The hepatitis B virus encoded oncoprotein pX amplifies TGF-β family signaling through direct interaction with Smad4: potential mechanism of hepatitis B virus-induced liver fibrosis

Dug Keun Lee, Seok Hee Park, Youngsuk Yi, et al.

*Genes Dev.* 2001 15: 455-466
Access the most recent version at doi:10.1101/gad.856201

This article cites 51 articles, 28 of which can be accessed free at:
[http://genesdev.cshlp.org/content/15/4/455.full.html#ref-list-1](http://genesdev.cshlp.org/content/15/4/455.full.html#ref-list-1)

Article cited in:
[http://genesdev.cshlp.org/content/15/4/455.full.html#related-urls](http://genesdev.cshlp.org/content/15/4/455.full.html#related-urls)

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](http://genesdev.cshlp.org/subscriptions)
The hepatitis B virus encoded oncoprotein pX amplifies TGF-β family signaling through direct interaction with Smad4: potential mechanism of hepatitis B virus-induced liver fibrosis


Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20892-5055, USA, 1Department of Biochemistry and Molecular Biology, Ajou University School of Medicine, Suwon 442-721, Korea; 2The Weizmann Institute of Science, Rehovot 76100, Israel

Hepatitis B, one of the most common infectious diseases in the world, is closely associated with acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Many clinical investigations have revealed that hepatic fibrosis is an important component of these liver diseases caused by chronic hepatitis B. TGF-β signaling plays an important role in the pathogenesis of fibrosis in chronic hepatitis and cirrhosis. As these diseases are associated with hepatitis B virus (HBV) infection, we examined the possibility that the HBV-encoded pX oncoprotein regulates TGF-β signaling. We show that pX enhances transcriptional activity in response to TGF-β, BMP-2, and activin by stabilizing the complex of Smad4 with components of the basic transcriptional machinery. Additionally, confocal microscopic studies suggest that pX facilitates and potentiates the nuclear translocation of Smads, further enhancing TGF-β signaling. Our studies suggest a new paradigm for amplification of Smad-mediated signaling by an oncoprotein and suggest that enhanced Smad-mediated signaling may contribute to HBV-associated liver fibrosis.

[Key Words: Hepatitis B virus pX, Smad, TGF-β, fibrosis, signaling]

Received October 3, 2000; revised version accepted December 27, 2000.
One of these cytokines, TGF-β, shown to be important in the regulation of the production, degradation, and accumulation of extracellular matrix proteins (Robert and Sporn 1990; Massague and Chen 2000), appears to have a major regulatory role in hepatic fibrosis and cirrhosis as shown in animal models and human hepatic injury (Castilla et al. 1991; Bedossa et al. 1995; Sanderson et al. 1995). Recent studies have identified novel Smad proteins as signal transducers for members of the TGF-β superfamily. On ligand stimulation, receptor-regulated Smads (R-Smads) are phosphorylated by the type I receptor serine/threonine kinase, form complexes with the common mediator, Smad4, and translocate into the nucleus, where they activate transcription of target genes. Whereas R-Smads are restricted to pathways downstream from particular TGF-β family ligands, Smad4 plays a pivotal central role in signaling from the entire set of ligands (Heldin et al. 1997; Massague and Chen 2000). The association between HBV infection and primary hepatocellular carcinoma (PHC), and the high frequency of detection of the pX antigen in liver cells from patients with chronic hepatitis, cirrhosis, and liver cancer, suggested that there may be an association between the expression of pX and TGF-β signaling in the liver. Based on these observations, we thought that pX might alter TGF-β signaling.

Transcriptional activation of a TGF-β-responsive gene by the pX

To examine the role of pX in TGF-β-induced transcriptional activation, we cotransfected HepG2 cells with a pX expression construct and with either the TGF-β-responsive 3TP-lux reporter construct, p800-Luc, a fragment of the PAI-1 promoter (Dennier et al. 1998), or SBE4-luc, which contains four Smad binding element (SBE) sites in tandem (Zawel et al. 1998). Introduction of pX greatly enhanced the TGF-β-dependent activities of all three of these reporter gene constructs (Fig. 1A–C), suggesting that pX enhances TGF-β-induced transactivation. Similar results were obtained in liver stellate cells and NMUMG human breast cancer cells (data not shown).

