressive liver disease leading to HCC development.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide (1). Chronic infection with hepatitis B virus (HBV) is the main causal factor for HCC (2). The underlying mechanisms in the multistep process of HBV-induced hepatocarcinogenesis may involve several pathways. A growing body of evidence suggests that HBV may have a direct oncogenic capacity and that expression of virally encoded proteins, in particular the HBV X protein (HBx), promotes cell growth and tumor development (3, 4). HBx is encoded by the smallest open reading frame of the HBV genome and, importantly, its expression is retained after viral integration into hepatocyte DNA (5). Furthermore, HBx is one of the most prevalent virus antigens in the liver and tumors of HBV carriers, and may induce humoral and cellular immune responses (4). It has been described that HBx alters a number of host functions that may lead to the carcinogenic process, including cell proliferation, viability, DNA repair, and genome stability (3, 4). Although HBx does not bind directly to DNA, it may activate the transcription of a wide range of cellular genes by different mechanisms involving activation of signal transduction pathways or direct interaction with components of the transcriptional machinery (6, 7). Recently, it has been proposed that HBx may also alter gene expression by promoting epigenetic changes in the DNA methylation profile (8) or by enhancing the stability of transcription factors such as HIF-1α (9) and c-myc (10). In this context, it has been shown that HBx may increase protein stability by downregulating proteasome activity (11) and by blocking protein ubiquitination (10). Thus, HBx expression results in transcriptional activation of a variety of cellular genes involved in inflammation, angiogenesis, fibrosis, oxidative stress and tumor development and progression (12-17).

Pituitary Tumor Transforming Gene 1-encoded protein (PTTG1), originally isolated from pituitary tumor cells (18), was later on identified as a human Securin, a protein implicated in inhibition of sister chromatid separation during mitosis which has been associated with malignant transformation and tumor development (19). Furthermore, PTTG1 plays key roles in cellular growth, DNA repair, development, and metabolism (20). Mechanisms of PTTG1 action include protein-protein interactions, transcriptional activity, and paracrine/autocrine regulation (20). During mitosis and following chromosome

alignment, PTTG1 is degraded by the proteasome at metaphase to anaphase transition via an E3 ubiquitin ligase, the anaphase-promoting complex/cyclosome (APC/C), releasing inhibition of separase, which in turn mediates the proteolysis of cohesins ring that holds sister chromatids together (21). In non-mitotic cells, the Skp1/Cul1/F-box protein (SCF) ubiquitin ligase complex is involved in the degradation of phosphorylated forms of PTTG1 (22). It has been shown that the protein phosphatase 2A (PP2A) regulates PTTG1 levels by counteracting its phosphorylation prior to its degradation by the proteasome (22). Furthermore, the SCF complex is involved in PTTG1 turnover in cycle arrested cells after UV radiation (23). PTTG1 overexpression has been reported in a great variety of tumors (20) in which it correlates with invasiveness (24, 25), and it has been identified as a key signature gene associated with tumor metastasis (26). In HCC, PTTG1 is overexpressed and its expression levels have prognostic significance for the survival of postoperative HCC patients (27). Interestingly, it has been proposed that PTTG1 might be critically involved in the development of HCC through the promotion of angiogenesis (27). PTTG1 specifically interacts with p53, both *in vitro* and *in vivo*, and inhibits the ability of p53 to induce cell death demonstrating its oncogenic potential (28). Additionally, PTTG1 overexpression in hepatoma cell lines negatively regulates the ability of p53 to induce apoptosis (29). These data suggest that PTTG1 gene silencing may be an effective approach to treat liver cancer in which is abundantly expressed (29).

Considering that HBV infection and HBx protein are associated with HCC and that a relationship between PTTG1 expression levels and HCC exists, we analyzed whether HBx may alter PTTG1 expression in chronic HBV infected patients, HBx transgenic mice and HBV-containing or HBx-expressing cell lines to provide new insights into our understanding of the molecular pathogenic mechanisms of advanced liver disease associated to HBV chronic infection.

MATERIALS AND METHODS.

A detailed description of the protocols and reagents employed has been included in supplementary materials and methods.

RESULTS.

PTTG1 expression level increases in HBx-immunoreactive cells as chronic hepatitis B progresses to cirrhosis and HCC.

We first investigated the expression of PTTG1 and HBx in human liver biopsies during the HBV-related hepatocarcinogenesis by staining serial liver sections with anti-PTTG1 and anti-HBx Abs. In specimens from patients with chronic hepatitis B, with a weak HBx expression, PTTG1 was not detected in hepatocytes (Figure 1A, chronic hepatitis panel). As chronic liver disease progressed from chronic hepatitis B to cirrhosis, PTTG1 protein appeared in HBx-immunoreactive hepatocytes (Figure 1A, cirrhosis panel). PTTG1 staining increased in HCC specimens showing high HBx expression (Figure 1A, HCC panel). Double immunofluorescence studies in HCC specimens showed that the distribution of PTTG1 fit well with the pattern shown by HBx immunolabeling (Figure 1B).

PTTG1 expression level increases as HBx transgenic mouse livers progress to hyperplasia and HCC

HBx, an essential factor for viral replication, is considered one of the most important determinants of HBV-induced hepatocarcinogenesis. We further investigated the expression of PTTG1 and HBx during HBx-induced hepatocarcinogenesis in HBx transgenic mouse livers. Beginning at the age of 2 months, HBx transgenic mouse liver showed centrilobular foci of cellular alteration with cytoplasmic vacuolation surrounding the central veins where hepatocytes with increased DNA synthesis were detected (30). PTTG1 and HBx were not detected in non-transgenic normal mouse livers. In hyperplastic HBx-transgenic mouse livers, expression of PTTG1 was found mainly in the cytoplasm of hepatocytes in the centrilobular region, and distribution of PTTG1 was similar to that of HBx (Figure 2, hyperplasia panel). Strong expression of both PTTG1 and HBx was observed diffusely in HCC specimens (Fig. 2, HCC

panel). Double immunofluorescence studies in transgenic mice-derived HCC specimens confirmed that PTTG1 and HBx are coexpressed in cancer cells (Suppl Figure S1).

HBx expression induces PTTG1 accumulation.

Given that PTTG1 expression was increased during both HBV and HBx-related chronic liver disease progression we speculated that HBV and more precisely HBx might induce PTTG1 expression. To address this issue, we first examined whether a HBV replicon could induce PTTG1 expression. We transfected the hepatic-derived Chang liver cells with the plasmid payw 1.2, harbouring 1.2 mer of the HBV genome that function as HBV replicon and then evaluated PTTG1 expression by Western blot. The complete replicon induced the expression of PTTG1 protein as shown in Figure 3A. Several evidences have suggested that the HBV oncogenic capacity relays in the HBx protein, which alters a number of host functions (3). Interestingly, PTTG1 expression in cells transfected with the HBx-defective whole-genome construct (payw*7) remained unchanged, indicating a role of HBx in PTTG1 induction (Figure 3A). To further explore the effects of HBx on PTTG1 expression we employed two hepatocyte derived cell lines, Chang liver p34X (p34X) and AML12 4pX (4pX), in which HBx expression was controlled by doxicycline treatment (Dox-on) or withdrawal (Dox-off) respectively. Western blot analysis showed increased PTTG1 expression upon induction of HBx during 48 h in both Dox-regulated systems (Figure 3B). Similar results were obtained after 24h of Dox treatment (Suppl Figure S2A). As controls, we included Chang liver and AML12 4p cells, the parental cell lines of p34X and 4pX cells respectively, and no PTTG1 variation after Dox challenge was observed. PTTG1 levels positively correlate with cell proliferation (31) and its expression is controlled in a cell-cycle dependent manner (32). Several studies have also shown that HBx promotes cellular proliferation by triggering DNA synthesis and speeding up cell-cycle progression (33) (34). However, evidences regarding the effects of HBx on liver cell proliferation and cell death are still controversial, depending on the experimental systems and cell lines employed (3). To assess the effect of HBx expression on cell cycle progression, we analyzed the growth profiles of Chang

liver p34X and AML12 4pX cells with or without Dox treatment by flow cytometry. In agreement with previous reports (35), our data showed that the percentages of p34X cells in G0/G1, S, and G2/M phases of the cell cycle displayed similar profiles 24 h (Suppl Figure S2B) and 48 h after induction of HBx expression (Figure 3D). Furthermore, 4pX cells displayed a significant increase in HBx-dependent S phase entry 24 h (Suppl Figure S2B) (36) but not 48 h after induction of HBx expression (Figure 3D). Additionally, transient transfection of Chang liver cells with the HBV wild-type and HBx-defective replicons did not induce changes in the cell cycle profile (Figure 3C). Given that HBx promoted PTTG1 accumulation without significantly affecting cell cycle (p34X and HBV complete replicon-transfected Chang liver cells), these results indicated that the HBx-promoted PTTG1 accumulation was not dependent on cell-cycle modifications.

HBx-mediated PTTG1 accumulation is regulated post-transcriptionally.

It is known that HBx transcriptionally induces the expression of a wide range of viral and cellular genes by activating promoter regulatory sequences (4). To determine whether HBx modulates PTTG1 transcription, its mRNA levels were measured by quantitative RT-PCR in p34x and 4pX cells. PTTG1 mRNA levels were unaffected by HBx expression in both p34X (Figure 4A) and 4px cells (Suppl Figure S3). As expected (13), RT-PCR analysis showed increased TNF-α mRNA levels upon induction of HBx (Figure 4A).

Aditionally, we transiently transfected Hela cells with both pPTTG1-CFP, an expression vector in which PTTG1-CFP transcription is controlled by the CMV promoter, and pHBx-HA plasmids. Western blot analysis using an anti-GFP antibody showed that PTTG1-CFP was clearly accumulated in HBx transfected cells (Figure 4B). Interestingly, the effect of HBx was not observed when cells were cotransfected with the control plasmid pCFP-N1, coding only for the CFP protein. These results were further confirmed by co-transfecting Hela cells with wild type HBV or HBx-defective replicons along with the pPTTG1-CFP vector (Figure 4C). These results strongly suggested that PTTG1 accumulation induced by HBx was not mediated by transcriptional activation.

We next examined whether HBx-induced PTTG1 upregulation could be explained through changes on protein stability by analyzing PTTG1 levels after blocking protein synthesis with cycloheximide. Western blot analysis showed that PTTG1 protein half-life increased in p34X cells after induction of HBx expression when compared to non-induced cells (Figure 4D and E). Taken together, these results indicated that HBx promoted PTTG1 accumulation by modulating its degradation.

HBx inhibits the ubiquitination of hyperphosphorylated PTTG1 forms.

