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Biochemical and Biophysical Research Communications 351 (2006) 253–258
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The interaction of hepatitis B virus X protein and protein phosphatase type 2 Cα and its effect on IL-6

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Received 18 September 2006
Available online 20 October 2006

Abstract

HBx has been suggested as an important determinant mediating the pathological effects of HBV via interacting with various cellular proteins. To identify new HBx-interacting proteins and elucidate a possible mechanism associated with HBx and HBx-interacting proteins in hepatocellular carcinoma, yeast two-hybrid screening was performed. We identified a novel HBx-interacting protein, serine/threonine protein phosphatase PP2Cα, and investigated the effects of PP2Cα on HBx-mediated IL-6 regulation. The interaction between endogenous PP2Cα, and HBx was confirmed by co-immunoprecipitation. Recombinant HBx dose-dependently reduced enzyme activity of recombinant PP2Cα *in vitro*. While ectopically expressed PP2Cα in Cos-7 and Huh-7 cells reduced the expression of IL-6, overexpressed HBx with recombinant HBx-expressing adenovirus overcame PP2Cα-mediated IL-6 downregulation. In the response of IL-6, HBx phosphorylated STAT3 and recovered PP2Cα-mediated dephosphorylation of STAT3. These results supported that HBx might play a crucial role in HBV-associated hepatocarcinogenesis even in cases where cells express a negative regulator, PP2Cα.

Keywords: PP2Cα; HBx; IL-6; Hepatocellular carcinoma

Hepatitis B virus (HBV) plays a major role in the development of hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [1]. Among the important etiologic factors for human hepatocarcinoma, the hepatitis B virus X protein (HBx) is one of the most important determinants mediating the pathological effects of HBV [2]. HBx can affect various signal transduction pathways such as NF-κB, Ras-Raf-MAPK, SAPK/JNK, PI3K-Akt, and JAK/STAT via protein–protein interaction [3,4]. While it has been reported that HBx plays an important role in hepatocarcinogenesis, further elucidation of the molecular mechanisms of HBx associated with hepatocarcinogenesis is required for understanding HBV-related HCC.

IL-6 is a pleiotropic cytokine that functions as an autocrine or paracrine growth factor in various tumor cells [5]. IL-6 regulates the synthesis of a broad spectrum of acute-phase proteins in the liver and is thought to be associated with the immunoregulatory perturbations in patients with chronic liver diseases. High levels of IL-6 are found in patients with chronic hepatitis B, liver cirrhosis, and HCC caused by HBV [6], and IL-6 can be induced in HBx overexpressed HepG2 cells [7]. Upregulated IL-6 phosphorylates and activates STAT3, which is transcription factor related with IL-6-mediated cell growth, differentiation, and survival, leading to cancer [8], and constitutively phosphorylated STAT3 is detected in HBx overexpressed HeG2 cells [9].

Protein phosphorylation/dephosphorylation is important in the regulation of cellular functions such as cell proliferation, differentiation, as well as cell death. Eukaryotic

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.10.028

protein phosphatases have been classified into two families, serine/threonine and tyrosine phosphatases. Serine/threonine phosphatases are further divided into four classes (PP1, PP2A, PP2B, and PP2C) [10]. Overexpression of PP2Cα shows anti-proliferative effect by inhibition of cell cycle progression and induction of apoptosis [11]. Although purified PP2A dephosphorylates STAT3 *in vitro* [12], and treatment of okadaic acid, an inhibitor of PP2A, increased IL-6 production in mast cells [13], the role of PP2Cα in IL-6 signal transduction is undetermined.

To investigate the newly possible role and molecular mechanisms of HBx in HBV-mediated hepatocarcinogenesis, we identified a novel HBx-interacting protein, PP2Cα, and determined the possible roles of HBx in cells overexpressing PP2Cα. We found that HBx interacted with PP2Cα and inhibited phosphatase activity of PP2Cα. HBx restored the PP2Cα-induced downregulation of IL-6, and dephosphorylation of STAT3 in Cos-7 and Huh-7 cells. HBx was shown to have a positive effect on the ability of colony formation that was suppressed by PP2Cα. Based on our results, we suggest that HBx may play an important role in hepatocarcinogenesis via inhibition of phosphatase activity of PP2Cα and its negative role in cell growth and proliferation.