The enhancement of the SBE4-luc reporter activity by pX suggests that it may directly amplify the transcriptional activation activity of Smad complexes. To determine the specificity of this interaction, we used two other Smad-dependent reporter constructs: ARE-lux, re-
responsive to activin (Chen et al. 1997), another tandemly repeated SBE reporter, and SBE-lux, derived from the Jun B promoter and responsive to BMPs [Jonk et al. 1998]. As with TGF-β1, introduction of pX enhanced both activin- and BMP-2-mediated activity (Fig. 1D,E), suggesting that pX may regulate the activity of a signaling component common to each of these pathways. Smad4 is a candidate for such a molecule, as it functions as a common mediator, or co-Smad, interacting with receptor-activated Smad downstream from TGF-β, activin, and BMP receptor complexes (Heldin et al. 1997; Massague and Chen 2000). As expected, pX failed to enhance the SBE4-luc activity experiment in the Smad4 null MDA-MB 468 human breast cancer cell line (Schutte et al. 1996). This result confirmed that enhancement of TGF-β-responsive SBE4-luc reporter activity by pX is dependent on Smad4 [Fig. 1F]. Consistent with the ability of pX to enhance basal transcription, we also observed a slight induction of the basal, uninduced transcription level of TGF-β-responsive reporters (Fig. 1). To show that pX has specific effect on Smad-mediated transcription, independent of its effects on the basal transcription factors, we examined the effect of pX on non-TGF-β responsive transcription factors, which depend on CBP/p300 as coactivator. PU.1 interacts with CBP (Yamamoto et al. 1999), and p300 interacts with the p65 subunit of NF-κB [Perkins et al. 1997]. In contrast to the strong transcriptional activation of Smad-dependent reporters by pX or by TGF-β, the transcriptional activity of Gal4-PU.1 and the C-terminal transcriptional activation domain of the p65 subunit of NF-κB linked to the Gal4 DNA-binding domain were not modulated by TGF-β1 treatment or pX [Fig. 1G].

**HBX interacts with Smads**

To examine the possibility that pX interacts directly with Smad proteins, we performed GST pull-down assays using HepG2 cells transfected with pX in a mammalian GST fusion vector along with Flag-tagged Smad expression constructs. Although there was a strong, ligand independent interaction between pX and Smad4 [Fig. 2A], pX formed complexes with Smad1, Smad2, or Smad3 only following ligand activation [Fig. 2B–D]. To examine the possibility that pX may interact directly with Smad1, Smad2, and Smad3 on ligand stimulation, we performed GST pull-down assays using Smad4-deficient cell lines: MDA-MB-468 human breast cancer cell line and SW-480 human colon cancer cell line (Zhang et al. 1996) transfected with GST-pX and flag-Smad3, and

![Figure 2](https://genesdev.cshlp.org/)

**Figure 2.** pX interacts with the Smad4. GST-pX was transfected into HepG2 cells with the Flag-tagged Smad1, Smad2, and Smad3 and Myc-tagged Smad4 constructs. Cells were treated with either BMP-2 [B] or TGF-β1 [A,C,D] for 1 h. Cell extracts were subjected to GST pull-down assay using glutathione-Sepharose beads followed by immunoblotting with anti-Flag or anti-Myc antibody. Expression of GST, GST-pX, and Smads was monitored as indicated. The interaction between Smads and pX was examined by GST pull-down assay in vitro. Biologically expressed GST-pX and GST alone were incubated with [35S]methionine-labeled Smad proteins [E]. Twenty-five percent of [35S]methionine-labeled Smad proteins used for the assay were applied as controls [Input].
treated with TGF-β. No significant association of Smad3 with pX was detected in MDA-MB-468 and SW-480 cells, although after long exposure we could detect a faint band in SW-480 cells (data not shown). This minimal association may represent a low affinity of the Smad3 for pX that is only observable following ligand stimulation and the subsequent conformational changes. The interaction between Smad proteins and pX was also studied by GST pull-down assays in vitro using 35S-labeled Smad1, Smad2, Smad3, and Smad4 proteins. Only pX interacted with 35S-labeled Smad4 [Fig. 2E].