Phosphorylation of PTTG1 leads to its ubiquitination and proteasomal degradation (22). Thus, we analyzed the levels of phosphorylated forms of PTTG1 in p34X cells treated with okadaic acid (OA), a PP2A inhibitor, and/or MG132, a proteasome inhibitor. As expected, proteasome inhibition by MG132 treatment promoted PTTG1 accumulation independently of HBx expression (Figure 5A, lanes 3 *vs* 1 and 7 *vs* 5). As previously described (22), MG132 plus OA co-treatment revealed the presence of slower migrating bands corresponding to phosphorylated forms of PTTG1 (P-PTTG1) in both control or Dox treated cells (lanes 4 and 8). OA treatment reduced PTTG1 levels in both HBx expressing and non-expressing cells (lanes 2 *vs* 1 and 6 *vs* 5). However, P-PTTG1 forms could be detected in the absence of MG132 after PP2A inhibition (OA treatment) only when HBx was expressed suggesting that HBx inhibited the degradation of P-PTTG1 (lanes 6 *vs* 2). In order to rule out that the differences observed between HBx-expressing and non-expressing cells could be due to undefined clonal properties of p34X cells or Dox-associated effects rather than the presence of HBx, parental Chang liver cells were included. As in HBx non-expressing p34X cells, P-PTTG1 forms in Chang liver cells were only detected after proteasome plus PP2A inhibition independently of Dox treatment (Suppl Figure S4A).

Additionally, we compared PTTG1 distribution between HBx-expressing *versus* non-expressing cells after OA treatment by immunofluorescence experiments. Of note, not all p34X cells expressed HBx in response to Dox treatment. In the absence of OA, PTTG1 was diffusely localized in both nucleus and cytoplasm of both HBx positive and negative p34X cells (Figure 5B top). As

aforementioned, OA treatment reduced PTTG1 levels (Figure 5B bottom). However, we observed a PTTG1 accumulation in HBx positive cells that co-localized with the viral protein. To quantify the effect of HBx on PTTG1 accumulation after OA treatment, Chang liver cells were transfected with bicistronic plasmids pCMS-EGFP-HBx (HBx-expressing vector; CMS-X) or pCMS-EGFP (control vector; CMS-O) and processed for immunofluorescence after PP2A inhibition. As shown in figure 5C, there was a marked increase of PTTG1 positive cells when transfected with HBx-expresing vector compared with control vector.

It has been shown that HBx is an inhibitor of both proteasome complex (11) and ubiquitin ligases (10). Therefore, HBx could promote PTTG1 accumulation through proteasome and/or ubiquitin ligase inhibition. Since ubiquitination targets proteins to proteasomal degradation, we analyzed the ubiquitination of PTTG1 in the presence of HBx. For this purpose, unstimulated or Dox treated p34X cells were incubated with the proteasome inhibitor MG132 and used for immunoprecipitacion using an anti-PTTG1 antibody. Membranes were blotted with anti-ubiquitin mAb to detect ubiquitinated forms of PTTG1. As expected, MG132-mediated proteasome inhibition promoted the accumulation of polyubiquinated PTTG1 forms in cells that did not express HBx (Figure 5D, lane 7 *vs* 5). In contrast, incubation of HBx-expressing cells with MG132 did not significantly increase the levels of ubiquitinated forms of PTTG1 (Figure 5D lane 8 *vs* 6). Similar results were obtained in Hela cells co-transfected with expression vectors coding for hemagglutinin (HA)-tagged ubiquitin (HA-Ubiquitin), PTTG1 (pcDNA-PTTG1) and either a HBx-coding vector (pSVX) or the control plasmid (pSVHygro) (Suppl Figure S4B). The tight junction-associated protein occludin is ubiquitinated and its degradation is sensitive to proteasome inhibition (37). To analyze whether HBx affected general ubiquitination events we determined the influence of HBx on occludin ubiquitination. As shown in figure 5E, the accumulation of polyubiquitinated occludin was not affected by HBx expression. All together, these results strongly suggested that HBx specifically reduced PTTG1 ubiquitination.

HBx disrupts the interaction between PTTG1 and the SCF protein complex.

It has been described that phosphorylated forms of PTTG1 are degraded by the proteasome after ubiquitination by SCF ubiquitin ligase complex (38). In agreement with our previous results using other cell lines (23), coimmunoprecipitation assays using lysates of unstimulated p34X cells treated with OA plus MG132 showed that the SCF core component Cul1 coimmunoprecipitated with PTTG1 (Figure 6A top, lane 4). Interestingly, treatment of p34X cells with Dox to induce HBx expression partially disrupted the interaction between PTTG1 and Cul1 (Figure 6A, lane 5 *vs* 4). GST-based pull-down assays showed that the fusion protein GST-PTTG1, but not GST, interacted with endogenous Cul1 from a cellular lysate of non-induced p34X (Figure 6B, top, lane 5). As above, this interaction was also reduced in the presence of HBx (Figure 6B, lane 6 *vs* 5 top). These data suggested that HBx could reduce PTTG1 ubiquitination, at least partially, by interfering the interaction between PTTG1 and SCF. In addition, these results indicated that the interaction of HBx with PTTG1 and/or SCF complex might be operating in the disruption of PTTG1/SCF association. To further explore this issue, additional pull-down assays were performed. As shown in Figure 6D, GST-HBx interacted with endogenous PTTG1 and GST-PTTG1 associated with HBx protein (Figure 6B bottom, lane 6). Furthermore, an interaction between GST-HBx and Cul1 could also be demonstrated (Figure 6D). The specificity of these GST-HBx interactions was confirmed by observing no interaction of HBx with occludin and other cell cycle-regulating proteins as cyclin B1 or STAG2/SA2 (Figure 6D). In adittion, the association between HBx and Cul1 was further confirmed by confocal double-label immunofluorescence in Chang liver p34X cells in which HBx significantly co-localized with Cul1 in dot-like structures (Figure 6E).

HBx does not affect the PTTG1 stabilization in Cul1 knockdown cells.

The SCF ubiquitin ligase complex is involved in the degradation of phosphorylated forms of PTTG1 (22). To analyze the specific role of Cul1 on HBx mediated PTTG1 accumulation a siRNAs-based knockdown approach was employed. First, we determined the levels of PTTG1 in Chang liver cells transiently transfected with control or Cul1-specifc siRNA, and then treated or not with OA, and/or MG132. Western blot analysis showed that Cul1

knockdown promoted PTTG1 accumulation in both control and OA treated cells. Additionally OA treatment of Cul1 knockdown cells resulted in the formation of phosphorylated PTTG1 forms (Suppl Figure S5). Next we analyzed the effect of HBx expression on PTTG1 accumulation in Cul1 silenced cells. Results showed that in both Chang liver and p34x cells, PTTG1 expression levels were increased after Cul1 silencing (Figure 7). As above, Dox-induced HBx increased PTTG1 levels in p34X control siRNA-treated cells (Figure 7, lanes 7*vs* 5). Interestingly, PTTG1 accumulation after Cul1 silencing was not further enhanced by HBx (Figure 7, lane 8 *vs* 6), suggesting that the stabilization of PTTG1 by HBx was Cul1-dependent, not being likely that other ubiquitin ligase was involved. Given that HBx expression mimicked the effects on PTTG1, it can be hypothesized that HBx interferes Cul1-asscociated functions. Overall, these data strongly suggest that HBx promotes the disruption of the PTTG1/SCF association and prevents its ubiquitination and it subsequent degradation by the proteasome (Figure 8).

DISCUSSION

HBV-associated carcinogenesis is a multi-factorial process. Liver inflammation results in hepatocellular death and regeneration processes that lead to the accumulation of critical mutations in the host genome. In addition, the regulatory protein HBx has been involved in hepatocarcinogenesis by altering cellular processes such as transcription, signal transduction, cell cycle progression, protein degradation, , apoptosis and genetic stability. In the present study, we have demonstrated that PTTG1 expression levels increase in HBx-immunoreactive cells as chronic hepatitis B progresses to cirrhosis and HCC. Furthermore, PTTG1 expression increases as HBx transgenic mouse livers progress through hyperplasia to HCC. In addition, PTTG1 accumulates in human and mouse HBx-expressing cell lines and in HBV replicon-containing cells, but not in cells harbouring a HBx-defective genome construct, All together, these data strongly suggest that PTTG1 accumulation is, at least partially, a HBx-mediated effect.

Several viruses, including HBV, are endowed with the ability to stimulate the cell cycle progression in order to facilitate their own replication. In doing so, viruses generally disrupt the normal cell cycle checkpoints and in turn extend proliferative signals to host cells to establish a carcinogenic environment (39). However, evidences regarding the effects of HBx protein on liver cell proliferation and death are controversial and depend on the experimental systems and cell lines analyzed. HBx has been demonstrated to suppress serum dependence for cell cycle activation (40). Furthermore, HBx has been shown to promote the transit through G1 in G0 arrested cells and to alter G1-to-S and G2-to-M progression (34, 36). However, in Chang liver p34X cells the cell cycle profile was unaffected after HBx induction (35). In addition, it is known that HBx transcriptionally induces the expression of viral and cellular genes (4). However, our data strongly suggest that HBx-promoted PTTG1 protein accumulation is not strictly dependent on neither cell cycle modifications nor transcriptional upregulation.

Through interactions with host factors, HBx alters different cellular processes implicated in the development of HCC such as cell cycle progression, genome stability, apoptosis and protein turnover. Protein degradation by the proteasome complex is a strictly regulated key event of cellular homeostasis.

Oncogenic viruses alter the proteasomal activity of target cells affecting viral entry, replication and release and enhancing cell survival (41). Targeting of proteins to the proteasome through interactions with ubiquitin ligases is essential for normal protein turnover. In this context, HBx is able to downregulate both proteasome (11) and ubiquitin ligase functions (10). Our data show that HBx induced a marked accumulation of PTTG1 protein by reducing its ubiquitination and subsequent degradation.

It has been demonstrated that the SCF ubiquitin ligase complex is involved in the degradation of phosphorylated forms of PTTG1 in non-mitotic cells. In addition, it has been shown that HBx affects SCF ubiquitin ligase functions through mechanisms involving protein-protein interactions (10). Confocal microscopy analysis and biochemical data strongly suggest that HBx may interact with both the SCF component Cul1 and PTTG1. Interestingly, the association between PTTG1 and Cul1 is disrupted in the presence of HBx. However, HBx expression does not enhance PTTG1 accumulation after Cul1 silencing. All together, these data suggest that HBx may alter the formation of the SCF/PTTG1 complex, leading to an impairment of PTTG1 ubiquitination. Thus, in the presence of HBx, PTTG1 is not targeted to proteasome-mediated degradation resulting in an abnormal protein accumulation (see model, Figure 8). It is tempting to speculate that by affecting the normal turnover of PTTG1, HBx could alter some of the PTTG1-related functions and promote cellular transformation.