Materials and methods

Cell culture. Human Chang liver (ATCC CCL13) and Chang X-34 cells were maintained in DMEM (Gibco-BRL, Grand Island, NY) containing 10% FBS (Gibco-BRL). The Chang X-34 cells are Chang cells expressing HBx under the control of tetracycline inducible promoter [14]. HBx was induced with 2 μg/ml doxycycline (Sigma, St. Louis, MO). Cos-7 (ATCC CRL 1651) and Huh-7 cells (JCRB 0403) (HSRRB, Osaka, Japan) were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere containing 5% CO_2.

Reagents. Rat anti-IL-6 and mouse anti-STAT3 antibodies were purchased from Pharmingen (San Jose, CA). Mouse anti-HA antibody and mouse anti-PP2Cα antibody were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and ATGen (Seoul, Korea), respectively. Anti-PP2Cα antibody for immunoprecipitation was purchased from Abcam (Cambridge, UK). Mouse anti-α tubulin antibody was purchased from Oncogene Research Products (Boston, MA). Recombinant human IL-6 (rhIL-6) was purchased from Biosource International Inc. (Camarillo, CA). Rabbit anti-phospho-STAT3 antibody was purchased from Cell Signaling Technology Inc. (Beverly, MA). GolgiStop was purchased from Pharmingen.

Yeast two-hybrid screening. The yeast two-hybrid interaction cloning protocol has been previously described [15]. Briefly, to express the HBx protein as a LexA DNA-binding domain fusion protein, the HBx gene derived from an HBV adr strain was subcloned into the *Mlu*I and *Not*I site of a bait pLexA202 vector. The HBx-bait plasmid (pLexA-HBx) was transformed into yeast strain EGY48 with lithium acetate method. Two reporter constructs were used for the selection of the candidate clones: one was based on complete minimal (CM) dropout media for auxotrophy and the second was based on β-galactosidase activity. For screening of targets, a human Daudi B cell cDNA library fused to B42 activator domain in the pCgatrp2 vector (pCgatrp2-cDNA) was transformed into the EGY48 expressing LexA-HBx protein. A pool of cells containing 2×10^6 primary library transformants was plated onto appropriate selection plates and grown clones were transferred onto the plates containing X-Gal. HBx-interacting cDNA sequences isolated from yeast clones were identified using the sequence database.

Cloning of human PP2Cα plasmid (pPP2Cα), and HIS-PP2Cα and -HBx for bacterial expression. For mammalian expression of PP2Cα, the human PP2Cα cDNA was obtained by RT-PCR using MMLV-RT (Gibco-BRL) of total RNA of Chang cells. The primers used for the cloning of PP2Cα cDNA were (sense) 5′-CGGATCCTAATGGGAG CATTTTTAGACAAGC-3′ and (antisense) 5′-CCCAAGCTTCCCA CATATCATCTGTTGATGT-3′. The resulting PCR product was cloned into the *Bam*HI and *Hin*dIII cloning sites of pcDNA3.1/Myc-His and confirmed by DNA sequencing. For bacterial expression of PP2Cα and HBx, RT-PCR was performed with the primers (sense) 5′-CGGATCC TAATGGGAGCATTTTTAGACAAGC-3 and (antisense) 5′-CCCAAG CTTTTACCACATATCTGTTGATGT-3′ for HIS-PP2Cα, and (sense) 5′-CGGAATTCCGATGGCTGCTAGGAT-3′ and (antisense) 5′-CCCCAAGCTTTTAGGCAGAGGTGAA-3′ for HIS-HBx. The resulting PCR products were cloned into the *Eco*RI and *Hin*dIII cloning sites of pRSET bacterial expression vector and confirmed by DNA sequencing.

