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Activation of calcium signaling by hepatitis B virus-X protein in liver cells

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Abbreviations: CREB, cyclic AMP response element binding protein; DMSO, dimethylsulfoxide; ECL, enhanced chemiluminescence. EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene-bis (oxyethylenenitrilo)tetraacetic acid; HCC, hepatocellular carcinoma; IP3, inositol-3-phosphate; MAPK (Erk), mitogen activated protein kinase; NF-AT, nuclear factor of activated T cells; PCNA, proliferative cell nuclear antigen; PI3K, phosphatidylinositol-3-kinase; PIP3, phosphatidylinositol-3-phosphate; SAPK/JNK, stress activated protein kinase/c-Jun N-terminal kinase

Abstract

Hepatitis B virus x gene product (HBx) is known to be a transactivator of transcriptional elements that regulate the expression of a variety of genes associated with the growth, differentiation, survival and the apoptosis of cells. However, the exact mechanism of the activation and inhibition of cellular events by HBx remains uncertain. The present study was designed to measure the effect of HBx, on the signal transduction pathways associated with intracellular Ca^{2+} mobilization following *HBx* transfection in the stable Chang liver cells (CHL-X). Enhanced cell proliferation by HBx in CHL-X was confirmed by MTT assay and by the immunodetection of PCNA. The transactivation of AP-1 by HBx induced in CHL-X was inhibited by cyclosporin A (CsA), a mitochondrial Ca^{2+} channel blocker and by BAPTA-AM, a cytosolic Ca^{2+} blocker. Activation of the SAPK/JNK signaling pathway by HBx was evidenced by the increased phosphorylations of c-Jun (Ser63) and of JNK (Thr183/Tyr185). Increased phospho-Erk/Erk and phospho-Raf1/Raf in HBx-induced CHL-X indicated that HBx might stimulate the MAPK pathway. PI3K activity and cytosolic free Ca^{2+} levels were elevated in HBx-induced CHL-X. These results imply that HBx transactivates both JNK and MAPK signal transduction pathways in association with the mobilization of cytosolic Ca^{2+}.

Keywords: AP-1; Ca^{2+}-signaling; HBx; MAPK; SAPK/JNK

Introduction

Hepatitis B virus (HBV) is a causative pathogen of liver cirrhosis and hepatocellular carcinoma (HCC), but the molecular events associated with pathogenes complex due to the involvement of multiple factors (Beasly *et al*., 1981; Ganem, 1982).

The x gene product of HBV (HBx) is known to be a transactivator of a variety of viral and cellular transcriptional elements, including AP-1, CREB, NFκB and C/EBP, but the mechanisms underpinning these transactivations by HBx remain controversial (Rossner 1992; Feitlson, 1997; Murakami, 1999; Diao *et al*., 2001). The association between HBx with the transactivation of transcription factors has been reported to be mediated through the up-regulation of the MAPK signal transduction pathway (Benn and Schneider, 1994; Doria *et al.,* 1995; Chirillo *et al*., 1996; Klein and Schneider, 1997).

The anti-apoptotic action of HBx *via* its interaction with the p53 molecule has been demonstrated in cultured human cells (Wang *et al*., 1995; Su and Schneider, 1997; Takada *et al*., 1997; Yun *et al*., 2000). Repression of apoptosis through the inhibition of caspase-3 activity or through the activation of PI3K by HBx in hepatocytes has been reported (Gottlob *et al*. 1998; Shih *et al*., 2000; Lee *et al*., 2001).

The SAPK/JNK pathway is a survival pathway for some cells undergoing Fas-mediated apoptosis, and its activity is upregulated in cells expressing HBx (Henkler *et al*., 1998). In addition to its known transcriptional activation function and its anti-apoptotic ability, HBx has been reported to be involved in cell cycle control and DNA repair (Baron *et al*., 1996; Becker *et al*., 1998; Matthews and Russe, 1998; Zwick *et al*., 1999; Ren and Nassal, 2001). Although HBx has been suggested to be a multifunctional factor, a mode of action of HBx in the regulation of these cellular events remains uncertain.

Recently, calcium signaling mediated by HBx in HBV-DNA replication has been reported (Bouchard *et*

al., 2001). In their report, the activation of HBV replication by HBx was blocked by inhibiting mitochondrial calcium channels, moreover, reagents that increased cytosolic calcium were found to have the ability to substitute for HBx in HBV replication. HBx has also been demonstrated to activate a calcium-stimulated transcription factor, and the nuclear factor of activated T cells (NF-AT) in T lymphocytes (Lara-Pezzi *et al*., 1998). The calcium ion is the most common signal transduction element in cells, which affect many signaling pathways regulating cell cycle progression, cell proliferation and apoptosis. Therefore, the maintenance of intracellular calcium level is critical for life, (Nicotera *et al*., 1994). The introduction of calcium ion into the cytosol is facilitated by the release of Ca^{2+} from two large calcium stores, the extracellular space and the endoplasmic reticulum (ER). Mitochondria also accumulates Ca^{2+} at up to 0.5 mM, due to the large electrochemical gradient created by mitochondrial hydrogen exchange, which thus may contribute to the intracellular Ca^{2+} level (Pozzan *et al*., 1994). Because the multi-cellular events observed in HBx expressing cells are similar to those in cells containing Ca^{2+} mobilization, studies on the role of HBx in the calcium signaling associated with cell proliferation and transformation are required.