The SCF ubiquitin ligases are mammalian cullin RING ubiquitin ligases in which F-box proteins provide the substrate targeting specificity of the complex (42). Skp2 is the F-box protein that targets key regulatory proteins, such as c-myc, for degradation (43). Since the sequence of events for the assembly of an active SCF complex is poorly understood, it is possible that binding of substrates to F box proteins could initiate the process. Interestingly, it has been shown that HBx is able to block ubiquitination of c-myc through a direct interaction with Skp2 and destabilization of the SCF/Skp2 complex. An association between HBx-mediated PTTG1 stabilization and HBx/Skp2 interaction may also exist, but this issue needs further studies.

PP2A is an important serine/threonine phosphatase family involved in essential cellular processes such as cell division, gene regulation, protein

synthesis and cytoskeleton organization. PP2A enzymes typically exist as heterotrimers comprising a common catalytic subunit (PP2Ac) and different structural and regulatory type subunits (44). It has been shown that hepatotropic viruses, including hepatitis C virus (45) and HBV, inhibit interferon-signalling pathways via upregulation of PP2Ac activity (46). HBx protein is the most likely candidate responsible for HBV-mediated PP2Ac modulation (46). Our results showed that HBx promoted PTTG1 accumulation inhibiting the degradation of phosphorylated forms of PTTG1 after chemical inhibition of PP2A. Further experiments will be necessary to analyze whether HBx could affect PTTG1 expression levels by upregulating PP2A activity.

Several lines of evidence suggest that an important transforming mechanism underlying PTTG1 overexpression is the induction of chromosomal instability (20). Thus, it has been demonstrated that PTTG1 accumulation inhibits mitosis progression and chromosome segregation, but does not directly affect cytokinesis, resulting in aneuploidy (47). It has been shown that HBx can transform cultured cells (33) and induce liver cancer in transgenic mice (48). Genetic instability is frequently accompanied with the acquisition of transformation ability and malignant progression of tumors. Moreover, recent reports have shown that HBx expression induces chromosomal aberrations such as chromosome rearrangements and micronuclei formation (49). Furthermore, HBx promotes multipolar spindle formation and chromosomal missegregation during mitosis, and increases multinucleated cells (50). Interestingly, it has been determined that HBx binds to BubR1, a component of the mitotic checkpoint complex, and attenuates the association between BubR1 and CDC20, an activator of the anaphase-promoting complex/cyclosome (APC/C), resulting in chromosomal instability (51). Our results demonstrated that HBx induced the accumulation of PTTG1 in interphase cells. Further experiments are necessary to study the effects of HBx on PTTG1 functions during mitotic events.

In summary, we propose that HBx promotes alterations of PTTG1 expression levels, which may provide new insights for our understanding of the molecular mechanisms of HBV-related pathogenesis of progressive liver disease leading to cirrhosis and HCC development.

REFERENCES

1. Bosch FX, Ribes J, Cleries R, Diaz M. Epidemiology of hepatocellular carcinoma. Clin Liver Dis 2005;9:191-211, v.

2. Kremsdorf D, Soussan P, Paterlini-Brechot P, Brechot C. Hepatitis B virus-related hepatocellular carcinoma: paradigms for viral-related human carcinogenesis. Oncogene 2006;25:3823-3833.

3. Bouchard MJ, Schneider RJ. The enigmatic X gene of hepatitis B virus. J Virol 2004;78:12725-12734.

4. Tang H, Oishi N, Kaneko S, Murakami S. Molecular functions and biological roles of hepatitis B virus x protein. Cancer Sci 2006;97:977-983.

5. Su Q, Schroder CH, Hofmann WJ, Otto G, Pichlmayr R, Bannasch P. Expression of hepatitis B virus X protein in HBV-infected human livers and hepatocellular carcinomas. Hepatology 1998;27:1109-1120.

6. Branda M, Wands JR. Signal transduction cascades and hepatitis B and C related hepatocellular carcinoma. Hepatology 2006;43:891-902.

7. Doria M, Klein N, Lucito R, Schneider RJ. The hepatitis B virus HBx protein is a dual specificity cytoplasmic activator of Ras and nuclear activator of transcription factors. EMBO J 1995;14:4747-4757.

8. Park IY, Sohn BH, Yu E, Suh DJ, Chung YH, Lee JH, Surzycki SJ, et al. Aberrant epigenetic modifications in hepatocarcinogenesis induced by hepatitis B virus X protein. Gastroenterology 2007;132:1476-1494.

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

10. Kalra N, Kumar V. The X protein of hepatitis B virus binds to the F box protein Skp2 and inhibits the ubiquitination and proteasomal degradation of c-Myc. FEBS Lett 2006;580:431-436.

11. Huang J, Kwong J, Sun EC, Liang TJ. Proteasome complex as a potential cellular target of hepatitis B virus X protein. J Virol 1996;70:5582-5591.

12. Yoo YD, Ueda H, Park K, Flanders KC, Lee YI, Jay G, Kim SJ. Regulation of transforming growth factor-beta 1 expression by the hepatitis B virus (HBV) X transactivator. Role in HBV pathogenesis. J Clin Invest 1996;97:388-395.

13. Lara-Pezzi E, Majano PL, Gomez-Gonzalo M, Garcia-Monzon C, Moreno-Otero R, Levrero M, Lopez-Cabrera M. The hepatitis B virus X protein up-regulates tumor necrosis factor alpha gene expression in hepatocytes. Hepatology 1998;28:1013-1021.

14. Lara-Pezzi E, Gomez-Gaviro MV, Galvez BG, Mira E, Iniguez MA, Fresno M, Martinez AC, et al. The hepatitis B virus X protein promotes tumor cell invasion by inducing membrane-type matrix metalloproteinase-1 and cyclooxygenase-2 expression. J Clin Invest 2002;110:1831-1838.

15. Lee SW, Lee YM, Bae SK, Murakami S, Yun Y, Kim KW. Human hepatitis B virus X protein is a possible mediator of hypoxia-induced angiogenesis in hepatocarcinogenesis. Biochem Biophys Res Commun 2000;268:456-461.

16. Majano P, Lara-Pezzi E, Lopez-Cabrera M, Apolinario A, Moreno-Otero R, Garcia-Monzon C. Hepatitis B virus X protein transactivates inducible nitric oxide synthase gene promoter through the proximal nuclear factor kappaB-binding site: evidence that cytoplasmic location of X protein is essential for gene transactivation. Hepatology 2001;34:1218-1224.

17. Martin-Vilchez S, Sanz-Cameno P, Rodriguez-Munoz Y, Majano PL, Molina-Jimenez F, Lopez-Cabrera M, Moreno-Otero R, et al. The hepatitis B virus X protein induces paracrine activation of human hepatic stellate cells. Hepatology 2008;47:1872-1883.

18. Pei L, Melmed S. Isolation and characterization of a pituitary tumor-transforming gene (PTTG). Mol Endocrinol 1997;11:433-441.

19. Zou H, McGarry TJ, Bernal T, Kirschner MW. Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science 1999;285:418-422.

20. Vlotides G, Eigler T, Melmed S. Pituitary tumor-transforming gene: physiology and implications for tumorigenesis. Endocr Rev 2007;28:165-186.

21. Pines J. Mitosis: a matter of getting rid of the right protein at the right time. Trends Cell Biol 2006;16:55-63.

22. Gil-Bernabe AM, Romero F, Limon-Mortes MC, Tortolero M. Protein phosphatase 2A stabilizes human securin, whose phosphorylated forms are degraded via the SCF ubiquitin ligase. Mol Cell Biol 2006;26:4017-4027.

23. Limon-Mortes MC, Mora-Santos M, Espina A, Pintor-Toro JA, Lopez-Roman A, Tortolero M, Romero F. UV-induced degradation of securin is mediated by SKP1-CUL1-beta TrCP E3 ubiquitin ligase. J Cell Sci 2008;121:1825-1831.

24. Heaney AP, Singson R, McCabe CJ, Nelson V, Nakashima M, Melmed S. Expression of pituitary-tumour transforming gene in colorectal tumours. Lancet 2000;355:716-719.

25. Boelaert K, McCabe CJ, Tannahill LA, Gittoes NJ, Holder RL, Watkinson JC, Bradwell AR, et al. Pituitary tumor transforming gene and fibroblast growth factor-2 expression: potential prognostic indicators in differentiated thyroid cancer. J Clin Endocrinol Metab 2003;88:2341-2347.

26. Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. Nat Genet 2003;33:49-54.

27. Fujii T, Nomoto S, Koshikawa K, Yatabe Y, Teshigawara O, Mori T, Inoue S, et al. Overexpression of pituitary tumor transforming gene 1 in HCC is associated with angiogenesis and poor prognosis. Hepatology 2006;43:1267-1275.

28. Bernal JA, Luna R, Espina A, Lazaro I, Ramos-Morales F, Romero F, Arias C, et al. Human securin interacts with p53 and modulates p53-mediated transcriptional activity and apoptosis. Nat Genet 2002;32:306-311.

29. Cho-Rok J, Yoo J, Jang YJ, Kim S, Chu IS, Yeom YI, Choi JY, et al. Adenovirus-mediated transfer of siRNA against PTTG1 inhibits liver cancer cell growth in vitro and in vivo. Hepatology 2006;43:1042-1052.

30. Koike K, Moriya K, Iino S, Yotsuyanagi H, Endo Y, Miyamura T, Kurokawa K. High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. Hepatology 1994;19:810-819.

31. Ramos-Morales F, Dominguez A, Romero F, Luna R, Multon MC, Pintor-Toro JA, Tortolero M. Cell cycle regulated expression and phosphorylation of hpttg proto-oncogene product. Oncogene 2000;19:403-409.

32. Dominguez A, Ramos-Morales F, Romero F, Rios RM, Dreyfus F, Tortolero M, Pintor-Toro JA. hpttg, a human homologue of rat pttg, is overexpressed in hematopoietic neoplasms. Evidence for a transcriptional activation function of hPTTG. Oncogene 1998;17:2187-2193.

33. Koike K, Moriya K, Yotsuyanagi H, Iino S, Kurokawa K. Induction of cell cycle progression by hepatitis B virus HBx gene expression in quiescent mouse fibroblasts. J Clin Invest 1994;94:44-49.

34. Benn J, Schneider RJ. Hepatitis B virus HBx protein deregulates cell cycle checkpoint controls. Proc Natl Acad Sci U S A 1995;92:11215-11219.

35. Yun C, Lee JH, Park H, Jin YM, Park S, Park K, Cho H. Chemotherapeutic drug, adriamycin, restores the function of p53 protein in hepatitis B virus X (HBx) protein-expressing liver cells. Oncogene 2000;19:5163-5172.

36. Lee S, Tarn C, Wang WH, Chen S, Hullinger RL, Andrisani OM. Hepatitis B virus X protein differentially regulates cell cycle progression in X-transforming versus nontransforming hepatocyte (AML12) cell lines. J Biol Chem 2002;277:8730-8740.

37. Traweger A, Fang D, Liu YC, Stelzhammer W, Krizbai IA, Fresser F, Bauer HC, et al. The tight junction-specific protein occludin is a functional target of the E3 ubiquitin-protein ligase itch. J Biol Chem 2002;277:10201-10208.