Purification of recombinant protein and in vitro phosphatase assay. The recombinant plasmids, HIS-PP2Cα and –HBx, were transformed into *Escherichia coli* BL21(DE3), and transformants were cultured at 37 °C with 1 mM IPTG to induce expression. The expression of recombinant proteins, rPP2Cα and rHBx, preparation of inclusion body, and refolding of denatured proteins were performed as previously described [16]. Purified recombinant proteins were verified with Western blot analysis with anti-HIS antibody. For the *in vitro* phosphatase assay, rPP2Cα and rHBx were diluted prior to use with 50 mM Tris, pH 7.5, 2 mM DTT, 10 mM $MnCl_2$, and 50 mM pNPP added as a substrate. The initial rate of liberation of *p*-nitrophenol was determined with spectrophotometry at 405 nm [17].

RT-PCR. Total RNA was isolated using an RNeasy kit (Qiagen, Santa Clarita, CA). cDNA was synthesized by reverse transcription with 5 μg total RNA, 0.5 μg of random hexamer (Promega), 1.25 mM dNTP (Boehringer-Mannheim, Mannheim, Germany), and 200 U MMLV-RT in a 20 μl reaction. PCR was performed with 3 μl cDNA, 10 pmol of primer sets, 0.25 mM dNTP, and 2 U of *Taq* polymerase (Perkin-Elmer, Norwalk, CT). PCR cycling conditions were as follows: 23–35 cycles of denaturation at 94 °C for 30 s, annealing at 56–60 °C for 30 s, and extension at 72 °C for 30 s. The primer sequences used were as follows. *HBx*, (sense) 5′-CGGAATTCCGATGGCTGCTAGGAT-3′ and (antisense) 5′-CGGGGTACCCCGTTAGGCAGAGGT-3′; *PP2Cα*, (sense) 5′-CGGATCCTAATGGGAGCATTTTTAGACAAGC-3′ and (antisense) 5′-CCCAAGCTTCCCACATATCATCTGTTGATGT-3′; *IL-6*, (sense) 5′-ATGAACTCCTTCTCCACAAGCGC-3′ and (antisense) 5′-GAAGAGCCCTCAGGGTGGACTG-3′; and *β-actin*, (sense) 5′-CGT GGGCCGCCCTAGGCACCA-3′ and (antisense) 5′-TTGGCTTAGG GTTCAGGGGGG-3′. The PCR products were analyzed by agarose gel electrophoresis.

Immunoprecipitation. Chang X-34 cells were treated with 2 μg/ml doxycycline for 24 h and lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4, 0.5 mM EDTA, 1.8 μg/ml aprotinin, 100 mM NaCl, 0.2% NP-40, 2 mM $MgCl_2$, and 0.5 mM PMSF). Cell lysates (500 μg) were incubated with anti-PP2Cα Ab, added with protein A–Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden), and Western blotted with an anti-HA antibody. For Western blotting, 50 μg of cell lysates was used on an 8–12% SDS–PAGE followed by immunoblotting with appropriate antibodies. Proteins were visualized by using Western Blotting Luminol Reagent (Amersham Pharmacia Biotech).

Preparation of HBx-recombinant adenovirus. The HBx-recombinant adenovirus (Ad-GFP-HBx) was used in the study as previously described [18]. Briefly, the cDNA of HBx was first subcloned into the shuttle plasmid pShuttle-CMV, thus generating pAdTrack-CMV-HBx. Homologous recombination of pAdTrack-CMV-HBx and pAdEasy-1 was performed in bacteria BJ5183, and pAd-GFP-HBx, the newly recombinant plasmid, was selected by kanamycin and confirmed by restriction endonuclease analysis. Recombinant pAd-GFP-HBx was linearized with *Pac*I and transferred into 293 cells to allow expression of the recombinant adenovirus Ad-GFP-HBx. To obtain virus stocks, the virus was purified from 293 cells. After six cycles of freezing and thawing, cell debris was removed by subjecting the lysed cells to cesium chloride gradient centrifugation. Concentrated virus was dialyzed and stored at −80 °C.