This study was designed to investigate the role of HBx in the regulation of signal transduction in association with cytosolic Ca^{2+} levels in Chang liver cells (CHL) and in CHL-X, a stable cell line expressing HBx protein. The transcriptional transactivating activity of HBx was evaluated by measuring luciferase activity in CHL-X transfected with vectors containing AP-1-linked reporter (luciferase) gene. In addition, the effects of HBx on the expressions of intracellular sigaling molecules and on the cytosolic Ca^{2+} level were determined.

These experiments show that HBx increases the cytosolic Ca^{2+} level and cell proliferation by activating both the JNK and MAPK pathways.

Materials and Methods

Cell culture

CHL and CHL-X cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). CHL are undifferentiated hepatocytes of human origin and the CHL-X cell line was established by transfecting a plasmid vector expressing HBx (pTet-X). CHL-X were found to express HBx in response to doxycycline treatment, since this expression is under the control of the tetracycline-inducible promoter (Figure 1A).

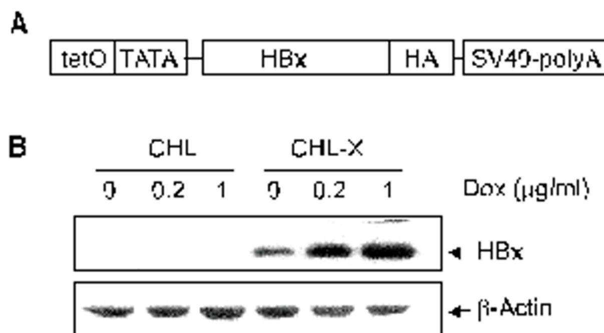

Figure 1. Schematic representation of the HBx expression vector (pTet-X) (A) and the expression of HBx in pTet-X transfected stable Chang liver cells (CHL-X) (B). The cells were treated with doxycycline (0.2 or 1.0 g/ml) to induce HBx, cultured for 24 h at 37°C and harvested. Cell lysates were prepared by sonicating the harvested cells, and these were subjected to SDS-polyacrylamide gel electrophoresis. Separated proteins were electrotransferred onto nitrocellulose membranes and blotted. β-actin and HBx were immunodetected using polyclonal antibodies against β-actin (Santa Cruz Biotech, Santa Cruz, CA) and rabbit antiserum against recombinant HBx protein (⟵). TetO, tetracycline operator; TATA, cytomegalovirus immediate early minimal promoter.

Cotransfection of plasmids and luciferase assay

CHL and CHL-X were plated into 6 well plates at 4×10^5 cells per well using DMEM supplemented with 10% FBS, 100 units/ml of penicillin and 100 g/ml streptomycin. After allowing 24 h for growth, cells were cotransfected with a control vector (pCMVβ) and a basal vector (pCMV) containing 4 copies of the consensus sequence for the AP-1 (5'-CGCTTGATGA-GTCAGCCGGAA) binding site linked to a TATA box and the luciferase gene using lipofectamine-plus reagent according to the manufacture's instructions. To normalize the transfection efficiency, β-galactosidase activity in the transfected cells was assayed by the method described in the kit manual (Invitrogene, Calsbad, CA). To determine the activation status of the AP-1 element, an AP-1 trans-reporting luciferase assay system was adopted. To evaluate the calcium dependent action of HBx in AP-1 transactivation, a mitochondrial Ca^{2+} channel blocker, cyclosporin A (CsA) and a cytosolic Ca^{2+} blocker, 1, 2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic tetra-(acetoxymethylester) (BAPTA-AM) were treated to HBx-induced CHL-X. The transfected cells were transferred to fresh media, and cultured for an additional 24 h. Then the cells were harvested by scraping from culture dishes, followed by centrifugation at 600 *g* for 10 min. Cell pellets were washed with PBS and lysed with the lysis buffer (20 mM Tris-Cl, buffer pH 7.4, containing 140 mM NaCl, 1% Nonodet p-40, 10 mM NaF, 1 mM Na_3VaO_4, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 5 g/ml of leupeptin, and 5 mM benzamidine). Luciferase activity in the cell extracts was measured

using a Microluminat plus (Bad Wildbad, Germany).

MTT assay

An MTT[3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] kit (Sigma Co., St Louis, Mo) was used to measure the metabolic activities of viable cells and the degree of cell proliferation. Subconfluent monolayer cultures of CHL and CHL-X were detached from the culture dishes by trypsin treatment and centrifuged at 200 *g* for 5 min. The cell pellets were resuspended in the fresh media (2.5×10^4 cells/ml) and 200 l of the cell suspension was transferred into each well of a flat-bottomed 96 well plate. HBx protein expression was induced by doxycycline (1.0 g/ml) treatment, and then incubated for 24 h at 37°C. At the end of the incubation, the cultured medium was removed and 200 l of fresh medium was added. MTT (2.5 mg dissolved in 50 l of dimethylsulfoxide) was added to each well and incubated for 4 h at 37°C. To remove formed MTT-formazan crystals, 200 l of DMSO was added. 25 l of glycine buffer (0.1 M, 0.1 M NaCl, pH 10.5) was added to each well and viable cells were detected by measuring absorbance at 570 nm. Proliferation activity is expressed as a percentage of the control (doxycycline untreated) cells.

