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NOTES

Hepatitis B Virus Replication Is Associated with an HBx-Dependent Mitochondrion-Regulated Increase in Cytosolic Calcium Levels[▽]

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The nonstructural hepatitis B virus (HBV) protein HBx has an important role in HBV replication and in HBV-associated liver disease. Many activities have been linked to HBx expression; however, the molecular mechanisms underlying many of these activities are unknown. One proposed HBx function is the regulation of cytosolic calcium. We analyzed calcium levels in HepG2 cells that expressed HBx or replicating HBV, and we demonstrated that HBx, expressed in the absence of other HBV proteins or in the context of HBV replication, elevates cytosolic calcium. We linked this elevation of cytosolic calcium to the association of HBx with the mitochondrial permeability transition pore.

Chronic infections with the human hepatitis B virus (HBV), a member of the hepadnavirus family, can lead to liver cirrhosis and hepatocellular carcinoma (2, 27). An estimated 350 million people worldwide are chronically infected with HBV; many of these individuals will eventually die from liver disease (38).

The HBV genome encodes three envelope proteins (36, 39) and core (capsid) protein (26), precore protein (25), polymerase (4), and X protein (HBx) (35). Woodchuck hepatitis virus, another hepadnavirus, requires the expression of its X protein for in vivo viral replication (12, 41); consequentially, it is thought that an X protein is required for the replication of mammalian hepadnaviruses.

HBx enhances HBV replication in HepG2 cells, a human hepatoblastoma cell line that is a model cell culture system for studying HBx-dependent HBV replication (6, 21). There are no available cell lines that can be directly infected with HBV (1). Therefore, in the studies described here, HBV was expressed from a greater-than-genome-length cDNA of either the wild-type HBV (payw1.2) or an HBx-deficient mutant HBV (payw*7) that was cloned into pGEM7Zf+ and was transfected into HepG2 cells (21, 31). These HBV constructs have been used to demonstrate HBx-dependent replication in HepG2 cells (6, 18, 21, 31). HBx activates the calcium-dependent Pyk2/focal adhesion kinase (FAK)-Src/Fyn-Ras-Raf–mitogen-activated protein kinase pathway (3, 6, 7, 9, 13, 20, 22). Blocking this pathway (by chelating cytosolic calcium or inhibiting Pyk2/FAK or Src/Fyn kinases) diminishes HBV replication in HepG2 cells (6, 7, 9, 19). Conversely, the mobilization of cytosolic calcium in HepG2 cells rescues replication from payw*7 (6, 7). These data suggested that HBx modulates cytosolic calcium to regulate HBV replication. Additionally, HBx-expressing Chang cells had elevated basal cytosolic calcium concentrations (23). Unfortunately, studies of Chang cells (American Type Culture Collection [http://www.atcc.org]; originally described as a human liver cell line) may not provide an ideal system; these cells may be contaminated with HeLa cells.

To test whether HBx increases cytosolic calcium levels in HepG2 cells, we designed pcDNA3.1− plasmids expressing HBx fused to the eight-amino-acid FLAG epitope at either the N terminus (FL1-154) or C terminus of HBx. FL1-154- and 1-154FL-transfected HepG2 cells were lysed in 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% deoxycholate, 50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, and 1 mM $MgCl_2$. Equal protein concentrations were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in a 15% polyacrylamide gel. FL1-154 and 1-154FL were detected as 17-kDa proteins by Western analysis using an anti-FLAG antibody (Stratagene) and visualized by using an enhanced chemiluminescence system (Bio-Rad) (Fig. 1A). For reasons that are currently unknown, FL1-154 was expressed at higher levels than 1-154FL was. We next verified that both HBx-expressing plasmids rescued replication from payw*7. HepG2 cells were cotransfected with payw1.2 and pcDNA3.1−, payw*7 and pcDNA3.1−, payw*7 and FL1-154, or payw*7 and 1-154FL. HBV replication was analyzed by Southern blotting as previously described (6). Replication from payw*7 was diminished compared to replication from payw1.2, and replication from payw*7 was rescued by both FL1-154 and 1-154FL (Fig. 1B).