HBX interacts with the Smad4 through the N-terminal domain

We next mapped the domain of pX responsible for interaction with Smad4 in vivo. Testing of pX deletion mutants in both binding and transcription assays showed that the full-length pX bound Smad4 (Fig. 3B), but that deletion of pX residues 1–72 abrogated both the direct interaction with Smad4 as well as the enhancement of TGF-β-induced transactivation of 3TP-lux (Fig. 3C). In contrast, deletion of only the first 51 or last 11 amino acids of pX did not inhibit its interaction with Smad4 and only slightly reduced transcriptional activation. These results suggest that the region of pX between residues 73 and 143 is required for interaction with Smad4 and transcriptional activation [Fig. 3C].

HBX interacts with the MH1 domain of Smad4

Similar GST pull-down assays were performed using GST-tagged pX along with various Flag-tagged Smad4 expression constructs to determine the domain of Smad4 interacting with pX. GST-pX was found to associate with the full-length and the N-terminal MH1 domain of Smad4, but not with the C-terminal MH2 or middle linker domains of this molecule [Fig. 3E], showing that the MH1 domain contained the pX interaction domain.

Induction of expression of the endogenous plasminogen activator inhibitor-1 (PAI-1) in NIH-3T3 cells stably expressing pX

One of the critical cellular activities of TGF-β is the transcriptional activation of matrix genes, including collagen, fibronectin, and plasminogen activation inhibitor-1 (PAI-1). To determine if pX could similarly induce the expression of PAI-1, NIH-3T3 cells were stably transfected with a pX expression vector. The expression of PAI-1 was measured by real-time PCR and Western blotting (Fig. 4A). The results showed that pX expression led to a significant increase in PAI-1 expression, with a peak at 24 h after transfection. These findings suggest that pX may play a role in regulating the expression of PAI-1 and other matrix genes in response to TGF-β stimulation.
1. Because pX activates TGF-β-induced transcriptional activation, we speculated that stable overexpression of pX in cells may augment the ability of these cells to induce expression of endogenous matrix genes in response to TGF-β and that this could be the molecular basis for the hepatic fibrosis associated with HBV infection. To test this hypothesis, we generated NIH3T3 cells stably expressing pX (Fig. 4A). The activation of the 3TP-Lux reporter in response to TGF-β was much greater in pX-expressing NIH-3T3 cells compared with control cells (Fig. 4B). We also confirmed that Smad4 interacted with pX in these cells by immunoprecipitation with Smad4 antibody and subsequent immunoblotting with anti-pX antibody (Fig. 4C). Although basal expression of PAI-1 mRNA was slightly higher in NIH-3T3-pX cells than in control NIH-3T3 cells, TGF-β-induced activation of PAI-1 mRNA expression was markedly enhanced in NIH-3T3-pX cells (Fig. 4D). The level of PAI-1 mRNA induced by either activin or BMP was also higher in pX expressing NIH-3T3 cells than in the control cells. Similar results were obtained with FAO rat hepatoma cells expressing pX (data not shown). These results suggest that pX can activate TGF-β-, activin-, or BMP-induced transcription in vivo.

**HBX enhances nuclear localization of Smads**

To determine whether pX might affect the subcellular localization of Smad4, we performed confocal microscopic analysis using anti-pX, anti-Smad4, and anti-Smad3 antibodies in NIH3T3 and NIH3T3-pX cells. In NIH3T3 cells, the TGF-β-induced nuclear accumulation of PAI-1 mRNA expression was markedly enhanced in NIH-3T3-pX cells (Fig. 4D). The level of PAI-1 mRNA induced by either activin or BMP was also higher in pX expressing NIH-3T3 cells than in the control cells. Similar results were obtained with FAO rat hepatoma cells expressing pX (data not shown). These results suggest that pX can activate TGF-β-, activin-, or BMP-induced transcription in vivo.

**HBX enhances nuclear localization of Smads**

To determine whether pX might affect the subcellular localization of Smad4, we performed confocal microscopic analysis using anti-pX, anti-Smad4, and anti-Smad3 antibodies in NIH3T3 and NIH3T3-pX cells. In NIH3T3 cells, the TGF-β-induced nuclear accumulation of PAI-1 mRNA expression was markedly enhanced in NIH-3T3-pX cells (Fig. 4D). The level of PAI-1 mRNA induced by either activin or BMP was also higher in pX expressing NIH-3T3 cells than in the control cells. Similar results were obtained with FAO rat hepatoma cells expressing pX (data not shown). These results suggest that pX can activate TGF-β-, activin-, or BMP-induced transcription in vivo.