Western blot analysis

Proteins were extracted from CHL and CHL-X and were subjected to Western blot analysis using specific antibodies for various signal molecules. In brief, the harvested cells (~2×10^8 cells) were resuspended in 200 l of PBS and sonicated 5 times (30 s/cycle). Cellular proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were electrotransferred onto nitrocellulose membrane and blotted. The membranes were then blocked with milk casein (5% by weight in PBS) and hybridized with each diluted primary antibody for 4 h at room temperature. The membranes were then washed 4 times for 15 min incubation with TBST (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and hybridized with horse radish-peroxidase (HRP)-conjugated secondary antibodies corresponding to each primary antibody. Protein bands specific for the antibodies were visualized by ECL-associated fluorography.

PI3K assay

PI3K activity was measured by using the method described by Johnson *et al*. (1993). Briefly, cell lysates used for PI3K assay were prepared in lysis buffer. PI3K present in the lysates was precipitated by incubating with a polyclonal anti-PI3K antibody (1 g; Santa Cruz Biotech., Santa Cruz, CA) overnight at 4°C. The immune complex was washed twice in lysis buffer, containing 0.5 M LiCl and 25 mM Hepes buffer (pH 7.2). The immunoprecipitates were dissolved in TNE buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA), and then phosphatidylinositol (Sigma Co. St. Louis, Mo) dissolved in TNE (10 mM Tris-Cl,100 mM NaCl, 1 mM EDTA, pH 7.4, 1 g/ l) was added to the reaction mixture. The kinase reaction was started by adding 20 l of a substrate mixture (20 mM Hepes, pH7.2, 5 mM $MgCl_2$, 20 M ATP, 10 Ci of [γ-^{32}P]ATP). The reaction mixture was incubated for 20 min at 25°C and the reaction was stopped by adding of 100 l of 1.0 N HCl. Lipids were extracted with 200 l of a 1:1 mixture of MeOH:$CHCl_3$ and spotted on silica gel coated TLC plate (Sigma Chem Co., St. Louis, MO). Chromatography was performed using a $CHCl_3$:MeOH:4 M acetic acid (9:7:2, V/V). The phosphorylated product of phosphatidylinositol (PIP_3) was identified by autoradiography.

The radioactivity of the phosphorylated products was quantitated on TLC plates by scanning the autoradiographic film with an imaging densitometer (Bio-Rad, Model GS690).

Measurement of cytosolic calcium level

The intracellular free calcium level was measured using the method of Pollock *et al*. (1986). CHL and CHL-X cells cultured in the presence or absence of doxycycline were detached from the culture dishes by trypsin treatment. The harvested cells were then centrifuged at 800 *g* for 10 min and washed with 1.0 ml of Krebs-Ringer solution. The cell pellets were resuspended in Krebs-Ringer solution (~2×10^8 cells/ml), Fura2AM (Sigma Co., St.Louis) was added to the cell suspension to a final concentration of 2 M, and incubated for 45 min at 37°C. The incubated cell suspension was centrifuged at 800 *g* for 10 min and the cell pellets were resuspended in 1 ml of Krebs-Ringer solution. Aliquots of the cell suspension (300 l) were dispended into cuvettes containing 2.7 ml of Krebs-Ringer solution supplemented with 1.1 mM $CaCl_2$ and incubated for 3 min at room temperature. EGTA (final 2 mM) was then added to chelate the extracellular Ca^{2+}, and Fura2-Ca fluorescence was measured using a fluorometer with an excitation wavelength of 360 nm and an emission wavelength of 500 nm. Ca^{2+} mobilized from internal storage sites was calculated using the general formula described by Pollock *et al*. (1986). $[Ca^{2+}]$ = Kd (F-Fmin/Fmax-F), where Kd for Fura2-Ca = 224 nM and Fmin = AF (autofluorescence) + (Fmax-AF)/7.4.