38. Romero F, Gil-Bernabe AM, Saez C, Japon MA, Pintor-Toro JA, Tortolero M. Securin is a target of the UV response pathway in mammalian cells. Mol Cell Biol 2004;24:2720-2733.

39. Feitelson MA, Reis HM, Liu J, Lian Z, Pan J. Hepatitis B virus X antigen (HBxAg) and cell cycle control in chronic infection and hepatocarcinogenesis. Front Biosci 2005;10:1558-1572.

40. Mukherji A, Janbandhu VC, Kumar V. HBx-dependent cell cycle deregulation involves interaction with cyclin E/A-cdk2 complex and destabilization of p27Kip1. Biochem J 2007;401:247-256.

41. Banks L, Pim D, Thomas M. Viruses and the 26S proteasome: hacking into destruction. Trends Biochem Sci 2003;28:452-459.

42. Frescas D, Pagano M. Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. Nat Rev Cancer 2008;8:438-449.

43. von der Lehr N, Johansson S, Wu S, Bahram F, Castell A, Cetinkaya C, Hydbring P, et al. The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. Mol Cell 2003;11:1189-1200.

44. Mumby M. PP2A: unveiling a reluctant tumor suppressor. Cell 2007;130:21-24.

45. Duong FH, Filipowicz M, Tripodi M, La Monica N, Heim MH. Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A. Gastroenterology 2004;126:263-277.

46. Christen V, Treves S, Duong FH, Heim MH. Activation of endoplasmic reticulum stress response by hepatitis viruses up-regulates protein phosphatase 2A. Hepatology 2007;46:558-565.

47. Yu R, Lu W, Chen J, McCabe CJ, Melmed S. Overexpressed pituitary tumor-transforming gene causes aneuploidy in live human cells. Endocrinology 2003;144:4991-4998.

48. Kim CM, Koike K, Saito I, Miyamura T, Jay G. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature 1991;351:317-320.

49. Livezey KW, Negorev D, Simon D. Increased chromosomal alterations and micronuclei formation in human hepatoma HepG2 cells transfected with the hepatitis B virus HBX gene. Mutat Res 2002;505:63-74.

50. Yun C, Cho H, Kim SJ, Lee JH, Park SY, Chan GK. Mitotic aberration coupled with centrosome amplification is induced by hepatitis B virus X oncoprotein via the Ras-mitogen-activated protein/extracellular signal-regulated kinase-mitogen-activated protein pathway. Mol Cancer Res 2004;2:159-169.

51. Kim S, Park SY, Yong H, Famulski JK, Chae S, Lee JH, Kang CM, et al. HBV X protein targets hBubR1, which induces dysregulation of the mitotic checkpoint. Oncogene 2008;27:3457-3464.

ACKNOWLEDGEMENTS

The authors express their gratitude to Drs. Andrisani, Cho, Lara-Pezzi , Levrero, Murakami, Nakayama, Slagle and Wands and for providing us with critical reagents. The authors also thank Rosario López-Rodríguez for statistical analysis.

CONFLICT OF INTEREST

There are no financial/commercial conflicts of interest.

FIGURE LEGENDS.

Figure 1. PTTG1 expression levels increase as human chronic hepatitis B progresses to cirrhosis and HCC. (A) PTTG1 and HBx proteins were detected in human cirrhotic liver and HCC liver biopsies. PTTG1 protein appeared in HBx-immunoreactive hepatocytes in cirrhotic liver, and both proteins were strongly expressed in HCC specimens. Formalin-fixed, paraffin-embedded liver sections were stained with anti-PTTG1 Ab and anti-HBx. The HBx section was paired with an adjacent section stained using anti-PTTG1 Ab. Abs were then bound by goat anti-rabbit IgG or by goat anti-mouse IgG conjugated with peroxidase-labeled polymer. Peroxidase activity was detected by 3, 3'-diaminobenzidine tetrahydrochloride (DAB). All sections were counterstained with hemotoxylin (blue). Brown color indicates specific Ab reactivity. Bar, 50 μm. We investigated 5 chronic hepatitis B, cirrhosis, and HCC specimens. Since all of the results were similar among the experiments, representative results are displayed. (B) Hepatocytic PTTG1 in human HCC specimens co-localized with HBx. Human HCC sections were stained for immunofluorescence to simultaneously detect PTTG1 (red) and HBx (green). Yellow color indicates overlap of proteins. Bar, 50 μm.

Figure 2. PTTG1 oncoprotein increases as HBx transgenic mouse livers progress through hyperplasia to HCC.(A) Distribution of PTTG1 and HBx in normal non-transgenic mouse liver, hyperplasia, and HCC specimens from HBx transgenic mice. PTTG1 was present mainly in the cytoplasm of hyperplastic hepatocytes immunoreactive for HBx oncoprotein surrounding central veins in HBx transgenic mouse liver. Strong staining of both proteins was observed diffusely in HCC specimens. Immunohistochemical analyses were performed as described above. We investigated 5 normal livers, hyperplasia and HCC liver biopsies. Since all of the results were similar among the experiments, representative results are displayed.

Figure 3. Effects of HBx on PTTG1 expression. (A) Chang liver cells were transfected with the whole HBV genome payw1.2 (HBx^+), a HBx-defective mutant payw7* (HBx^-) or control plasmid (pcDNA 3.1), and PTTG1 expression was monitored by Western blot. Tubulin expression was assessed to ensure

equal protein loading of all samples. (B) PTTG1 protein levels were analyzed in Chang liver, Chang liver p34X, AML12 4p and AML12 4pX grown with or without Dox for 48 h by Western blot. (C) Flow cytometry analysis of cell cycle progression in Chang liver cells 24 h after transient transfection with payw1.2(HBx^+), payw*7(HBx^-) or control plasmid (pCDNA3.1). (D) Cell cycle progression in p34X and 4pX cells 48 h after induction of HBx expression. Values represent the mean ± SD of six independent experiments (*p≤0,05 *vs* HBx non-expressing cells, Mann-Whitney U test).

Figure 4. HBx increases PTTG1 expression levels post-transcriptionally. (A) PTTG1 (grey bars) and TNF-α (black bars) mRNA levels were measured in p34X cells 24 and 48 h after induction of HBx expression by quantitative RT-PCR. Results were normalized with histone H3 and represented as fold induction over control. Results are presented as the mean value ± SD of three independent experiments. (B) Hela cells were transfected with pPTTG1-CFP or pECFP-N1 plus pHBx-HA or empty vector (pcDNA 3.1). Cell lysates were analyzed by Western blot. (C) Hela cells were transfected with pPTTG1-CFP or pCFP-N1 plus payw1.2, payw*7 or control plasmid. Cell lysates were analyzed by Western blot. (D) Control or Dox-induced p34x cells (48 h) were treated with 20 μM cycloheximide for different time intervals. Equal amounts of protein, were subjected to Western blot analysis. Representative results of three independent experiments are shown.(E) Analysis of PTTG1 relative levels, assessed by scanning densitometry, was plotted (black, control cells; grey, HBx expressing cells). Results are expressed as percentage of the values obtained without cycloheximide treatment for each experimental condition analyzed. Values represent the mean ± SE of three independent experiments.

Figure 5. HBx reduces the ubiquitination of hyperphosphorylated PTTG1 forms. (A) Chang liver p34x cells were treated with okadaic acid (OA) (1μM), MG132 (10 μM) or both for 2 h after 48 h of doxycycline induction. Lysates were subsequently analyzed by Western blot for the detection of PTTG1. (B) Confocal immunofluorescence analysis of the distribution of PTTG1 (green) and HBx (red) in OA treated (OA+) or not (OA-) p34x cells. DAPI staining is shown

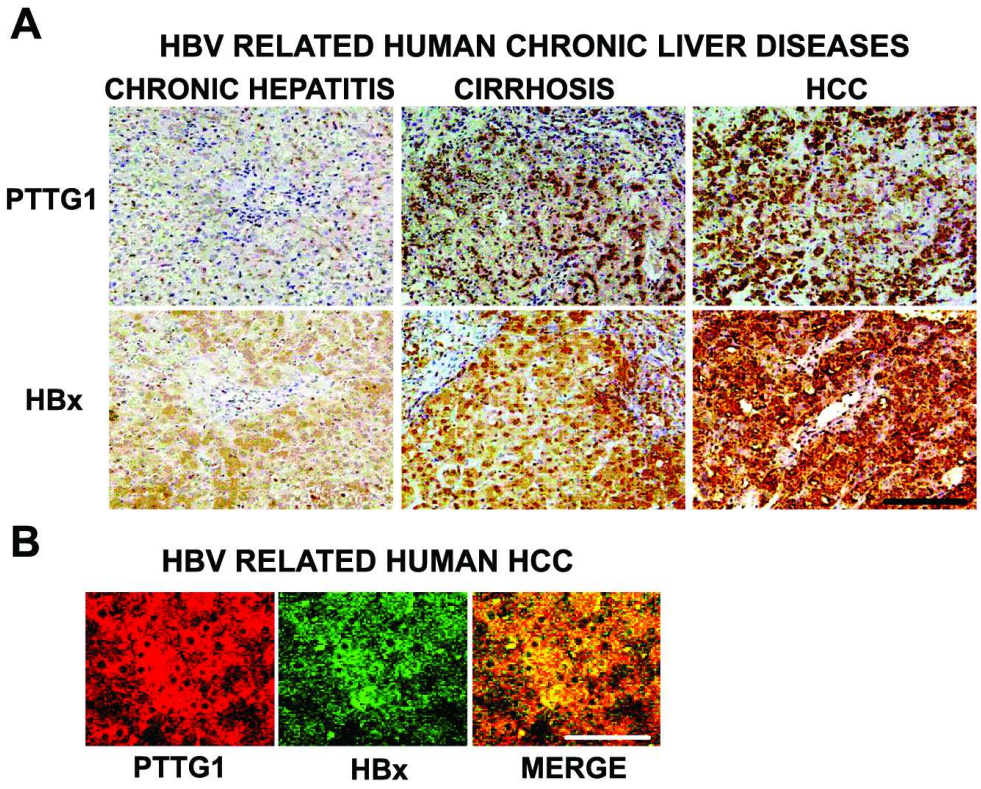
in blue. Bar, 5 µm. (C) 24 h after transfection of Chang liver cells with pCMS-EGFP-HBx (CMS-X) or pCMS-EGP (CMS-0) plasmids, cells were treated with OA and PTTG1 accumulation was compared by immunofluorescence. Percentages of transfected cells (GFP positive) displaying PTTG1 accumulation are shown. Values represent the mean ± SD of four independent experiments in which at least 50 GFP-positive cells were analyzed (D) Control or Dox-induced p34X cells were treated or not for 4 h with MG132 (10 µM). Cell lysates were immunoprecipitated (IP) with anti-PTTG1 pAb and immunoblotted with anti-PTTG1 (bottom) and anti-Ubiquitin (top) Abs. (E) Cells were treated as in (D) and lysates were immunoprecipitated with anti-Occludin pAb and immunoblotted with anti-occludin (bottom) and anti-Ubiquitin (top). Representative results of at least two independent experiments are shown.