**HBX enhances nuclear localization of Smads**

To determine whether pX might affect the subcellular localization of Smad4, we performed confocal microscopic analysis using anti-pX, anti-Smad4, and anti-Smad3 antibodies in NIH3T3 and NIH3T3-pX cells. In NIH3T3 cells, the TGF-β-induced nuclear accumulation of PAI-1 mRNA expression was markedly enhanced in NIH-3T3-pX cells (Fig. 4D). The level of PAI-1 mRNA induced by either activin or BMP was also higher in pX expressing NIH-3T3 cells than in the control cells. Similar results were obtained with FAO rat hepatoma cells expressing pX (data not shown). These results suggest that pX can activate TGF-β-, activin-, or BMP-induced transcription in vivo.

**HBX enhances nuclear localization of Smads**

To determine whether pX might affect the subcellular localization of Smad4, we performed confocal microscopic analysis using anti-pX, anti-Smad4, and anti-Smad3 antibodies in NIH3T3 and NIH3T3-pX cells. In NIH3T3 cells, the TGF-β-induced nuclear accumulation of PAI-1 mRNA expression was markedly enhanced in NIH-3T3-pX cells (Fig. 4D). The level of PAI-1 mRNA induced by either activin or BMP was also higher in pX expressing NIH-3T3 cells than in the control cells. Similar results were obtained with FAO rat hepatoma cells expressing pX (data not shown). These results suggest that pX can activate TGF-β-, activin-, or BMP-induced transcription in vivo.
of endogenous Smad4 peaked at 1 h of TGF-β treatment and was maintained even at 2 h after TGF-β treatment (Fig. 5A,b and C). In NIH3T3-pX cells, pX is found in both nuclear and cytoplasmic compartments, with a roughly equal distribution. Considerable variability to this distribution was observed, however, and Fig. 5 shows a cell showing predominantly a nuclear localization (Fig. 5B,b). In the NIH3T3-pX cells, most Smad4 also localized to the nucleus even without TGF-β treatment (Fig. 5B,C), suggesting that pX may facilitate the nuclear translocation of Smad4 protein both in the presence and absence of TGF-β signaling. Comparison of the subcellular distribution of endogenous Smad 4 and pX by confocal microscopy revealed extensive overlap in NIH3T3-pX cells (Fig. 5B,d). After TGF-β treatment, Smad 3 translocates to the nucleus both in NIH3T3 and NIH3T3-pX cells (Fig. 5D,E). However, more Smad3 was translocated into the nucleus in the presence of pX (Fig. 5E). This

---

**Figure 5.** Localization of the endogenous Smad and pX in NIH3T3 and NIH3T3-pX cells. (A) NIH3T3 cells were cultured for 2 h in the presence (b) or absence (a) of 5 ng/mL TGF-β1. The cells were incubated with rabbit anti-Smad4 antibody. TRITC-conjugated [red] anti-rabbit IgG was used as secondary antibody. Localization is shown by the red signal. (B) NIH3T3-pX cells were cultured in the absence of TGF-β. The cells were incubated with rabbit anti-Smad4 antibody [a] and mouse anti-pX monoclonal antibody [b]. TRITC-conjugated [red] anti-rabbit IgG [a] and FITC-conjugated [green] anti-mouse IgG [b] were used as secondary antibodies. Nuclei were visualized with DAPI [c]. Colocalization is shown by the yellow signal, generated by the overlay of the red and green signal (d). (C) The percentage of NIH3T3-pX cells showing nuclear staining of Smad4 in the absence of TGF-β. (D) NIH3T3-pX cells were cultured for 2 h in the presence (c) or absence (a) of 5 ng/mL TGF-β1 and incubated with rabbit anti-Smad4 antibody. TRITC-conjugated [red] anti-rabbit IgG was used as secondary antibody. Localization is shown by the red signal. Nuclei were visualized with DAPI [b,d]. (E) The percentage of NIH3T3 and NIH3T3-pX cells showing nuclear staining of Smad3 in the presence or absence of 5 ng/mL TGF-β.
enhancement of Smad-nuclear translocation by pX may contribute in part to effect of pX on basal transcription of Smad-dependent reporters.