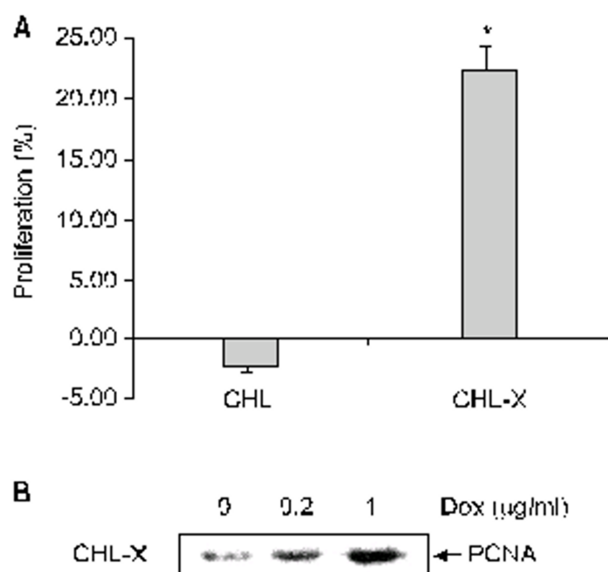

Figure 2. Induction of proliferation by HBx induced in CHL-X cells. MTT assay for the metabolic activity of CHL and CHL-X treated with doxycycline (1.0 g/ml) was performed and relative proliferations (%) were calculated from the OD_{570} values for each group of cells (A). To detect PCNA expressed in CHL-X, HBx was induced in CHL-X cultured for 24 h after treating with doxycycline (0.2 and 1.0 g/ml), and cells were harvested. Proteins in harvested cells were extracted by sonication and subjected to SDS-polyacrylamide gel electrophoresis. Proteins in gel were electrotransferred onto nitrocellulose membranes, and blotted. PCNA was detected by western blot immunochemical analysis using monoclonal antibody against PCNA (Santa Cruz Biotech., Santa Cruz, CA) (B). $^*P < 0.01$ (difference between CHL and CHL-X).

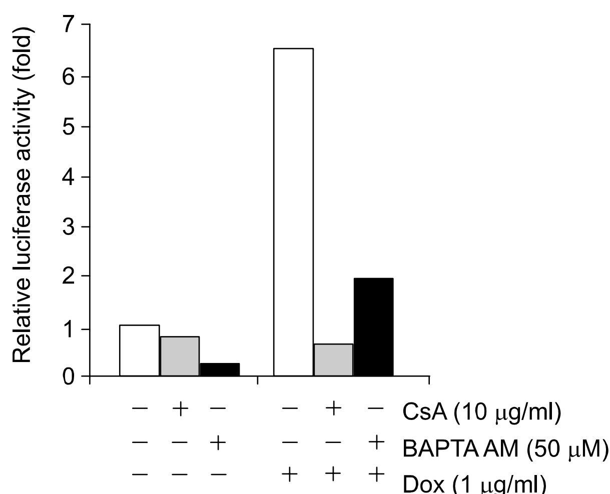

Figure 3. Inhibition of the HBx-induced transcriptional activity of AP-1 in CHL-X by calcium channel blockers. CHL-X cells cotransfected with 10 ng of pCMVβ and the plasmid containing AP-1-linked luciferase gene were cultured for 24 h in the presence or absence of CsA (10 g/ml) or BAPTA-AM (50 M). Luciferase activity was determined as-described by the manufacturer (Promega Co. Madison, WI).

Results

Induction of HBx expression in the CHL-X cell line

CHL-X is a Chang liver cell line, stably established by HBx gene insertion. It expressed HBx protein upon treating with doxycycline in a dose dependent manner, upto 1.0 g doxycycline/ml culture medium (Figure 1B). The expression of the hemagglutinin (HA) gene in CHL-X by treating doxycycline confirmed that the induction system for HBx in CHL-X cells could be used to test the effect of HBx on the cellular events associated with various signal molecules. The amounts of β-actin in the cell extracts were invariable and HBx was not detected in control CHL cells regardless of doxycycline treatment.

Proliferation of CHL-X by HBx induction

The induction of HBx in CHL-X by the treatment with doxycycline improved cell proliferation significantly versus control CHL cells. The MTT value was higher in HBx-induced CHL-X than in control CHL cells (Figure 2A). Western blot immunochemical analysis of PCNA in the cell extract of CHL-X revealed that the HBx induction by doxycycline increased PCNA expression in a dose dependent manner (Figure 2B). These results indicate that HBx stimulated the metabolic activity and the viability of liver cells (CHL-X).

Transcriptional activation of AP-1 in HBx-induced CHL-X

An AP-1-controlled luciferase expression system was employed to test the transactivating activity of HBx upon the transcriptional element (AP-1) in CHL-X. HBx induction by doxycycline treatment in CHL-X increased AP-1-regulated luciferase gene expression (Figure 3). Treatment of CsA (10 g/ml), a mitochondrial calcium channel blocker, inhibited the stimulating effect of HBx on AP-1 controlled luciferase expression in CHL-X. Treatment with BAPTA-AM (50 M), a cytosolic Ca^{2+} blocker, also inhibited the transactivating effect of HBx on the AP-1element (Figure 3). These results imply that the transactivation of AP-1 by HBx induced in CHL-X might be mediated through calcium mobilization into the cytosol from intracellular calcium stores.

Activation of the SAPK/JNK pathway by HBx in CHL-X

c-Jun is a component of AP-1, a transcription factor which binds at the AP-1 element and activates transcription of AP-1 controlled genes. The transcriptional activity of c-Jun is regulated by its phosphorylation at Ser63 and Ser73. The phosphorylation of