Figure 6. HBx inhibits the interaction between PTTG1 and SCF complex. (A) Lysates from p34X cells, grown with or without Dox for 48 h, were immunoprecipitated (IP) using anti-PTTG1, or rabbit immunoglobulins (IgG) as control. Western blot analysis of cell lysates and immunoprecipitates was performed with anti-Cul1 (top) or anti-PTTG-1 (bottom) Abs. (B) Pull-down assay with GST or GST-PTTG1 and Dox treated (+) or untreated (-) p34X extracts. Cell lysates and bound proteins were subjected to Western blot analysis using anti-Cul1 (top) or anti-HA Abs (bottom). (C) Coomassie brilliant blue staining of 1/10 of the GST proteins used is shown. Molecular weight markers (kDa) are indicated on the left. (D) Pull-down assay with GST or GST-HBx and non-induced p34X cell extracts. Cell lysates and bound proteins were subjected to Western blot analysis using anti-Cul1, anti-PTTG1, anti-occludin, anti-SA2 and anti-Cyclin B1 Abs. (E) Confocal immunofluorescence analysis of the distribution of Cul1 (green; monoclonal antibody anti-Cul-1) and HBx (red; byotinilated antibody anti-HA epitope) in OA treated (OA+) or not (OA-) p34x cells. DAPI staining is shown in blue. Bar, 7,5 µm. All results shown are representative of at least of two independent experiments.

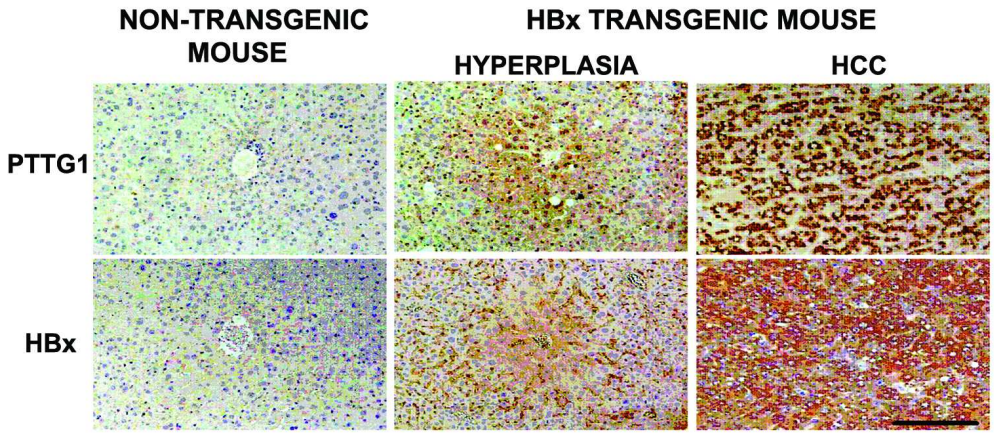
Figure 7. HBx does not enhance PTTG1 stabilization after Cul1 knockdown. Chang liver and p34x cells were transfected with control or Cul1

siRNA and treated with Dox for 48 h. PTTG1, Cul1 and tubulin protein levels were analyzed by Western blot. Results are representative of two independent experiments.

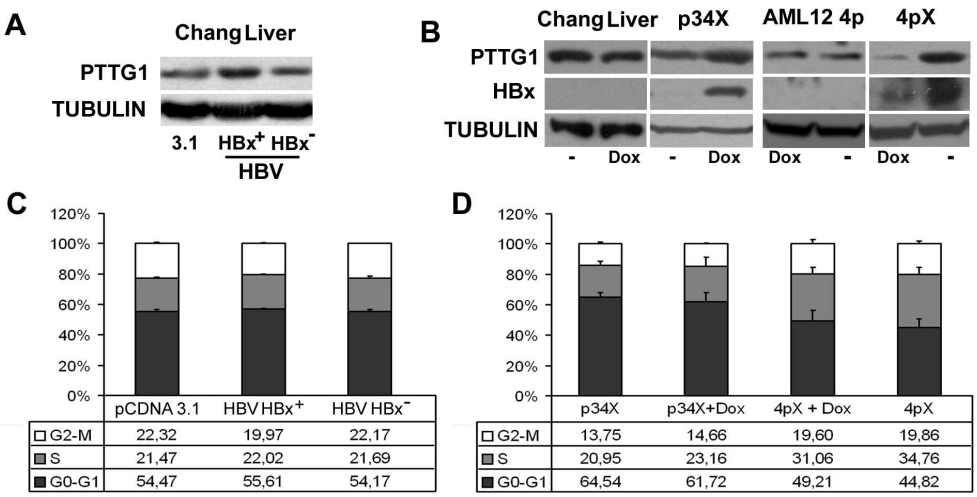
Figure 8. Possible mechanism for HBx-mediated PTTG1 stabilization. PTTG1 undergoes proteasomal degradation via ubiquitination by the SCF (SKP1/Cul1/Fbox) ubiquitin ligase complex (A. PTTG1 degradation). Cul1 interacts with PTTG1 and in the presence of HBx this interaction is disrupted. As a result, there is an impairment of PTTG1 ubiquitination that leads to an increase of its half-life (B. HBx-induced PTTG1 accumulation). The proliferative actions of PTTG1 and HBx could act synergistically in cell transformation.



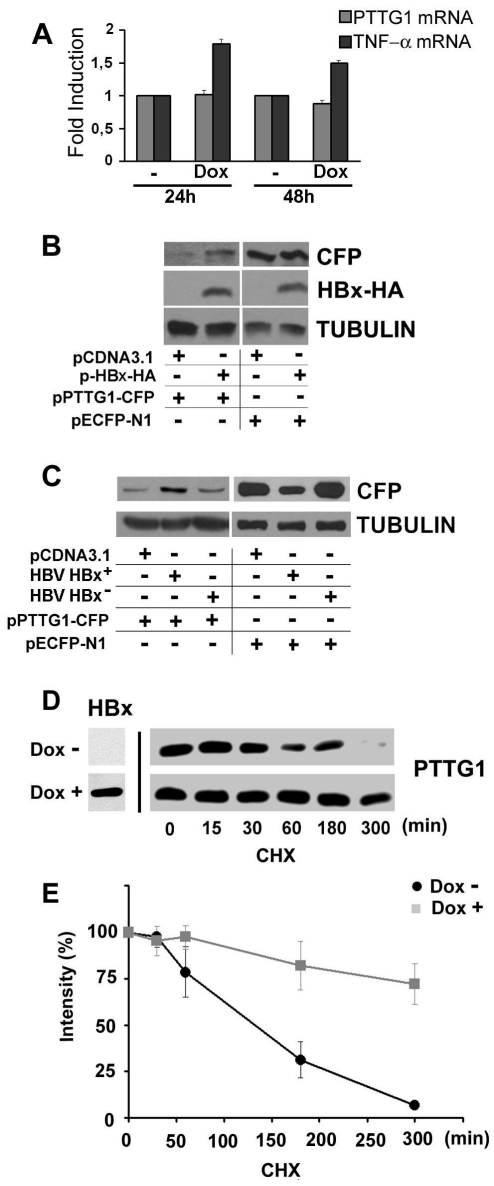
Molina-Jimenez et al. FIGURE 1
115x90mm (300 x 300 DPI)



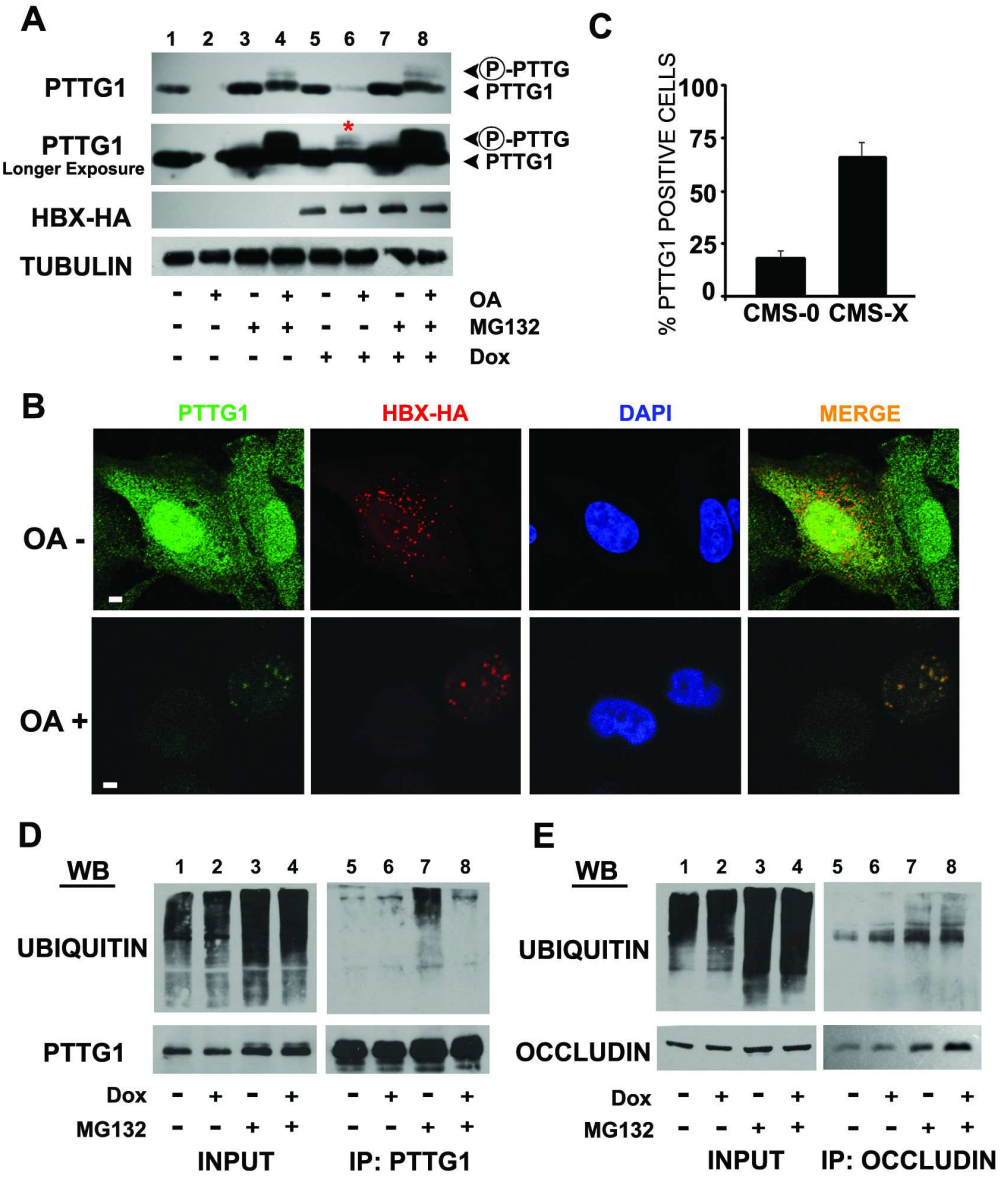
Molina-Jimenez et al. FIGURE 2
129x58mm (300 x 300 DPI)