**pX stabilizes the association of Smad proteins with TFIIB**

The transcriptional coactivators, CBP/p300, have been shown to interact with the Smads in the nucleus and to be required for their transcriptional activating activity (Feng et al. 1998). pX has been shown to bind to TFIIB and to target TFIIB in transcriptional activation, and the domain of pX implicated in this activity overlaps with that we have shown to be essential for Smad4 interaction and transcriptional activating activity (Feng et al. 1998). Therefore, we postulated that pX might stabilize the complex between TFIIB and Smads, thereby enhancing Smad-mediated transcription. To test this possibility, we examined whether TFIIB, p300, and Smads can be found in pX complexes using pull-down assays coupled with immunoblotting in HepG2 cells. As shown in Figure 6A, lysates of cells pulled down with glutathione-Sepharose beads and subjected to Western blotting using antibodies against either the HA-, Flag-, or Myc-epitope tags showed that TFIIB, Smad3, and Smad4 were each found in pX complexes. These data also suggest that the

---

**Figure 6.** pX interacts with both Smads and TFIIB. (A) HA-TFIIB was cotransfected with Flag-Smad3 or Myc-Smad4 either alone or together (lanes 4 and 8) or together with GST (lanes 1–4) or GST-pX (lanes 5–8). Whole cell lysates were reacted with glutathione-Sepharose beads for overnight, washed with lysis buffer containing 200 mM NaCl and 75 mM KCl, and detected by Western blotting with anti-Flag, anti-myc, or anti-HA antibody. Cell lysates were also blotted with anti-Flag, anti-myc, anti-GST, and anti-HA antibodies for control of Smads, TFIIB, and pX expression. (B) GST-pX or GST was coexpressed with Flag-Smad3 and Myc-Smad4. The cell lysates of transfected HepG2 cells were subjected to immunoprecipitation with anti-TFIIB antibody followed by immunoblotting with anti-Flag, anti-Myc, or anti-GST antibody. The upper three panels show the interaction and the lower two panels show the expression of each protein as indicated. I.p., immunoprecipitation. (C) HepG2 cells were transfected with the indicated combinations of plasmids. The cell lysates were subjected to GST pull-down assay followed by immunoblotting with antibodies indicated in the figure.
interaction of pX with TFIIB and Smad4 occurs in a ligand-independent manner. To examine whether Smad4 and TFIIB might interact, and whether this interaction is affected by pX, flag-tagged Smad3 and Myc-tagged Smad4 were transfected into HepG2 cells in the absence or presence of pX. The level of Smad4 co-purified with endogenous TFIIB was much higher in cells cotransfected with pX (Fig. 6B). Because it is known that Smads interact with p300 directly, we also investigated whether endogenous p300 might also be present in this protein complex. The lysates of cells transfected with Smad3, Smad4, and GST-pX and pulled down with glutathione-Sepharose beads contained p300 (Fig. 6C).

Taken together, these data suggest that pX has the capacity to form quaternary complexes containing pX-Smads-TFIIB and p300. To determine the functional significance of these complexes, we examined the ability of pX on TGF-β-dependent transcriptional responses. E1A is known to repress TGF-β-induced, Smad-dependent transcriptional activation by blocking the interaction of p300 with the receptor activated Smads (Nishihara et al. 1990). Because our data show that pX can mediate interaction of Smad4 with TFIIB, we hypothesized that pX would be able to bypass E1A-mediated suppression of Smad-dependent transcriptional activity. Cotransfection of E1A with the 3TP-lux reporter construct showed that full-length E1A is able to specifically suppress the TGF-β-induced reporter activity, whereas introduction of increasing amounts of pX resulted in a recovery in TGF-β-induced reporter back to the levels observed in the absence of E1A (Fig. 7A). However, given the continuing presence and inhibitory activity of the E1A protein in these cells, the maximal enhancement of reporter activity observed with pX alone (as shown in Fig. 1A) was no longer attainable. A deletion construct of E1A, in which the p300 interaction domain (residues 2–35) has been removed, was used as a control.

Similar results were obtained with the 4xSBE-lux reporter construct (Fig. 7B). This suggests that even though E1A inhibits the interaction between p300 and the Smads, pX can overcome this block, presumably by catalyzing and stabilizing the formation of a TFIIB/Smad complex in the absence of p300, resulting in TGF-β- and Smad-dependent transcriptional activation.

Discussion

In this study, we have shown that pX can stabilize the association of the Smad complex with the transcriptional machinery, including TFIIB (Fig. 8). Our data additionally suggest that pX facilitates the nuclear translocation of Smad4 protein even in the absence of TGF-β signaling, and it enhances the nuclear translocation of Smad1, 2, and 3 on ligand stimulation. Both of these effects likely contribute to the ability to amplify Smad-dependent signaling.