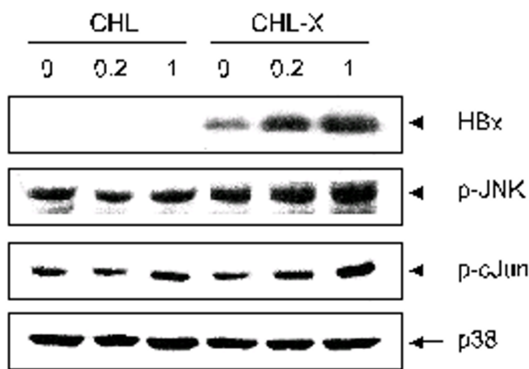

Figure 4. Activation of the SAPK/JNK signal transduction pathway by HBx induced in CHL-X. CHL and CHL-X were treated with doxycycline (1.0 g/ml) and cultured for 24 h. Cells harvested by trypsinization were washed with PBS by centrifugation (600×*g*, 10 min). Cell pellets were dissolved in lysis buffer and sonicated (4×30 s) using a sonicator (Fisher Co., Model 300). Lysates were subjected to SDS-polyacrylamide gel electrophoresis and proteins in the gel were electrotransferred onto nitrocellulose membranes and immunodetected using monoclonal antibody against phospho-c-Jun, phospho-JNK or p38 as primary antibodies (Santa Cruz Biotech., SantaCruz, CA) and HRP-conjugated anti-mouse IgG antibody (Sigma Chem. Co., St. Louis, Mo) as a secondary antibody. Fluorography was performed using ECL detection kit (Amersham Phamacia Biotech Inc., Piscataway, NJ).

c-Jun is mediated by SAPK/JNK, which is activated by the same signal that activates c-Jun. SAPK/JNK is activated by its phosphorylation at Th183/Tyr185 and this activity can be measured by the determining the level of c-Jun phosphorylation.

Western blot analysis for the phosphorylation of c-Jun and SAPK/JNK using antibody against phospho-c-Jun (Ser63) and phospho-SAPK/JNK (Thr183/Tyr185) showed that HBx induction in CHL-X caused a slight increase in the expression of both phospho-c-Jun (Ser63) and phospho-SAPK/JNK content in the cell extracts (Figure 4). This result indicates that the SAPK/JNK pathway is stimulated by HBx induction in CHL-X cells.

Activation of the MAPK pathway by HBx in CHL-X

Erk (Erk1/Erk2) is a MAPK that plays a role in the regulation of cell growth and differentiation. The activation of MAPK (42, 44 KDa) occurs through phosphorylations of the threonine (202) and tyrosine (204) of MAPK by MAPK kinase. Activated Erk may activate a variety of transcription factors, such as, c-*Myc*, *Elk*, CREB and p90RSK. HBx induction in CHL-X increased the phospho-Erk level in CHL-X and this peaked at 24 h after doxycycline treatment (Figure 5A). The ratio of p-Erk to Erk in the cells also elevated by doxycycline treatment (Figure 5B). Treatment of CHL-X with doxycycline (1 g/ml) to CHL-X increased the expression of phospho-Raf1 (p-Raf1) in

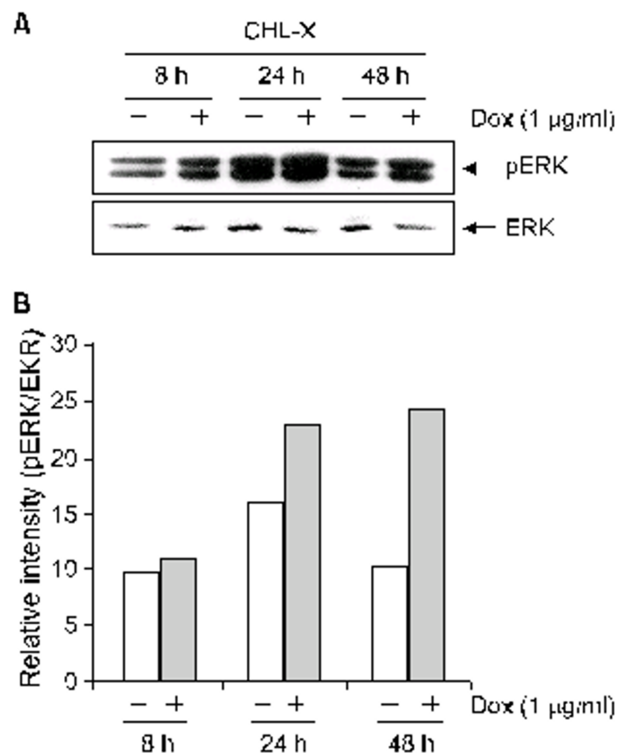

Figure 5. Activation of the MAPK pathway by HBx induced in CHL-X. Phospho-Erk (P-Erk) and Erk expressed in CHL-X treated with doxycycline (1.0 g/ml) were detected by western blot immunodetection method using anti-Erk or anti-phospho-Erk antibodies (Santa Cruz Biotech., Santa Cruz, CA) as primary antibodies and HRP-conjugated anti-rabbit IgG as a secondary antibody. Cells were treated with doxycycline (1 g/ml) and cultured for the indicated times (A). The ratio of pERK to ERK was calculated from the intensities of the corresponding bands on the desitogram obtained by scanning the fluorographic film (B).

both CHL and CHL-X (Figure 6A) and the ratio of p-Raf1 to Raf1 was higher in doxycycline-treated (1 g/ml) CHL-X than that in the control CHL (Figure 6B). This result suggests that the induction of HBx in CHL-X might activate Ras-Raf downregulation of Erk through MEK (Erk kinase).

Effect of HBx on PI3K in CHL-X

PI3K catalyzes the phosphorylation of phosphatidylinositol to PIP_3 which is then further converted to inositol-3-phosphate (IP3), a signal molecule that regulates cellular events, such as, the mobilization of calcium from the ER membrane or the activation of Akt signaling pathway, which regulates cell survival and apoptosis. Intracellular free Ca^{2+} can affect many signaling pathways, which suggest extensive inter-pathway crosstalk. In the present study, PI3K activity in CHL-X was increased by doxycycline (1 g/ml) treatment, but no significant change in PI3K activity by doxycycline treatment was observed in CHL control (Figure 7).