We next determined whether HBx expression elevates cytosolic calcium levels in HepG2 cells. Basal cytosolic calcium levels were analyzed in HepG2 cells transfected with FL1-154, 1-154FL, or pcDNA3.1−. Transfected HepG2 cells were trypsinized and washed in loading buffer (Eagle's minimal essential medium supplemented with nonessential amino acids, 1 mM sodium pyruvate, and 10 mM HEPES, pH 7.4). Cells were then incubated with 2 μM Fura-2 acetoxymethyl ester (2AM)

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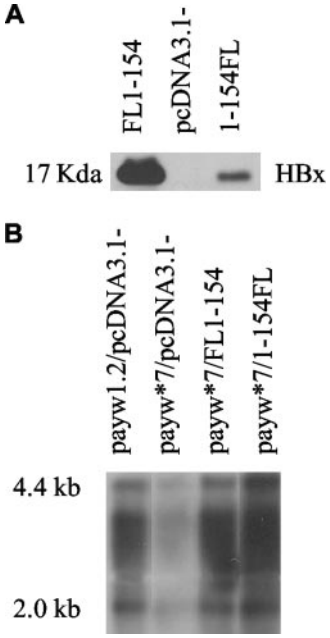

FIG. 1. Analysis of HBx expression and function. (A) HepG2 cells lysates, tranfected with pcDNA3.1−, FL1-154, or 1-154FL, were subjected to Western blot analysis with anti-FLAG antibody. (B) Capsid-associated DNA isolated from HepG2 cells transfected with payw1.2 plus pcDNA3.1−, payw*7 plus pcDNA3.1−, payw*7 plus FL1-154, and payw*7 plus 1-154FL were detected by Southern blot analysis using a ^{32}P-labeled full-length HBV DNA probe.

(Molecular Probes) or with dimethyl sulfoxide (vehicle control) in loading buffer for 20 min, washed in loading buffer, and then suspended in 1.8 mM $CaCl_2$, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4. Fura-2 is a ratiometric calcium indicator that is used to determine cytosolic calcium levels. When bound to calcium, Fura-2 exhibits a shift in its excitation wavelength from 380 nm (calcium free) to 340 nm (calcium bound). This change is observed by recording the emission at 510 nm of samples excited at 340 or 380 nm; the ratio of these emissions is used to calculate the calcium concentration.

Cytosolic calcium concentrations were determined in a Jobin Yvon HORIBA FluoroMax-3 spectrofluorometer by analyzing 200,000 cells/ml in a continually stirred suspension at 37°C. Emission intensities (510 nm) at 340-nm and 380-nm excitations were recorded. We determined basal cytosolic calcium levels for each sample with the following formula: $[Ca^{2+}]_i = K_d$ (Fura-2) $\times (R - R_{min})/(R_{max} - R) \times 380_{min}/380_{max}$. The derivation of this formula and a description of its components have been described extensively previously (34). In brief, R_{min} is the fluorescence ratio observed when the cytosolic Fura-2 in the sample is calcium free and R_{max} is the fluorescence ratio when the cytosolic Fura-2 is saturated with calcium. R is the 340/380 intensity ratio before the addition of any agents to the cell suspension and is the ratio that should be altered by the transfected expression plasmids. The intensity at the 380-nm excitation after the addition of EGTA-Tris is 380_{min}, and 380_{max} is the intensity after the addition of $CaCl_2$. To obtain R_{min} and R_{max} values, 0.3 uM ionomycin, 14.4 mM EGTA-108 mM Tris-HCl, pH 8.7, and 30 mM $CaCl_2$ were