Colony formation assay. Cells (1×10^5 cells) were transiently transfected with 0.5 μg pPP2Cα using Lipofectamine (Invitrogen, Carlsbad, CA), incubated in complete media for 24 h, and transduced with Ad-GFP (mock control) or Ad-GFP-HBx. After 48 h of incubation in low serum condition, cells were washed with PBS and fixed with 4% paraformaldehyde [19]. The fixed cells were washed with PBS and stained with 1% crystal violet.

Results

Identification of a novel HBx-interacting protein, PP2Cα

To identify novel HBx-interacting proteins and obtain new insights in its putative cellular function of the interacting protein, we screened a Daudi cDNA library using a yeast two-hybrid system. Full length of HBx gene prepared by PCR was subcloned into pLexA202 vector resulting in bait pLexA-HBx. The cDNA library was fused with the B42 activator domain in the pCgatrp2 vector. Yeast transformants expressing HBx protein were transformed with the B42 fusion cDNA library. Yeasts forming colonies, which carried the pLexA-HBx and pCgatrp2-cDNA in the CM dropout plate, were transferred onto plates containing X-Gal. Of the numerous double positive clones which expressed HBx-interacting cDNAs, 50 clones were clustered by the same patterns based on restriction fragment size analysis, and the genes were identified by DNA sequencing. Based on the sequencing analysis, 3 of 50 double positive clones shared an identical sequence that encoded PP2Cα. To exclude effects of bait vector sequence on the interaction of between HBx and PP2Cα, we changed bait vector (pCgatrp3) and constructed another bait plasmid, pCgatrp3-PP2Cα, which included a full length PP2Cα. HBx interacted with PP2Cα as determined by a β-galactosidase activity (Fig. 1A). To confirm the specificity of the interaction between HBx and PP2Cα in mammalian cells, an immunoprecipitation assay was performed. Compared to Chang cells, HBx interacted with endogenous PP2Cα in Chang X-34 cells treated with doxycycline (Fig. 1B).

Effect of HBx on in vitro phosphatase activity of PP2Cα

To investigate the functional interaction of HBx and PP2Cα, an *in vitro* phosphatase assay was performed with rPP2Cα and rHBx. Recombinant proteins were verified by Western blot with anti-HIS antibody (Fig. 1D). The enzyme activity of rPP2Cα appeared to be dose-dependent and the activity was saturated at almost 10 μg rPP2Cα (Fig. 1C). When rHBx was added, the enzyme activity of rPP2Cα was suppressed in a dose-dependent manner (Fig. 1D). These data suggest that HBx might inhibit the enzyme activity of PP2Cα via direct or indirect association with PP2Cα, but ultimately functioning as a negative regulator of PP2Cα.

Effect of HBx and PP2Cα on expression of IL-6

High levels of IL-6 are found in patients with HBV infection and acute hepatitis [20], and are correlated with clinical severity of chronic HBV infection [21]. HBx induces IL-6 by transactivating the IL-6 promoter through the NF-κB-binding site [7]. To study the effect of HBx and PP2Cα on IL-6, we investigated transcripts and protein levels of IL-6 using RT-PCR and Western blot, respectively. To achieve expression of HBx, an HBx recombinant adeno