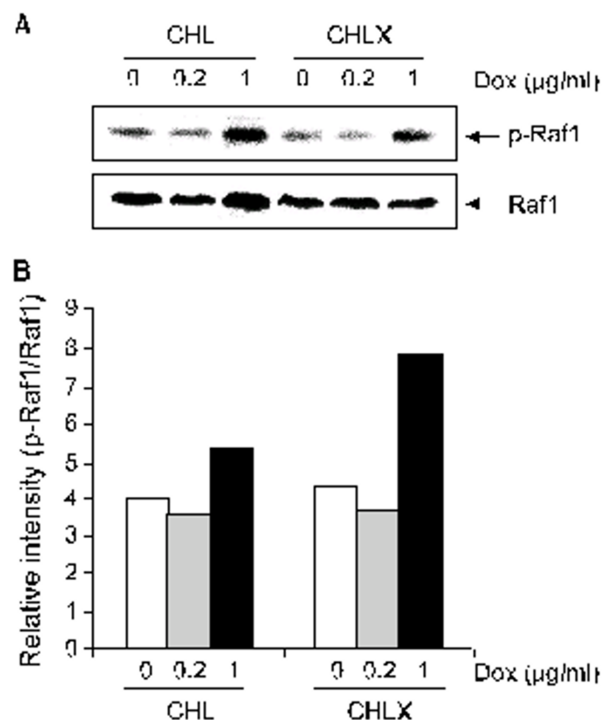

Figure 6. Western blot immunodetection of Raf and p-Raf1 (ser-338) in CHL-X treated with doxycycline (1 g/ml). Raf1 and p-Raf1 were immunodetected by the same method using anti-p-Raf1 antibody and anti-Raf antibody (Santa Cruz Biotech., Santa Cruz, CA) as primary antibodies. The ratio of p-Raf1 to Raf1 was determined by the method described in Figure 5B.

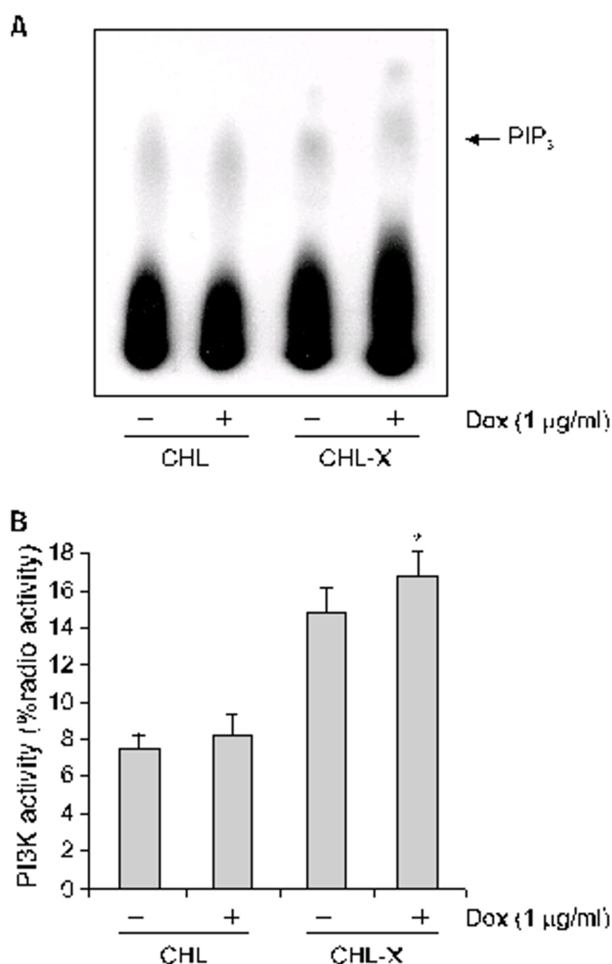

Figure 7. PI3-kinase activity in CHL and CHL-X. Cells were cultured for 24 hr in the presence or absence of doxycycline (1.0 g/ml) and then detached from the culture dish by additing lysis buffer, and scraping. PI3-kinase activity in the cell extracts was assayed by the method described by Pollock *et al.* (1986). Autoradiography was performed to detect ^{32}P-labeled PIP_3 (←) separated by TLC (A). The detailed methods used are described in the PI3K assay section. PI3K activity was quantified after scanning the TLC plate by a Phosphoimaging analysis program (Fuji Co.) (B). *, Significantly different from Dox group ($P < 0.05$).

Cytosolic Ca^{2+} levels in HBx-induced CHL-X

Cytosolic Ca^{2+} levels were measured using Fura2AM, which is permeable to cells and binds to free Ca^{2+} only after its cleavage by intracellular esterase. Since the intracellular free Ca^{2+} level in mammalian cells is tightly regulated, and extracellular Ca^{2+} level (~2 mM) is much higher (about 20,000 times), various intracellular signal molecules as well as environmental factors may affect the balance of cytosolic Ca^{2+} in cells.