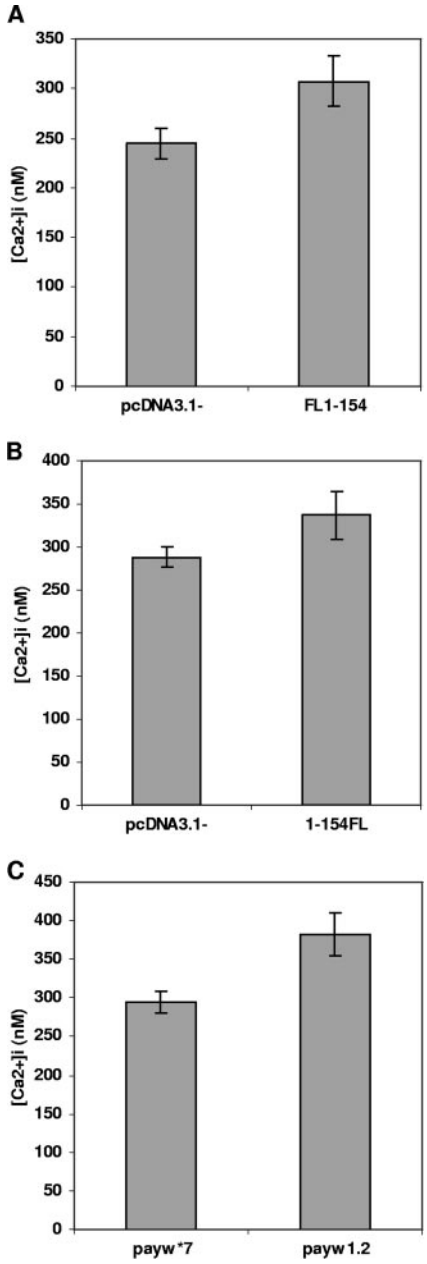

FIG. 2. HBx caused an increase in cytosolic calcium concentrations. HepG2 cells, transfected with (A) pcDNA3.1− and FL1-154, (B) pcDNA3.1− and 1-154FL, and (C) payw1.2 and payw*7, were loaded with Fura-2AM, and cytosolic calcium concentrations were determined. Mean cytosolic calcium concentrations are depicted. Error bars represent standard errors of the means. Statistical analysis, conducted using the Wilcoxon matched-pairs signed-rank test, verified that these increases are statistically significant ($P \leq 0.05$). Matched pair analyses were performed 10 times for the experiments depicted by panels A and B, and 12 times for the experiment depicted by panel C.

added sequentially to the Fura-2-loaded cell suspension. Ionomycin allows calcium exchange between the cell suspension buffer and the cytosol, EGTA chelates calcium to generate calcium-free Fura-2, and the 30 mM $CaCl_2$ is added to saturate cytosolic Fura-2 with calcium. Both FL1-154 and 1-154FL elevated cytosolic calcium concentrations compared to results with match-paired pcDNA3.1− (Fig. 2A and B). For many cell

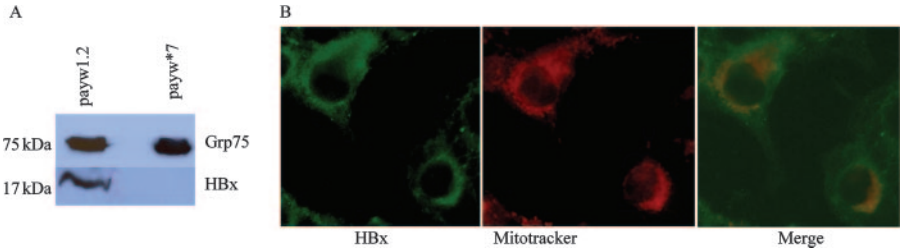

FIG. 3. HBx expressed in the context of HBV is localized to mitochondria. (A) Western blot analysis of mitochondrial-rich fractions isolated from payw1.2- or payw*7-transfected HepG2 cells with anti-Grp-75 (a mitochondrial marker) and anti-HBx antibodies. (B) Immunofluorescence analysis of mitochondria and HBx colocalization. payw1.2- or payw*7 (not shown)-transfected HepG2 cells were labeled with MitoTracker and an anti-HBx antibody and secondary fluorescein isothiocyanate-conjugated antibody. The HBx panel refers to localization of HBx, the MitoTracker panel refers to mitochondria localization with MitoTracker, and the merge panel is an overlay of the two images. There was no anti-HBx background staining in the payw*7-transfected cells.