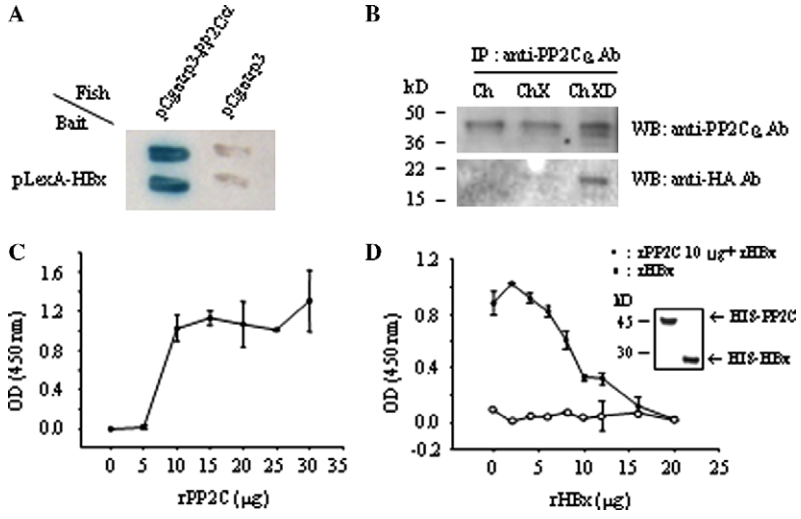

Fig. 1. A novel HBx-interacting protein, PP2Cα. (A) A human Daudi B cell line cDNA library was screened with bait HBx by using a yeast two-hybrid screening system. Yeast carrying PP2Cα cDNA formed blue colonies on the plate containing X-Gal. (B) Cell lysates from Chang and Chang X-34 cells treated with or without 2 μg/ml doxycycline for 24 h were immunoprecipitated with anti-PP2Cα antibody and western blotted with anti-HA and anti-PP2Cα antibodies. (C) Hydrolysis of pNPP by rPP2Cα was determined in the presence of 10 mM $MnCl_2$. (D) Added to the reaction mixture were increasing amounts of rHBx, and then *in vitro* phosphatase assay was performed. Purified rPP2Cα and rHBx were detected by Western blot with anti-HIS antibody. IP, immunoprecipitation; Ch, Chang cells; ChX, Chang X-34 cells; ChXD, Doxycycline-treated Chang X-34 cells; rPP2Cα, recombinant PP2Cα; rHBx, recombinant HBx.

virus, Ad-GFP-HBx was transduced in Cos-7 and Huh-7 cells. Forty-eight hours after infection of Ad-GFP and Ad-GFP-HBx, the expression of GFP, which was used to determine transduction efficiency of recombinant viruses, was visualized by fluorescence microscopy. Significant amounts of viruses were present in both cell lines (Fig. 2A, lower panel). To confirm the expression of HBx, RT-PCR was performed with primer set for HBx. HBx transcripts were detected in both cell lines transduced with Ad-GFP-HBx, but not in cells transduced with Ad-GFP (Fig. 2B). HBx induced IL-6 transcripts, while transiently overexpressed PP2Cα reduced this induction of IL-6 in Cos-7 and Huh-7 cells. HBx restored the level of IL-6 transcripts suppressed by PP2Cα (Fig. 2C). The effect of HBx on IL-6 protein expression was assessed by Western blot with GolgiStop treatment, which blocks intracellular transport processes. HBx significantly increased IL-6, and consistent with the above results, was able to recover IL-6 in both cell lines after transient overexpression of PP2Cα (Fig. 2D). These results indicate that PP2Cα functions as a negative regulator in IL-6 expression. HBx induced IL-6 might play a positive role in both the inflammation and proliferation of HBV-infected cells even under the conditions where PP2Cα is highly expressed.

Effect of HBx and PP2Cα on phosphorylation of STAT3

IL-6 has been reported to induce STAT3 activation, which is related with cell growth and survival via phosphorylation [8]. Studies have also shown that activation of STAT3 is correlated with the progression of HBV infections to chronic hepatitis and HCC, and that HBx constitutively activates STAT3 in response to IL-6 [9]. STAT3 has been shown to possess oncogenic potential and is constitutively activated in a variety of tumors and transformed cells [22]. To determine the functionality of IL-6 in HBx expressing cells, we treated Huh-7 cells with recombinant human IL-6 (rhIL-6) and determined the phosphorylation of STAT3. rhIL-6 played a functional role in the phosphorylation of STAT3 (Fig. 3A). To investigate the functional activity of increased IL-6 by HBx, we determined phosphorylation of STAT3 in Cos-7 and Huh-7 cells. HBx and PP2Cα phosphorylated and dephosphorylated STAT3 in both cell lines, respectively. In HBx expressing cells, phosphorylation of STAT3 was somewhat maintained (Fig. 3B). These data suggest that the HBx-induced increase of IL-6 might play a role in the phosphorylation and activation of STAT3 and HBx sustains STAT3 activation to some extent in PP2Cα overexpressed cells, thus promoting cell survival and proliferation.