The basal cytosolic Ca^{2+} levels in CHL and CHL-X cells were 133.2 and 127.7 nM, respectively, and treating CHL and CHL-X cells with doxycycline (1 g/ml) increased these levels to 364.5 and 523.0 nM, respectively (Figure 8). These results indicate that HBx induction has a stimulatory effect on Ca^{2+} mobilization from intracellular calcium stores into the cytosol of CHL-X cells.

Discussion

Number of investigators (Wang *et al.*, 1991; Diamantis *et al.*, 1992; Paterilini *et al.*, 1995) reported that HBx has oncogenic potential, but the direct involvement of HBx in the pathogenesis of HCC developed in HBV-infected patients remains controversial.

In the present study, HBx was induced in CHL-X cells by doxycycline treatment, and the effect of HBx on Ca^{2+}-mediated signaling pathways was studied. HBx induction in CHL-X caused an increase in cellular proliferation, as evidenced by increased PCNA expression and a higher MTT value versus the HBx-negative control CHL. The present results suggest that HBx is involved in the proliferation of non-dividing cells (a typical phenomenon observed in HCC) and in liver cell survival. These results are consistent with the results by other investigators (Kim *et al.*, 1991; Koike *et al.*, 1994; Koike *et al.*, 2002).

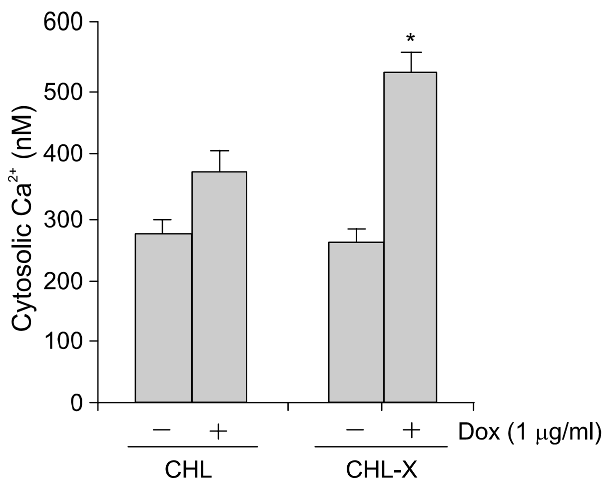

Figure 8. Cytosolic calcium level in CHL and HBx-induced CHL-X cells. Both CHL and CHL-X cells were treated with doxycycline (1.0 g/ml) and cultured for 24 h. Cells were detatched from the culture plate by trypsin treatment and resuspended in Krebs Ringer solution (2×10^8 cells/ml). Aliquots of cells (1 ml) were incubated with 2 M of fura2-am for 45 min at 37°C and then the external calcium concentration was adjusted to 1 mM with $CaCl_2$, and the cells were further incubated for another 3 min at room temperature. EGTA (2 mM) was added to chelate extra free Ca^{2+} and the fluorescence (360 nm excitation, 500 nm emission) was measured. The intracellular calcium level (nM) was calculated using the general formula: $[Ca^{2+}]$ = Kd (F-Fmin/Fmax-F), where Kd = 224 nM, Fmin = AF (autofluorescence)+ (Fmax-AF)/7.4. $*P < 0.05$ (difference between CHL and CHL-X).

It has been demonstrated that HBx transactivates the cis-acting elements of genes and interacts with components of the basal transcription machinery, where by it promotes cell survival and growth by activating signal transduction pathways (Benn and Schneider, 1994; Su and Schneider, 1996). A pro-oncogenic role has been proposed for HBx due to its ability to activate several signal transduction cascades and the related transcription factors (Maguire *et al.*, 1991; Kekule *et al.*, 1993; Natoli *et al.*, 1994; Truant *et al.*, 1995; Klein *et al.*, 1997; Lee and Yun, 1997; Larapezzi *et al.*, 1998).

In the present study, the transcriptional activation of AP-1 by HBx induction was observed by cotransfecting a pCMV vector, containing AP-1-linked luciferase reporter gene and pCMVβ, into CHL-X.

Treatment with CsA (10 g/ml), a mitochondrial calcium channel blocker inhibited the AP-1 directed transactivating activity of HBx in CHL-X cells, indicating that the action of HBx on the AP-1 element is cytosolic Ca^{2+}-dependent to some extent, and that the stimulation of Ca^{2+} release from mitochondria by HBx might contribute to the transcriptional activation of the AP-1-linked reporter gene. We tested the inhibitory effect of BAPTA-AM, a cytosolic calcium channel blocker on the AP-1 transactivating activity by HBx in CHL-X, and found that treatment with 50 M of BAPTA-AM to both CHL and CHL-X could reduce the AP-1 transactivating activity upregulated by doxycycline-induced HBx.

The increased phosphorylations of c-Jun, SAPK/JNK, Erk and Raf-1 by HBx in CHL-X cells in the present study imply that HBx might activate both the JNK and the MAPK signal transduction pathways. The activation of the JNK pathway might be reflected by the increased AP-1-controlled expression of luciferase reporter gene (Figure 3).