types, the basal cytosolic calcium levels are maintained at around 100 nM (10); however, hepatocytes have higher cytosolic basal calcium levels, and our calculated basal level of 250 to 275 nM in cells transfected with pcDNA 3.1− is consistent with those in previous studies of HepG2 cells (15, 30, 32). Matched-pair experiments were repeated a minimum of 10 times, and statistical analysis using the Wilcoxon matched-pair signed-rank test confirmed that the frequency at which an HBx-induced increase in cytosolic calcium levels was observed and the increase itself were statistically significant. The presence of an N- or C-terminal FLAG epitope did not hinder HBx modulation of cytosolic calcium (Fig. 2A and B). The differences observed for basal calcium levels in pcDNA3.1− transfected cells (Fig. 2A and B) reflect changes that were detected over the weeks required to acquire these data and may indicate changes in basal calcium levels that are affected by cell passage number. Importantly, these data of matched pairs of pcDNA3.1−- and HBx-transfected cells support the conclusion that HBx expression consistently increases cytosolic calcium levels. While the increase is small, even slight changes in cytosolic calcium levels can affect cellular processes (reviewed in reference 10) and we previously demonstrated that HBV replication and HBx expression activate Pyk2, FAK, and Src kinases in a calcium-dependent manner, supporting the conclusion that small HBx-induced changes in cytosolic calcium levels activate cellular signaling cascades (6, 9).

We next determined whether HBV replication is associated with an HBx-dependent elevation of cytosolic calcium levels. Cytosolic calcium concentrations were determined in HepG2 cells 72 h posttransfection with payw1.2 or payw*7 (Fig. 2C), the time point at which we observed maximum levels of HBV replication (data not shown). We observed an increase in cytosolic calcium levels when HBx was expressed in the context of HBV replication (Fig. 2C); this is the first direct demonstration that HBx increases cytosolic calcium concentrations in liver cells when it is expressed in the context of HBV replication.

The regulation of intracellular calcium involves numerous cellular signaling pathways and organelles. Mitochondria are regulators of cellular calcium signaling; one component of mitochondria involved in this process is the mitochondrial permeability transition pore (MPTP), a multiprotein complex that extends from the mitochondrial matrix through the outer mitochondrial membrane. Opening and closing of the MPTP can regulate cytosolic and mitochondrial calcium levels (24). HBx interacts with the voltage-dependent anion channel, a component of the MPTP, suggesting that HBx could modulate cytosolic calcium by regulating the MPTP (29). HBx was shown to localize to mitochondria when expressed in the absence of other HBV proteins, and one study of HepG2215 cells, a cell line that constitutively expresses replicating HBV from an integrated cDNA of the genome, demonstrated that HBx could be observed in the mitochondrion-enriched fraction of these cells (11, 17, 28, 33). We used biochemical purification and immunofluorescence analyses to demonstrate that HBx, expressed in the context of HBV replication, localizes to mitochondria. HepG2 cells were transfected with payw1.2 or payw*7, collected, and dounce homogenized, and cellular organelles were fractionated by differential centrifugation as previously described (40). The mitochondrion-rich fractions were lysed in Laemmli-SDS-PAGE loading buffer, proteins were separated by SDS-PAGE on a 15% polyacrylamide gel, and Western blot analyses were performed using HBx-specific (Affinity BioReagents) and Grp75-specific (Santa Cruz Biotechnology) antibodies (14). Grp75, a mitochondrial protein, was detected in fractions isolated from both payw1.2- and payw*7-transfected cells, whereas HBx was detected in mitochondrion-rich fractions isolated from payw1.2-transfected cells but not payw*7-transfected cells, demonstrating that HBx, expressed in the context of HBV replication, interacts with mitochondria (Fig. 3A). Because this purification method produces a crude mitochondrial fraction and may contain low levels of contamination from other cellular organelles, we also analyzed HBx localization to mitochondria by immunofluorescence analyses. HepG2 cells were transfected with payw1.2 or payw*7, and 3 days after transfection, the cells were incubated for 30 min with MitoTracker (Molecular Probes) and then fixed and permeabilized with 95% ethanol–5% acetic acid. Cells were stained with the anti-HBx antibody, followed by a fluorescein isothiocyanate-conjugated second antibody as previously described (5), and mitochondria colocalization was analyzed by comparing MitoTracker and HBx localization. Initially, we observed very weak HBx expression and therefore capitalized on the known stabilization of HBx by proteasome inhibitors such as MG132 (16). Transfected cells were treated with MG132 for 12 h prior to immunostaining. For FL1-154-transfected cells, where HBx expression is more easily detected, we determined that treatment with MG132 for this short duration had no effect on HBx localization or mitochondrial morphology compared to the effect from no treatment of HBx-transfected cells