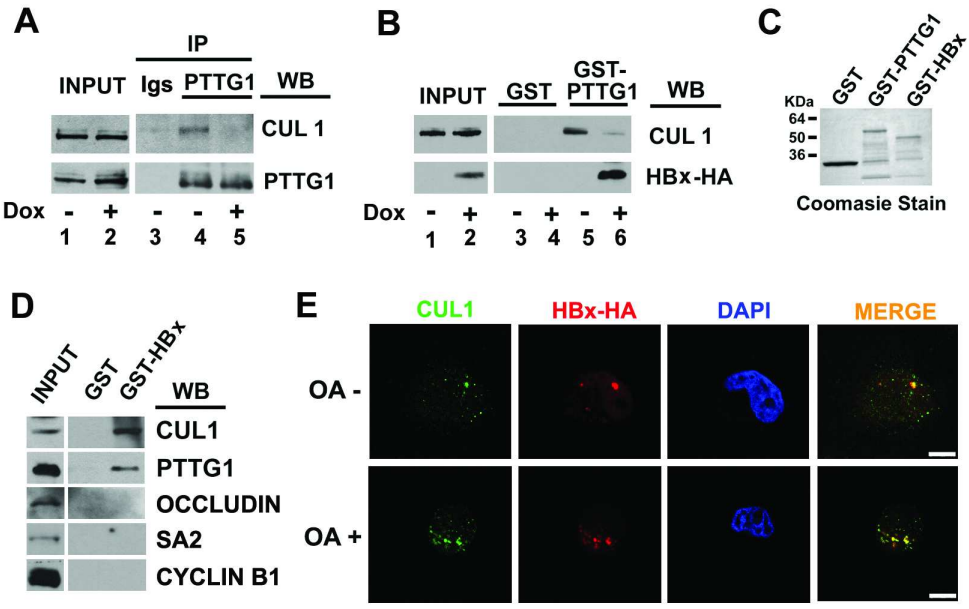
Molina-Jimenez et al. FIGURE 3
163x84mm (300 x 300 DPI)



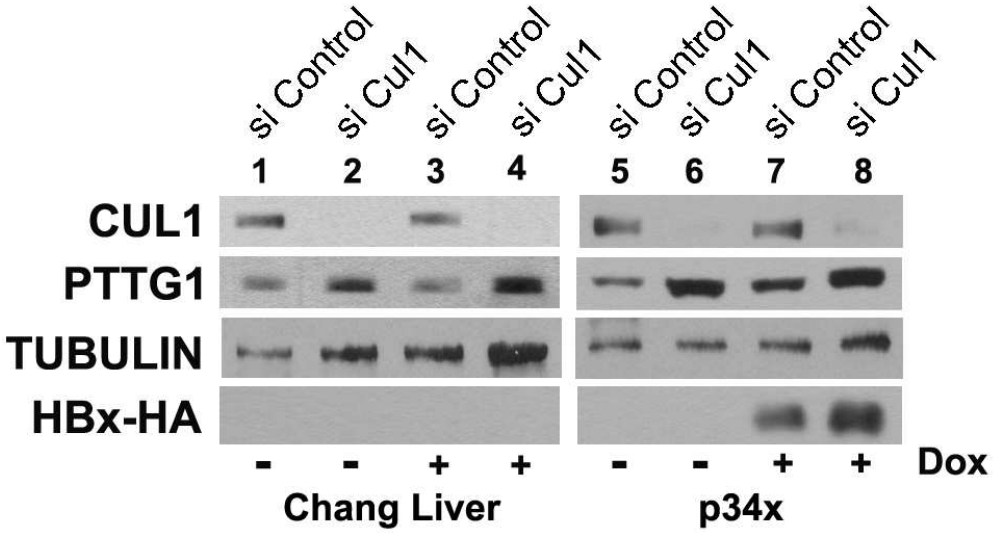
Molina-Jimenez et al. FIGURE 4
83x203mm (300 x 300 DPI)



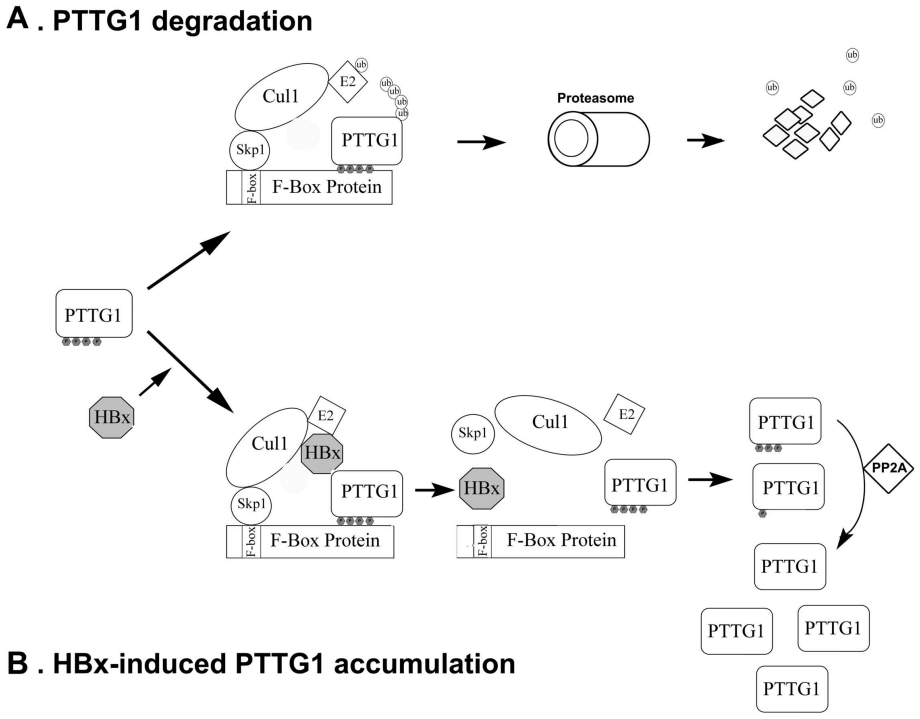
Molina-Jimenez et al. FIGURE 5
142x169mm (300 x 300 DPI)



Molina-Jimenez et al. FIGURE 6
141x86mm (300 x 300 DPI)



Molina-Jimenez et al. FIGURE 7
81x46mm (300 x 300 DPI)



Molina-Jimenez et al. FIGURE 8
105x80mm (600 x 600 DPI)

SUPPLEMENTARY DATA.

SUPPLEMENTARY MATERIALS AND METHODS.

Patients and transgenic mice.

Fifteen patients with HBV-related chronic liver disease underwent liver biopsy at the Department of Gastroenterology and Hepatology of Kansai Medical University Hospital between 1992 and 1994. All patients were seropositive for hepatitis B surface antigen (Abbott Laboratories, North Chicago, IL) and were seronegative for anti-HCV antibody (Ortho Diagnostics, Tokyo, Japan). Patients included 5 with chronic hepatitis, 5 with cirrhosis, and 5 with HCC. Written informed consent was obtained from each patient according to the Helsinki Declaration. We also obtained approval for this study from our ethics committee (52).

HBx transgenic mice were derived by microinjection of an 1151-bp HBV-DNA fragment containing the HBx gene with its own regulatory elements and polyadenylation signal into fertilized eggs of CD-1 mice. An independent line (H9) was derived from founders (30).

Immunohistochemical and immunofluorescence analyses.

After formalin fixation, specimens of human or mouse livers were dehydrated through graded alcohol series, embedded in paraffin, and sectioned at a thickness of 4 μm. Paraffin sections were then deparaffinized in xylene and rehydrated as described previously (53). Primary Abs used in this study included mouse monoclonal anti-HBx Ab (2 μg/mL; Abcam, Cambridge, UK), and rabbit polyclonal anti-PTTG1 Ab (2 μg/mL; Zymed Laboratory Inc., South San Francisco, CA).

For immunohistochemical analyses, sections exposed to primary Abs were then incubated with peroxidase-labeled polymer conjugated to goat anti-mouse or anti-rabbit IgG (DAKO, Glostrup, Denmark). Finally, sections were developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA), counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany), and mounted under coverslips.

For double-labeling immunofluorescence analyses, sections exposed to a pair of primary Abs (rabbit plus mouse) were then incubated in a 1:500 dilution of goat anti-rabbit IgG conjugated with a red fluorophore (Alexa Fluor 594; Molecular Probes, Eugene, OR) and goat anti-mouse IgG conjugated with a green fluorophore (Alexa Fluor 488; Molecular Probes). Images were obtained with a fluorescence microscope (Carl Zeiss Microimaging, Oberkochen, Germany).

Cell culture.

Chang liver and HeLa cells (CCL13 and CCL-2, respectively; American Type Culture Collection, Manassas, VA) were maintained at 37ºC with a 5% CO2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. It is important to point out that despite the ATCC catalogue states that Chang liver cells derive from cross-cell contamination with HeLa cells, it has been demonstrated that Chang liver cells are hepatic-derived cells, based on microarray experiments containing more than 6700 genes (54). Chang liver pX-34 (p34x) cells were kindly provided by Dr. Cho (Ajou University School of Medicine, Suwon, South Korea). These cells were grown as described (50), and HBx expression was induced by adding doxycycline (Dox), a tetracycline derivative, at 2 µg/ml for the indicated time. The stable transfectants AML12 4pX cells (4pX), derived from the parental AML12 4p cells established from the immortalized murine hepatocyte cell line AML12, were kindly provided by Dr. Andrisani (55). HBx expression in 4pX cells was induced by Dox removal.

Plasmid constructs, transfections and reagents.