Effect of HBx and PP2Cα on colony formation

To investigate the effect of HBx and PP2Cα on cell growth, we performed colony formation assays with

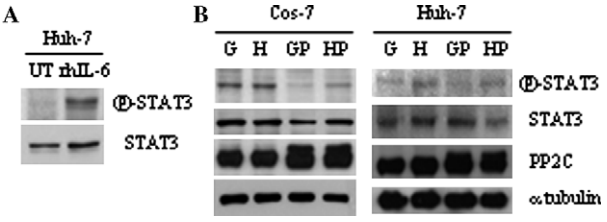

Fig. 3. Effect of HBx on the PP2Cα-mediated dephosphorylation of STAT3. (A) After treatment of 50 ng/ml rhIL-6 for 40 min, Huh-7 cells were harvested, and total lysates were used for Western blot with anti-phospho-STAT3 antibody. (B) Cells were transiently transfected with 0.5 μg pPP2Cα, incubated in complete media for 24 h, and then starved with low serum media (0.1% FBS) for 15 h to dephosphorylate STAT3 which is phosphorylated in complete media [24]. After 48 h of transduction of Ad-GFP or Ad-GFP-HBx, cells were harvested for Western blot analysis of phospho-STAT3.

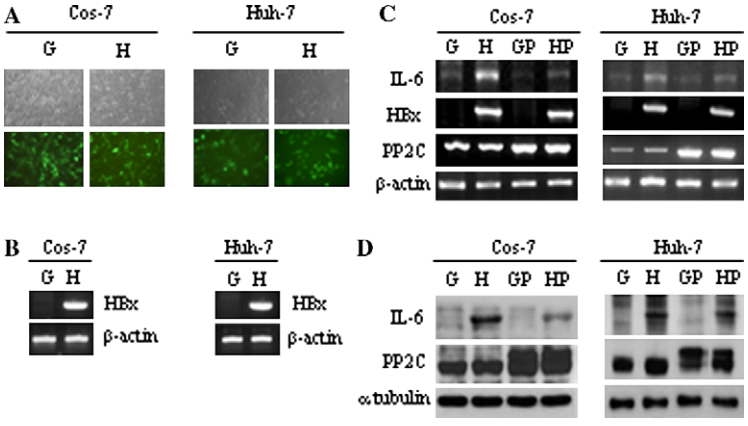

Fig. 2. Effect of HBx on the PP2Cα-mediated downregulation of IL-6. (A) After 48 h of transduction with recombinant adenovirus, Ad-GFP or Ad-GFP-HBx, transduction efficiency was determined by GFP expression (×100). (B) For the detection of HBx in Ad-GFP-HBx transduced cells, RT-PCR was performed with the primer set of HBx. (C,D) Effect of HBx on the PP2Cα-mediated downregulation of IL-6. Cells were transiently transfected with 0.5 μg pPP2Cα using Lipofectamine. After 24 h of transfection, Ad-GFP or Ad-GFP-HBx was transduced. After 48 h of transduction, cells were treated with GolgiStop (1 μl/ml) for 9 h and lysed for RT-PCR (C) and Western blotting (D) of IL-6. G, Ad-GFP + pcDNA3.1; H, Ad-GFP-HBx + pcDNA3.1; GP, Ad-GFP + pPP2Cα; HP, Ad-GFP-HBx + pPP2Cα.