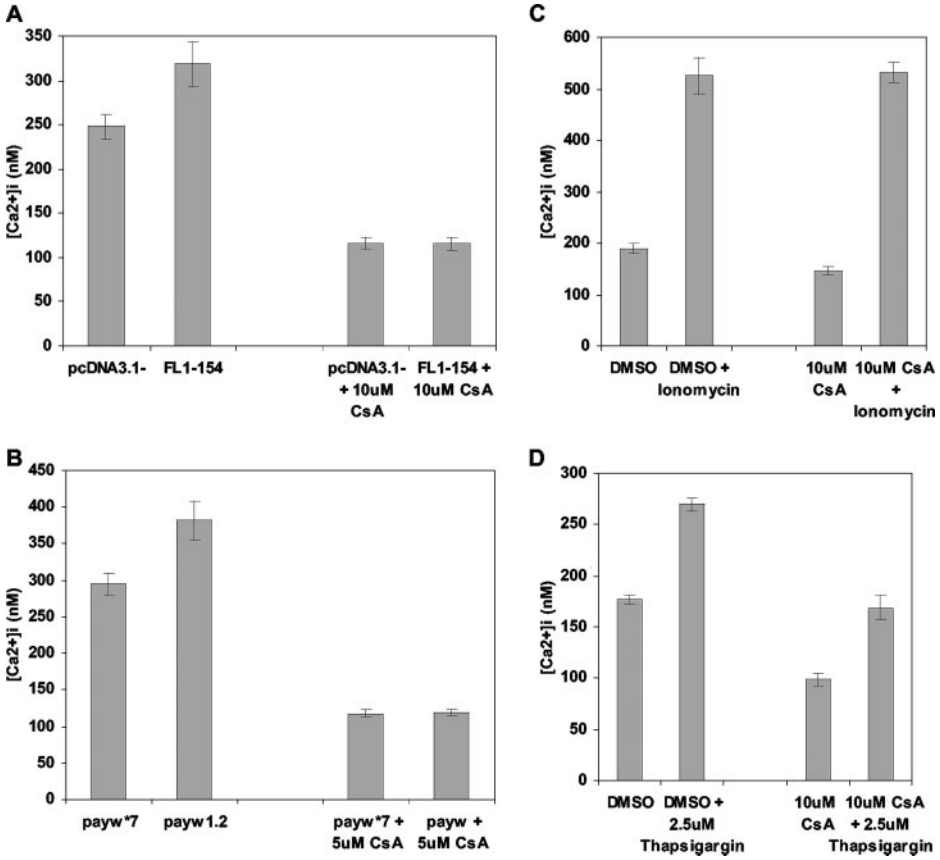

FIG. 4. CsA blocked the HBx-dependent increase in cytosolic calcium. HepG2 cells, transfected with (A) pcDNA3.1− and FL1-154 or (B) payw1.2 or payw*7 and treated with CsA or dimethyl sulfoxide (DMSO), were loaded with Fura-2AM, and cytosolic calcium concentrations were determined. Nontransfected HepG2 cells, loaded with Fura-2AM, were treated with (C) ionomycin or (D) thapsigargin, and cytosolic calcium concentrations were determined. Mean cytosolic calcium concentrations for matched pairs are depicted. Error bars represent standard errors of the means. Statistical analysis, conducted using the Wilcoxon matched-pairs signed-rank test, verified that the increase in the cytosolic calcium concentrations in HBx-expressing cells is statistically significant ($P \leq 0.05$). For panel A, matched pair analyses were performed 10 times without CsA and 7 times with CsA. For panel B, matched pair analyses were performed 12 times without CsA and 8 times with CsA. Matched pair analyses were performed two times for the experiments depicted by panels C and D. Because these CsA-blocking experiments were conducted concurrent with experiments shown in Fig. 2, in Fig. 4A and B, the data shown for samples not treated with CsA are the same as data shown in Fig. 2A and C, respectively.

(data not shown). Significantly, these studies showed that in the context of HBV replication, a fraction of HBx localizes to mitochondria (Fig. 3B); no signal was detected with the anti-HBx antibody in payw*7-transfected cells (data not shown). A comparison of HBx levels in the mitochondrion-enriched fraction to HBx expression in the total cell lysate suggests that approximately 5% of HBx localized to mitochondria (data not shown).

To determine whether this association with mitochondria is involved in the HBx-induced elevation of cytosolic calcium, we treated FL1-154- and payw1.2-transfected HepG2 cells with cyclosporine (CsA), an inhibitor of the MPTP, and evaluated cellular basal calcium levels. A total of 5 μM CsA partially blocked and 10 μM CsA completely blocked FL1-154 from increasing cytosolic calcium (Fig. 4A and data not shown); 5 μM CsA completely prevented payw1.2 from increasing cytosolic calcium (Fig. 4B). The requirement for a higher concentration of CsA to completely block FL1-154 likely reflects the higher levels of HBx expression from this plasmid relative to the payw1.2. Because FL1-154 and 1-154FL gave identical results in our previous studies, we analyzed only the