It has been shown that Smads cooperate with DNA-binding partners to regulate transcription of target genes (Heldin et al. 1997; Derynck et al. 1998; de Caestecker et al. 2000). Smad2 and/or Smad3 can associate with AP-1, ATF2, TFE3, PEBP2/CBF, and the vitamin D receptor. The transcriptional activity of Smads also depends on interactions with coactivators within the complex. CBP and p300 proteins are important transcriptional coactivators for Smad activity (Feng et al. 1998; Pouponnot et al. 1998; Topper et al. 1998). Both R-Smads and Smad4
can activate transcription by recruiting the coactivators CBP/p300 and MSG1, respectively [Janknecht et al. 1998; Pouponnot et al. 1998; Shiota et al. 1998; Yahata et al. 2000].

Alternatively, they can also recruit corepressors, such as TGFβ or Ski family members, which, in turn, bind histone deacetylases [Akiyoshi et al. 1999; Luo et al. 1999; Sun et al. 1999; Wotton et al. 1999]. A Ski-related protein, Snon, represses transactivation of Smads by recruitment of the transcriptional corepressor N-CoR [Stroscio et al. 1999]. The activity of Smad3 can also be blocked by interactions with the nuclear oncproteins, E1A or Evi-1 [Kurokawa et al. 1998; Nishihara et al. 1999], which inhibit the interaction of Smad3 and p300. Thus, Smad-interacting proteins can both positively or negatively regulate transcription of specific genes in response to TGFβ family signaling. In this study, we have shown that another Smad-interacting protein, the viral oncprotein pX, can function as a transcriptional coactivator for Smad activity by stabilizing the Smad complex with the general transcriptional machinery, thereby amplifying the transcriptional activity of the complex.

pX activates transcription of a vast number of promoters of cellular genes through a distinct DNA cis element [Seto et al. 1990; Maguire et al. 1991; Yoo et al. 1996]. We have shown that pX enhances transcriptional activity of the PAI-l promoter in response to TGFβ. In contrast, another group has recently reported that the stable overexpression of pX has no effect on the TGFβ-mediated induction of the PAI-l p800-luc reporter in Hep 3B human hepatoma cell line [Shih et al. 2000]. This apparent discrepancy may be because the Hep 3B cells contain an integrated HBV genome, whereas NIH3T3 and Hep G2 cells do not carry the HBV genome. Thus, the introduction of pX may not have the same effect in Hep 3B cells because of the potential role of HBV genome and the potential levels of pX already existing in those cells.

pX has been reported to bind p53, TFIIB, and a cellular protein associated with DNA repair [Wang et al. 1994; Truant et al. 1995; Haviv et al. 1998]. These findings suggest a possible role of pX in HBV replication, as well as the potential to disturb growth control and DNA repair in host cells. Malignant transformation has been observed in some pX-transfected cell lines and in HBV-X transgenic mice, suggesting that the HBV-X gene plays an important role in neoplastic transformation of hepatocytes in HBV-infected livers [Chisari et al. 1989; Kim et al. 1991]. Recent study showed that the expression of pX among the three HBV antigens (pX, surface antigen, and core antigen) examined was preferentially maintained throughout the multistage process from foci and nodules of altered hepatocytes, for which a preneoplastic nature has been shown, to HCC [Su et al. 1998], suggesting the important role of the pX in HBV-associated hepatocarcinogenesis in humans. The strong association of persistent HBV infection and HCC is intriguing, yet the mechanism of oncogenesis has not been elucidated. The risk of HCC is increased 10-fold to 390-fold in patients chronically infected with HBV. Cirrhosis of the liver is present in more than 90% of HBV-related HCC, and the chronic inflammation and cellular proliferation and regeneration associated with cirrhosis may lead to a predisposition to cellular transformation and frank malignancy. An alternative and intriguing possibility is that pX may be augmenting specific oncogenic activities of TGFβ such as may result from synergistic interaction between Smad and MAPK signaling pathways, including activation of API sites, important in invasion and metastases, as well as the transduction of signals mediating autoinduction of TGFβ, itself associated with malignant transformation [de Caestecker et al. 1998].