Erk is an important signal molecule which down regulated the transcription of the CRE-controlled gene through the MAPK cascade, for which the phosphorylation of Ras-Raf-MEK is a prerequisite. In the present study, the ratios of p-Erk to Erk and of p-Raf1 (Ser388) to Raf1 were increased by doxycycline (1 g/ml) treatment in CHL-X (Figure 5B, 6B), indicating that HBx induced in CHL-X may activate c-Raf-1 down regulation and Erk activation. This result is consistent with the results of a previous study (Lee *et al.*, 2001).

Recently, the involvement of HBx in transcriptional activation and apoptosis in various cells has been suggested, and the interaction of HBx with a variety of proteins associated with cell cycle check points and apoptotic pathways has been reported (Truant *et al.*, 1995; Gottlob *et al.*, 1998; Shih *et al.*, 2000). But the molecular mechanism for the development of hepatocellular transformation by HBx remains uncertain.

PI3K activity was more elevated in CHL-X than in CHL, and its activity was further enhanced by doxycycline treatment (Figure 7), indicating that the HBx induced in CHL-X had a stimulating effect on PI3K activity. PI3K catalizes the phosphorylation of phosphatidylinositol to produce PIP_3. This is an intermediate for IP_3, an important signal molecule required for Ca^{2+} mobilization into the cytosol which is required for the regulation of cell survival or apoptosis (Berridge and Irvine, 1989). The release of Ca^{2+} from ER membrane is triggered by G-protein-coupled and tyrosine kinase-linked IP_3 receptor. Moreover, the IP_3-mediated pathway predominates in non-excitable cells such as, hepatocytes (Berridge and Irvine, 1989), Therefore, it is possible that HBx is involved in IP_3 receptor-mediated Ca^{2+} release into cytosol from the ER membrane.

It is noteworthy that HBx induction in CHL-X cells was found to increase the cytosolic Ca^{2+} level with the activation of PI3K, and that AP-1 transactivation by HBx was inhibited by CsA or BAPA-AM. In HBx-induced CHL-X, the cytosolic Ca^{2+} level is likely to be regulated by the release of free Ca^{2+} from the ER, the largest calcium store, and from mitochondria, a source of a significant amount of the cytosolic Ca^{2+}, and one that is probably sufficient to activate the Ca^{2+}-dependent signal transduction pathways directly

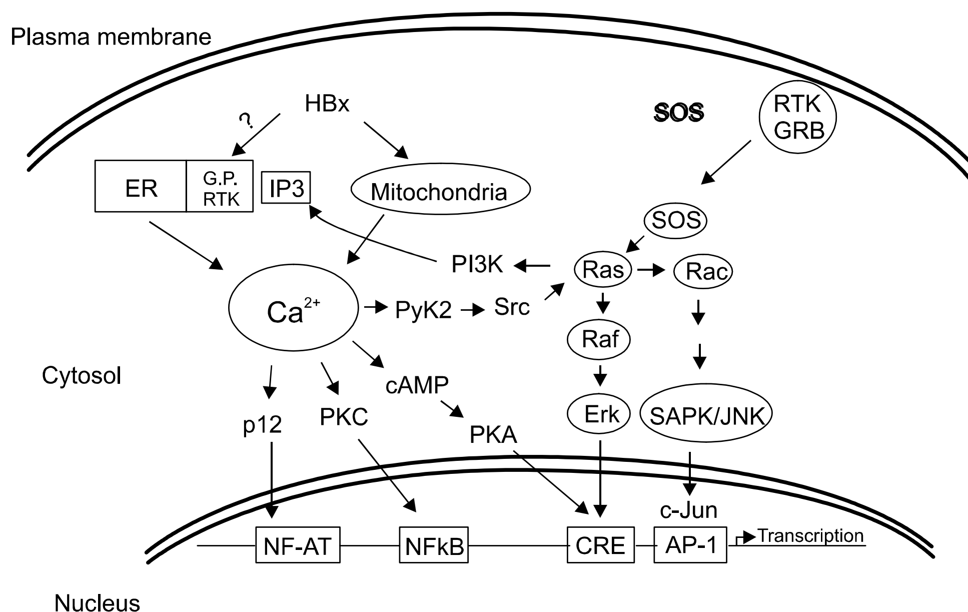

Figure 9. Schematic representation of the proposed calcium signaling cascades in the CHL-X cell line.

or indirectly (Figure 9).

It has been suggested that HBx activated calcium signaling is mediated by mitochondrial calcium channels (Bouchard *et al*., 2001). In their study, HBx was found to be required for HBV DNA replication by activating the cytosolic calcium-dependent proline-rich tyrosine kinase-2 (Pyk2), a *Src* kinase activator. In another study, it was found that NF-AT is activated by HBx protein *via* a CsA-sensitive pathway (Lara-Pezzi *et al*., 1998), and that cytoplasmic calcium in T cells, induced by human T-cell lymphotropic virus type 1. p12 expression could activate NF-AT (Ding *et al*., 2002). These results suggest that HBx is involved in the cytoplasmic Ca^{2+} mobilization that triggers Ca^{2+}signaling in T cells.

The increased level of cytosolic Ca^{2+} in HBx-induced CHL-X, which occurred in parallel with the activations of PI3K, SAPK/JNK and MAPK pathways, suggest that HBx is involved in the activation of signaling molecules in association with intracellular Ca^{2+} mobilization.

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