The expression vector pcDNA-PTTG1 and the plasmid encoding GST-PTTG1 were previously described (28). The pPTTG1-CFP construct was generated by insertion of a PCR product coding for full length PTTG1 into the *Eco*RI and *Bam*HI sites of pECFP-N1, encoding the cyan fluorescent protein (CFP), a variant of green fluorescent protein (GFP) (Clontech Laboratories Inc, Mountain View, CA). The primers used were 5´-TAAAGAATTCGCC ACCATGGCTACTCTGA-3´ (forward) and 5´-TAATGGATCCCGAATATC TATGTCACA-3´ (reverse). The plasmids payw1.2 coding for a replication-

competent construct that contains 1,2 copies of the HBV genome and payw*7 containing a HBx-minus mutant under the control of their endogenous promoters have been described elsewhere (56). The expression vector pHBx-HA was obtained by subcloning into the *Eco*RI site of pcDNA3.1 a PCR product encoding the HBx open reading frame, cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA). The PCR was carried out with the primers 5'-ATTAGAATTCGCCACCATGGCTGCTAGGCTG-3' (forward) and 5'-TAAATTGGGCCCTTTAAGCGTAGTCTGGGACGTCGTATGGGT ATCTAGAGGCAGAGGTGAAAAAGTTGC-3' (reverse), using the expression vector pSLN-X-Flag as template (7). The expression vectors pSV-HBx and pSV-hygro have been previously described (16). The bicistronic expression vector pCMS-EGFP-HBx, a plasmid that constitutively expresses EGFP as a transfection marker and HBx protein, was generated by subcloning a product encoding the HBx open reading frame obtained from the pSV-X vector into pCMS-EGFP plasmid (Clontech).

The plasmid encoding GST-HBx was described elsewhere (57). The expression vector pCGN-HA-ubiquitin was kindly provided by Dr. Keiichi Nakayama (Kyushu University, Fukuoka, Japan). Transfections were performed employing Lipofectamine Transfection Reagent (Invitrogen) according to manufacturer's instructions. Doxycycline, cycloheximide and MG132 were purchased from Sigma (Sigma Aldrich, St. Louis, Missouri) and okadaic acid (OA) was obtained from Calbiochem (Cambridge, MA, USA).

Western blot.

Cells were lysed and protein levels were analyzed as previously described (58). Membranes were incubated overnight at 4ºC with the following primary antibodies: rabbit polyclonal anti-PTTG1 (Zymed, San Francisco, CA), rabbit polyclonal anti-occludin (Zymed), monoclonal anti-Cul-1 (Zymed), rabbit polyclonal anti-Cul1 (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-HA (12CA5; Boehringer Mannheim GmbH, Mannheim, Germany), monoclonal anti-ubiquitin (Santa Cruz); monoclonal anti-tubulin (Sigma Aldrich); rabbit anti-cyclin B1 (Santa Cruz Biotecnology) and rabbit policlonal anti-SA2 (59).To detect PTTG1-CFP protein an anti-GFP antibody was used (Santa Cruz). This antibody recognizes multiple GFP variants including CFP. Anti-HBx

antiserum was kindly provided by Dr B. Slagle (Baylor College of Medicine, Houston, TX). After washing, membranes were incubated with the corresponding peroxidase-labeled goat anti-mouse or anti-rabbit IgG (Pierce, Rockford, IL) and visualized with the SuperSignal West Pico Chemiluminiscent Substrate (Pierce).

Cell cycle analysis by flow cytometry.

Chang liver p34x and AML12 4px cells were grown with or without Dox for 24 and 48 h. Additionally, Chang liver cells were transfected with payw1.2 or pawy*7 plasmids and cultured for 24 h before processing. Cells were trypsinized, pelleted, and fixed with 70% cold ethanol for 30 min. After washing, samples were resuspended in PBS at a density of 2×10^6 cells/ml. An equal volume of propidium iodide solution, containing 200 µg/ml RNase (Sigma Aldrich), 20 µg/ml propidium iodide (Sigma Aldrich), and 0.1% Triton X-100 in PBS, was added to the cell suspension for 30 min at room temperature. A FACSCalibur flow cytometer (BD Bioscience, Franklin Lakes, NJ) was used to analyze DNA content; emitted light was measured at 675 nm.

Real-time quantitative RT-PCR analysis.

Total RNA was extracted with TRI Reagent (Ambion Inc., Austin, TX), and the cDNA was obtained from 1 µg of RNA by reverse transcription. Quantitative PCR was carried out in a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) using a SYBR Green kit (Roche Diagnostics GmbH) and three specific primer sets (5'-CAGGCACCCGTGTGGTTGCT-3' and 5'-AGGCTGGTGGGGCATCGAAC-3' for PTTG1 (27); 5'-GGCCTGTACCTCATCTACT-3' and 5'-CTCCCAGATAGATGGGCTCATA- 3' for TNF-α; 5'-AAAGCCGCTCGCAAGAGTGCG-3' and 5'-ACTTGCCTCCTGCAAAGCAC-3' for histone H3).

Immunofluorescence analysis and confocal microscopy

Cells were grown on coverslips, fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.1% NP-40 in PBS for 10 minutes at room temperature. Cells were blocked with TNB [0.1 M Tris-HCl, 0.15 M NaCl, 0.5% blocking reagent (Boehringer Mannheim GmbH)] for 30 minutes at 37ºC and

incubated with the indicated antibodies for 1 h at 37ºC. After washing, cells were incubated with the secondary antibodies Alexa 488, Alexa 568, conjugated goat anti-mouse or anti-rabbit, and streptavidin Rhodamine Red-X (Molecular Probes, Inc., Eugene, OR) for 20 minutes at 37ºC followed by counterstain with DAPI (Pierce). The coverslips were mounted on DakoCytomation Fluorescent Mounting Medium (DAKO A/S, Glostrup, Denmark). The preparations were analyzed with a Leica TCS-SP5 confocal microscope (Leica Microsystems, Heidelberg, Germany).

Detection of PTTG1 ubiquitination.

Chang liver p34x with or without Dox treatment and HeLa cells transiently transfected with pCGN-Ub-HA, pCDNA3.1-PTTG1 and pSV-hygro or pSV-HBX were cultured for 48 h. MG132 (10μM), a proteasome inhibitor, was added 4 h before lysis. Cells were washed with ice-cold PBS and lysed in buffer containing 150mM NaCl, 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.1% SDS, 1mM NaF, 1mM Na_3VO_4, 1mM PMSF and 2 μg/ml leupeptin. Following centrifugation (23,440 × g) at 4ºC for 20 minutes, the protein concentration of the supernatants was measured with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Cleared lysates (1 mg of protein) were then subjected to immunoprecipitation with 2 μg of anti-PTTG1, anti-occludin, and rabbit IgG (DAKO A/S) overnight at 4ºC. The immunocomplexes were captured with Protein A-Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 1 hour at 4ºC. Precipitates were washed three times with ice-cold lysis buffer, resuspended in Laemmli buffer and boiled for 10 minutes. Bound proteins were separated on a SDS-polyacrylamide gel and analyzed by Western blotting using the indicated antibodies.

Purification of recombinant proteins and pull-down assays.

GST, GST-PTTG1 and GST-HBx were expressed in *E. coli*, purified with glutathione-Sepharose 4B (Amersham Biosciences, Buckinghamshire, UK) and incubated with Dox induced Chang liver p34x cellular extracts as previously described (58). Bound proteins were analyzed by Western blot using the indicated antibodies.

Coimmunoprecipitation.

Chang liver p34x cells were grown with or without Dox for 48 h and were treated with okadaic acid (1 μM) and MG132 (10 μM) 2 h before lysis. Cleared lysates were subjected to immunoprecipitation as described above.

Small interference RNAs (siRNAs) and transfections.

Cells were transfected using the Dharmafect 1 protocol with 100 nM ON-TARGETplus SMARTpool siRNAs directed against human Cul1 or a nonspecific control siRNA (Dharmacon, Lafayette, CO). After 48 h, cellular extracts were used in pull-down assays or lysed to check knockdown efficiency by Western blot (see above).

Statistical analysis.

Results are given as mean ± SD or ± SE. After performing normality tests, comparison between groups was done by using the nonparametric Mann-Whitney U-test . P less that 0.05 was considered statistically significant. The statistical program GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA) was used.

SUPPLEMENTARY REFERENCES

52. Murata M, Matsuzaki K, Yoshida K, Sekimoto G, Tahashi Y, Mori S, Uemura Y, et al. Hepatitis B virus X protein shifts human hepatic transforming growth factor (TGF)-beta signaling from tumor suppression to oncogenesis in early chronic hepatitis B. Hepatology 2009;49:1203-1217.

53. Yoshida K, Matsuzaki K, Mori S, Tahashi Y, Yamagata H, Furukawa F, Seki T, et al. Transforming growth factor-beta and platelet-derived growth factor signal via c-Jun N-terminal kinase-dependent Smad2/3 phosphorylation in rat hepatic stellate cells after acute liver injury. Am J Pathol 2005;166:1029-1039.

54. Lee JS, Thorgeirsson SS. Functional and genomic implications of global gene expression profiles in cell lines from human hepatocellular cancer. Hepatology 2002;35:1134-1143.

55. Tarn C, Bilodeau ML, Hullinger RL, Andrisani OM. Differential immediate early gene expression in conditional hepatitis B virus pX-transforming versus nontransforming hepatocyte cell lines. J Biol Chem 1999;274:2327-2336.

56. Melegari M, Scaglioni PP, Wands JR. Cloning and characterization of a novel hepatitis B virus x binding protein that inhibits viral replication. J Virol 1998;72:1737-1743.

57. Murakami S, Cheong JH, Kaneko S. Human hepatitis virus X gene encodes a regulatory domain that represses transactivation of X protein. J Biol Chem 1994;269:15118-15123.

58. Benedicto I, Molina-Jimenez F, Barreiro O, Maldonado-Rodriguez A, Prieto J, Moreno-Otero R, Aldabe R, et al. Hepatitis C virus envelope components alter localization of hepatocyte tight junction-associated proteins and promote occludin retention in the endoplasmic reticulum. Hepatology 2008;48:1044-1053.

59. Prieto I, Pezzi N, Buesa JM, Kremer L, Barthelemy I, Carreiro C, Roncal F, et al. STAG2 and Rad21 mammalian mitotic cohesins are implicated in meiosis. EMBO Rep 2002;3:543-550.

SUPPLEMENTARY FIGURE LEGENDS.

Supplementary figure S1. HCC sections of HBx transgenic mouse livers were stained for immunofluorescence to simultaneously detect PTTG1 (red) and HBx (green). Yellow color indicates overlap of proteins. Bar, 50 μm.

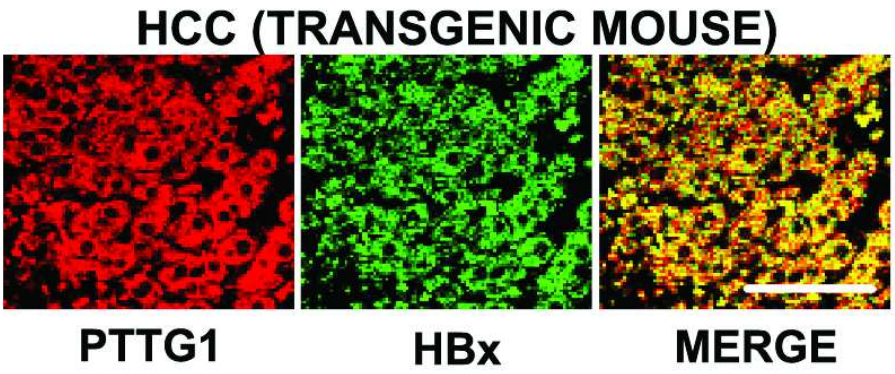
Supplementary figure S2. (A) Chang liver, p34X, AML12 4p and 4pX cells were cultured with or without Dox for 24 h, and HBx and PTTG1 protein levels were analyzed by Western blot. Tubulin expression was assessed to ensure equal protein loading of all samples. (B) Flow cytometry analysis of cell cycle

progression of p34X and 4pX cells 24 h after induction of HBx expression. Values represent the mean ± SD of three independent experiments performed at least in duplicate. (*p≤0,05 *vs* HBx non-expressing cells, Mann-Whitney U test).