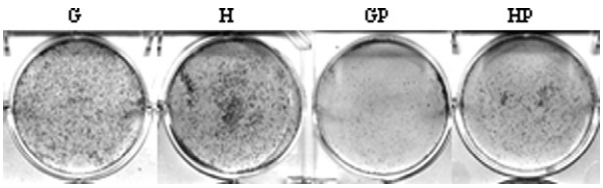

Fig. 4. Effect of HBx on PP2Cα-mediated suppression of colony formation. After 48 h of transduction of Ad-GFP or Ad-GFP-HBx into PP2Cα transfected Cos-7 cells, cells were stained with 1% crystal violet.

Cos-7 cells. Consistent with previous findings that PP2Cα has an anti-proliferative effect through the induction of apoptosis [11,23], PP2Cα dramatically reduced colony formation in Cos-7 cells. However, HBx rendered Cos-7 cells resistant to PP2Cα-mediated cell death, thus leading to cell survival and restoring colony formation ability of Cos-7 cells overexpressing PP2Cα (Fig. 4). These data indicate that HBx might play a critical role in cell growth and proliferation by negating the effects of PP2Cα on cell survival.

Discussion

HBx has been well known to play an important role in various signal transduction pathways related with cell proliferation and oncogenesis via direct or indirect protein–protein interaction [3,4]. Despite extensive research to date, fundamental molecular mechanism of HBx-mediated hepatocarcinogenesis is not completely understood.

To identify novel cellular targets of HBx and elucidate a more understandable mechanism-associated with HBx in hepatocarcinogenesis, we screened HBx-interacting proteins with a yeast two-hybrid system and demonstrated PP2Cα as a novel HBx-interacting protein (Fig. 1). With respect to effects on cell survival, activation of PP2Cα is correlated with G2/M cell cycle arrest and apoptosis via activating p53 pathway in 293 cells [11]. Thus, more thorough characterization of between PP2Cα related with anti-cell proliferative and proapoptotic effect and its functional association with oncogenic HBx may be important in understanding HBV-related hepatocarcinogenesis.

In vitro phosphatase assays showed that rHBx suppressed enzyme activity of rPP2Cα (Fig. 1D). These results suggest that the inhibitory effect of rHBx on phosphatase activity of rPP2Cα may be caused by direct or indirect interaction. The phosphorylation status of STAT3 was analyzed to the functional response of IL-6 induced by HBx. HBx phosphorylated STAT3 in Huh-7 cells, which suggested that HBx-induced IL-6 plays a role in the phosphorylation of STAT3. PP2Cα dephosphorylated STAT3, and HBx sustained somewhat extent of the phosphorylation of STAT3 in Huh-7 cells. Although STAT3 was not dephosphorylated under low serum condition in Cos-7 cells, when PP2Cα was present, it effectively dephosphorylated STAT3 and HBx maintained phosphorylation of STAT3 in Cos-7 cells (Fig. 3B). As IL-6-mediated STAT3 phosphorylation and activation are related to cell survival and proliferation [8], we speculated that HBx-induced IL-6 expression and phosphorylation and activation of STAT3 might function against PP2Cα-mediated cell growth arrest and apoptosis. To examine what effect HBx has on the PP2Cα-mediated anti-proliferative function, we performed colony formation assay. Although HBx did not dramatically increased colony formation, it inhibits PP2Cα-mediated cell death, thus preserving the number of colonies (Fig. 4). These results suggest that HBx might play a crucial role in the development of cancer by negating the ability of PP2Cα to induce growth inhibition and cell death during and after HBV infection as well as during viral replication.

Together, the data showed that PP2Cα is a novel target of HBx that it has a negative effect on IL-6 upregulation following phosphorylation of STAT3 that contribute to cell proliferation and oncogenesis. HBx was able to suppress the PP2Cα-mediated effects on cell survival by interacting with PP2Cα and suppressing its phosphatase activity. Thus, these results support the idea that HBx play a pivotal role in HBV-associated hepatocarcinogenesis even in cells where PP2Cα, a negative regulator of cell survival, is highly expressed.

Acknowledgment

This work was supported by the Brain Korea 21 Project in 2005.

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