TGFβ is an important cytokine in the pathophysiology of liver fibrosis, stimulating the production of extracellular matrix [Robert and Sporn 1990]. In a number of epithelia, repeated or prolonged injury leads to progressive fibrosis and subsequent development of excessive, unwanted scarring. The late stage of this process in the liver is termed cirrhosis. TGFβ appears to have a major regulatory role in this process [Castilla et al. 1991], Nage et al. 1991; Bodoessa et al. 1995; Sanderson et al. 1995; Yoo et al. 1996; De Bleser et al. 1997; Qi et al. 1999]. In hepatic fibrosis, the hepatic extracellular matrix proteins, including collagens, glycoproteins, and glucosaminoglycans, are markedly increased. In both experimental models of hepatic fibrosis and in patients with liver cirrhosis, increased expression of type I collagen genes is detected [Castilla et al. 1991], Nage et al. 1991]. Transgenic mice overexpressing TGFβ1 are characterized by fibrosis in many organs, including the liver [Sanderson et al. 1995]. The TGFβs also stimulate type I collagen gene expression in primary cultures of hepatocytes, Ito cells, and fibroblasts. Castilla et al. 1991] have shown that the level of TGFβ1 mRNA in liver biopsy specimens correlated positively with hepatic fibrosis in a large group of patients with chronic viral
hepatitis, suggesting that TGF-β1 may play a role in the pathogenesis of hepatic fibrosis.

Our data now suggest new insights into the mechanisms of disease pathogenesis resulting from HBV infection. Our data suggest that pX, which is present in HBV-infected liver cells including hepatocytes and Kupffer cells, binds to Smad4 and enhances its transcriptional activating activity, including, especially, its activation of genes involved in extracellular matrix production and, possibly, also expression of the TGF-β1 gene, which we previously showed to be enhanced by pX (Yoo et al. 1991). pX-dependent increases in TGF-β1 levels may further activate liver cells in an autocrine or paracrine manner, increasing extracellular matrix production and leading eventually to fibrosis and cirrhosis. A recent report suggests that activin is also involved in liver fibrosis and cirrhosis, because activin is overexpressed in rat cirrhotic and fibrotic livers (Sugiyama et al. 1998). Our results suggest that pX can amplify signaling not only by TGF-β, but also by activin and BMP, and that this, in turn, may contribute to hepatic fibrosis associated with the hepatitis B virus.

Materials and methods

Constructs

Flag-tagged Smad 4 deletion constructs and GST-pX deletion constructs were generated by polymerase chain reaction (PCR) using a proofreading polymerase and subcloned into pcDNA3 (L-MH2 and MH2 domains of Smad4), EF-Flag (MH1 and L-MH1 domains of Smad4), or mammalian GST fusion vectors. All PCR-generated products were sequenced using the dideoxynucleotide method.

Generation of NIH-3T3 cell lines expressing pX

The HBV-X of hepatitis B virus was PCR amplified, restriction digested, and purified to be subcloned into the MFG vector (Chang et al. 1997). An IRES-NEO cassette was also subcloned into the constructs to obtain the stable transfectants. The control vector, MFG-CAT, was described previously (Ohashi et al. 1992).

Northern blot analysis

Total RNA was isolated using TRIZOL reagent (GIBCO BRL), and 10 µg of each sample was separated on 1% agarose 0.66 M formaldehyde gels, transferred onto Zeta-Probe (Bio-Rad) in 10× SSC for 4 h, and covalently bound using a ultraviolet Stratagene formaldehyde. After transfection, all cell lines were washed four times with the buffer containing 200 mM NaCl and 75 mM KCl. Western blots were performed. Immunoprecipitation were performed by incubation with anti-HA (Santa Cruz) for 1 h. After immunoprecipititates were washed four times with the buffer containing 200 mM NaCl and 75 mM KCl, Western blot were prepared.

Immunofluorescence

NIH3T3 and NIH3T3-pX cells were cultured in the presence or absence of 5 ng/mL TGF-β1 for 2 h. Endogenous Smad 3/4 proteins or pX protein were detected by incubating with anti-rabbit Smad3/4 antibodies or pX mouse monoclonal antibodies overnight at room temperature, followed by incubation with goat anti-mouse FITC or TRITC-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature. The cells were mounted with medium containing DAPI (Vector Labs). Cells were visualized by use of a fluorescence microscope.

Acknowledgments

We would like to thank Drs. S. Kern, J. Wrana, K. Miyazono, D. Luskutof, and J. Massague for reagents. We also thank Genetics Institute for BMP2.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.

References


Lee et al.