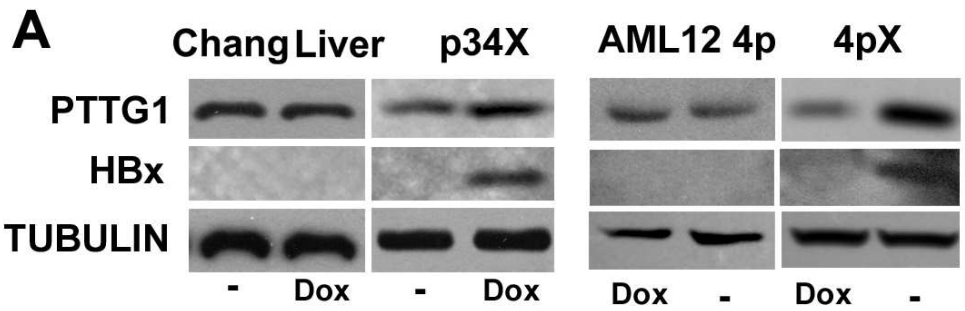
Supplementary figure S3. PTTG1 mRNA levels in 4pX cells in response to HBx expression were measured by quantitative RT-PCR. The results were normalized with histone H3 and represented as fold induction over control. Results are presented as the mean ± SD of three independent experiments.

Supplementary figure S4. (A) Chang liver cells were treated with okadaic acid (OA) (1 µM), MG132 (10 µM) or both for 2 h after 48 h of doxycycline treatment. Lysates were subsequently analyzed by Western blot for the detection of PTTG1. Tubulin expression was assessed to ensure equal protein loading. (B) Hela cells were co-transfected with pCGN-HA-ubiquitin, pcDNA-PTTG1 and either pSV-HBx or pSV-hygro plasmids and treated for 4 h with MG132 (10 µM). Cell lysates were immunoprecipitated with anti-PTTG1 mAb and immunoblotted with anti-PTTG1 (bottom) and anti-HA (top) Abs. Representative results of at least two independent experiments are shown.

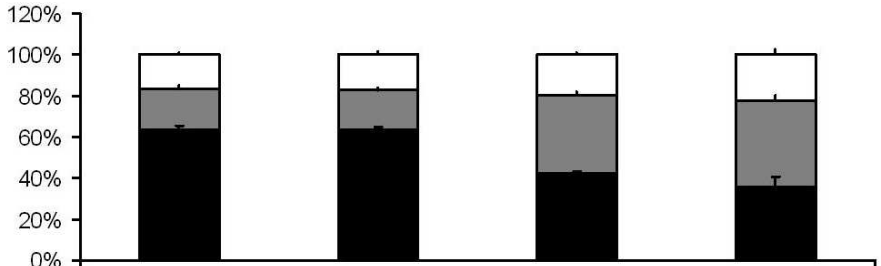
Supplementary figure S5. Chang liver cells were treated for 2 h with okadaic acid (OA; 1µM) and/or MG132 (10µM) after 48 h of transfection with control or Cul1 siRNA. Lysates were subsequently analyzed by Western blot for the detection of PTTG1 and Cul1. Tubulin expression was assessed to ensure equal protein loading.



75x34mm (300 x 300 DPI)

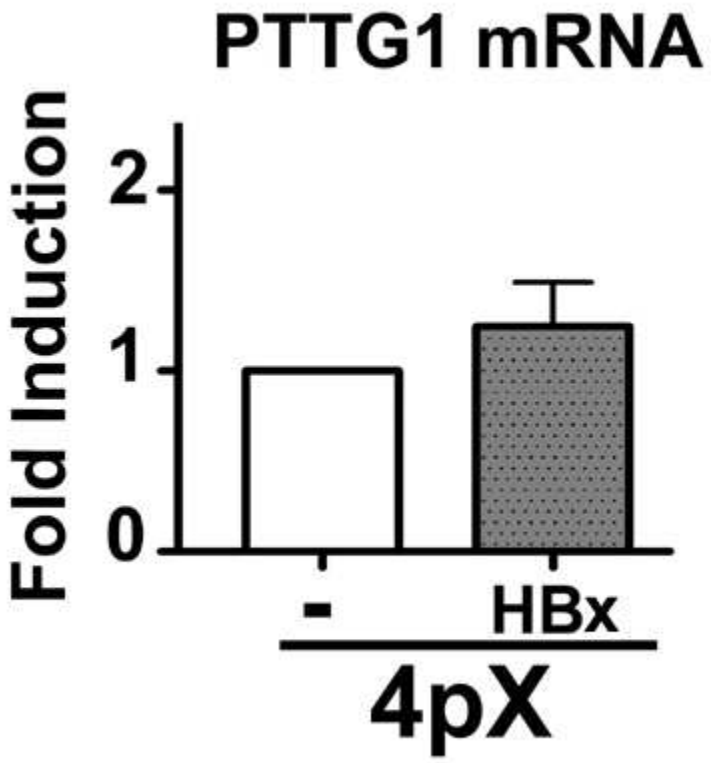


B

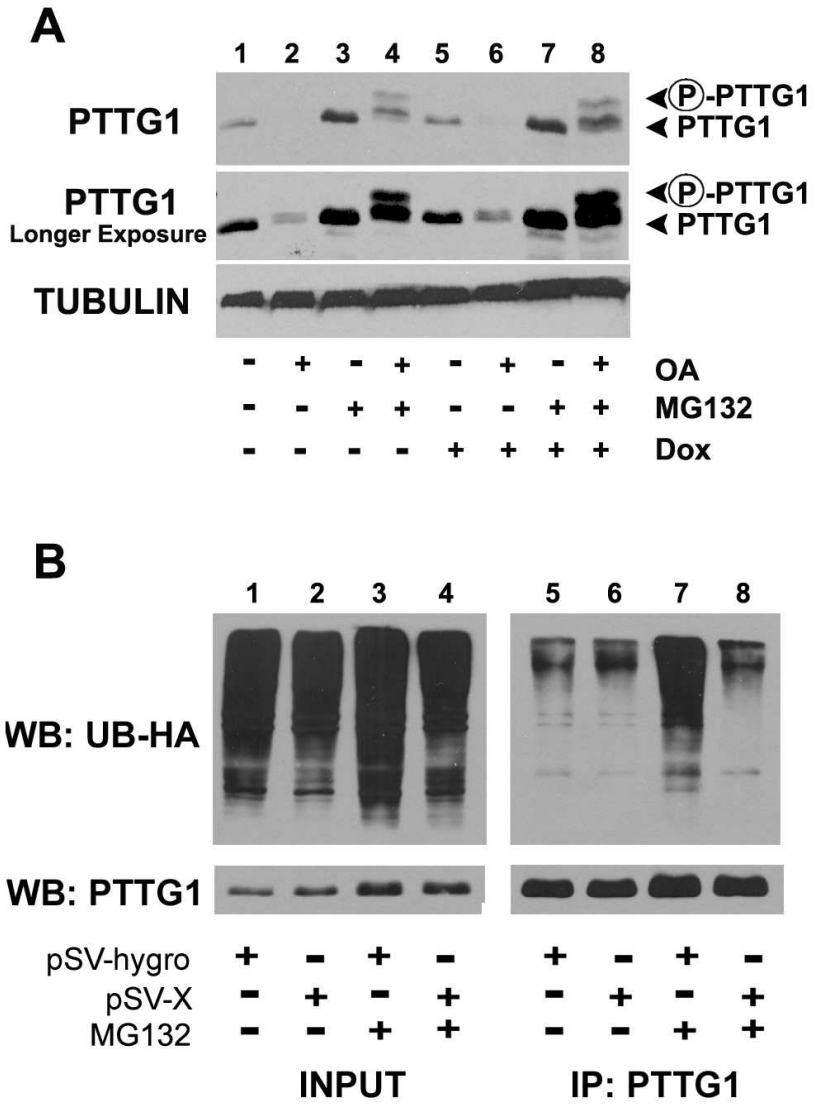


	p34X	p34X+Dox	4pX+Dox	4pX
G2-M	16,77	17,05	19,56	22,36
S	19,30	19,04	38,42	41,66 *
G0-G1	63,08	62,77	42,01	35,86 *

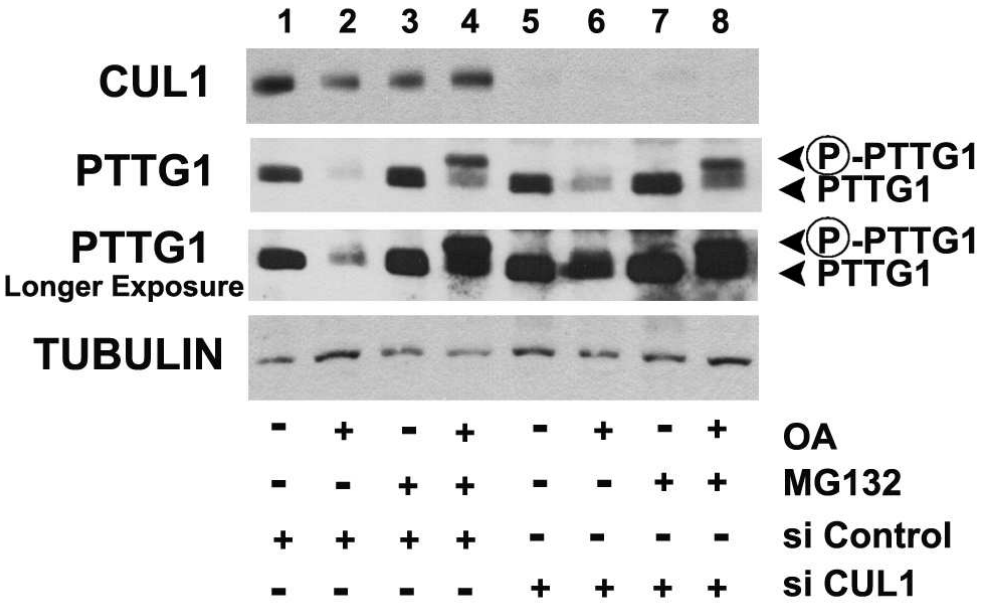
93x76mm (300 x 300 DPI)



35x40mm (300 x 300 DPI)



84x122mm (300 x 300 DPI)



84x54mm (300 x 300 DPI)