effect of CsA in FL1-154-transfected cells. To verify that CsA was not inhibiting all cytosolic calcium signaling in HepG2 cells, we showed that 10 μM CsA could not prevent ionomycin from increasing cytosolic calcium levels (Fig. 4C). To further demonstrate that CsA specifically inhibited mitochondrial regulation of cytosolic calcium, HepG2 cells were treated with 2.5 μM thapsigargin alone or in combination with 10 μM CsA (Fig. 4D). Thapsigargin, which inhibits endoplasmic reticulum uptake of calcium, caused an increase in cytosolic calcium. HepG2 cells treated with CsA and thapsigargin had decreased cytosolic calcium concentrations relative to those of cells treated with thapsigargin alone, but not to the extent that CsA treatment decreases cytosolic calcium. The observed cytosolic calcium levels in these cells are the results of the two drugs exerting competing effects (Fig. 4D), and these studies show that CsA is not simply inhibiting all modes of cytosolic calcium signals. Collectively, these studies suggest that HBx interacts with mitochondria to elevate cytosolic calcium.

Various activities have been attributed to the expression of

HBx in liver cells, yet the underlying molecular mechanisms responsible for many of these activities remain undefined (8). One potential mechanism that could explain HBx regulation of HBV replication, cell cycle progression, apoptotic pathways, reactive oxygen species levels (37), and transcription is the modulation of cytosolic calcium signals. Previous studies either have indirectly assessed this HBx activity or were conducted using cells of nonliver origin (6, 11, 23). HepG2 cells are a model liver cell culture system to study HBx-dependent HBV replication. Significantly, because this system recapitulates the in vivo requirement for X protein expression that is likely essential for the replication of mammalian hepadnaviruses, studies of HepG2 cells can identify key functions of HBx that are required for HBV replication (21). We have demonstrated that HBx, expressed in the absence of other viral proteins or in the context of HBV replication, increases basal cytosolic calcium. Moreover, we show that HBx, expressed during HBV replication, localizes to mitochondria and that inhibition of the MPTP blocks the HBx-induced increase of cytosolic calcium levels. Collectively, these studies suggest that a fundamental activity of HBx is to increase cytosolic calcium levels through modulation of the MPTP.

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