Caspase-dependent Alterations of Ca$^{2+}$ Signaling in the Induction of Apoptosis by Hepatitis B Virus X Protein*

Received for publication, April 22, 2003, and in revised form, June 9, 2003

Published, JBC Papers in Press, June 10, 2003, DOI 10.1074/jbc.M304202200

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The hepatitis B virus X protein (HBx) is a multifunctional protein, acting on different targets (e.g. transcription factors, cytoplasmic kinases, and mitochondrial proteins) and exerting cellular effects as diverse as stimulation of cell proliferation and apoptosis. In its biological effects, the modulation of cellular Ca$^{2+}$ signals has been proposed to be involved, but the direct assessment of Ca$^{2+}$ homeostasis in HBx-transfected cells has not been carried out yet. In this work, we have employed for this purpose aequorin-based recombinant probes specifically targeted to intracellular organelles and microdomains. Using these probes, we observed that overexpression of HBx enhanced agonist-evoked cytosolic Ca$^{2+}$ signals in HepG2 and HeLa cells, without affecting either the steady state of endoplasmic reticulum Ca$^{2+}$ concentration or the kinetics of Ca$^{2+}$ release. Rather, caspase-3-dependent cleavage of the plasma membrane Ca$^{2+}$ ATPase could be demonstrated, and larger rises were detected in the cytoplasmic rim beneath the plasma membrane. In mitochondria, major morphological (fragmentation and swelling) and functional (reduced Ca$^{2+}$ uptake) alterations were detected in HBx-expressing cells. As to the cellular consequences, we observed that HBX-induced apoptosis was markedly reduced when the alterations in Ca$^{2+}$ signaling (e.g. by loading a Ca$^{2+}$ chelator or preventing PMCA cleavage) or the downstream effects (e.g. by inhibiting mitochondrial permeability transition) were prevented. Overall, these results indicate that HBx perturbs intracellular Ca$^{2+}$ homeostasis, acting on the extrusion mechanisms, and that this effect plays an important role in the control of HBX-related apoptosis.

Infection by human hepatitis B virus (HBV)$^{1}$ is a major risk factor for the development of liver cirrhosis and hepatocellular carcinoma (1). Among the proteins encoded by the HBV genome, the X protein (HBx) has been shown to have a central role in HBV replication and a potential role in liver oncogenesis (2, 3). Overexpression of HBx induces transformation of some cell types (4, 5). Moreover, some HBx transgenic mouse strains develop liver cancer (6). However, the tumorigenic effect of HBx is still incompletely understood, because in different genetic contexts HBx transgensics exhibit no obvious pathology (7) but cause an increased susceptibility to chemical carcinogens (8) or an acceleration of the development of c-Myc-driven hepatocellular carcinoma (9). Different animal viruses have been shown to have the ability to induce apoptosis, in addition to their ability to induce cell growth (for review see Ref. 10). Depending on the cell type and experimental procedure, HBx has been reported either to inhibit (11) or promote (12–14) cell death. Actually, an integrated view of the role of HBx on apoptosis has been proposed. In this model, high levels of HBx, which are present during the acute phase of HBV infection, cause cell cycle block and apoptosis, whereas low HBx levels, such as those observed in chronically infected humans, would allow cell liver proliferation (1). However, the molecular mechanism involved in the HBX-related control of cell death is still unknown. HBx is a multifunctional protein initially described as a transcriptional transactivator capable of stimulating a variety of viral and host gene promoters through its interaction with transcription factors, including AP-1, ATF/CREB, ERCC, and RPB5 of RNA polymerase and recently with NFAT-1 (3, 15). Various groups have shown that HBx expression deregulates cell growth (16). In fact, HBx has been demonstrated to directly interact with the proteasome (17) and with cellular proteins controlling cell growth, apoptosis (p53, DNA repair), and senescence (18, 19). HBx protein is predominantly localized in the cytoplasm, with a partial nuclear distribution (20, 21). Through its cytosolic localization, HBx has been shown to participate in a wide range of cellular signal transduction cascades, including Ras-Raf-mitogen-activated protein kinase, c-Jun N-terminal Kinase, NF-xB, Src, and JAK1-STAT signaling pathways (3).

Recent data have shown that the HBx protein is also targeted to mitochondria (21) and colocalizes with the voltage-dependant anion channel 3 (22). This observation is consistent with further data showing that HBx expression alters mitochondrial membrane potential (22) and activates STAT-3 and NF-xB transcription factors via mitochondrial oxidative stress (23).

Calcium is a ubiquitous intracellular signal responsible for...
controlling numerous cellular processes including fertilization, proliferation, differentiation, development, and cell death (24).

Two lines of evidence support a role for Ca\(^{2+}\) in apoptosis. First, elevation of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_\text{cyt}\)) induces activation of Ca\(^{2+}\)-dependent protein kinases, proteinases, and endonucleases leading to apoptosis (25). Second, the anti-apoptotic oncogene Bcl-2 has been suggested in a recent work of Bouchard et al. (28).

Treatment with agents that in different ways reduce or buffer Ca\(^{2+}\) extrusion has been demonstrated in an in vitro model of HBV replication to counteract the effect of HBx on Ca\(^{2+}\) homeostasis in control and HBx-transfected cells. Cytosolic [Ca\(^{2+}\)] in control and HBx-transfected HeLa cells (CCL-2; American Type Culture collection) derived from human cervix epithelioid carcinoma were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HeLa cells (CCL-2; American Type Culture collection) derived from human cervix epithelioid carcinoma were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Celbio, Milan, Italy). HepG2 cells, derived from human hepatocellular carcinoma, were transfected with cytAEQ and HBx (HBx, gray traces). 48 h after transfection, measurements of aequorin luminescence were carried out and calibrated into [Ca\(^{2+}\)] values, as described under “Experimental Procedures.” Where indicated, cells transfected with KRBI (mM CaCl\(_2\)) were challenged with 100 \(\mu\text{M}\) histamine or 100 \(\mu\text{M}\) ATP added to the same buffer. These data and those of the following figures are representative of at least three independent transfections that gave similar results.

**Experimental Procedures**

**Reagents**—Ionomycin, histamine, ATP, digoxin, and cyclosporin A (CsA) were purchased from Sigma; 2-Valine-alanine-aspartate fluoromethyl ketone (zVAD-fmk) was from Calbiochem (INALCO S.P.A., Milan, Italy); BAPTA-AM and coelenterazine were from Molecular Probes, and ExGen transfection solution was from Euromedex (Souffelweyersheim, France).

**Cell Lines and Constructs**—HepG2 cells, derived from human hepatocellular carcinoma, were transfected in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal calf serum. HeLa cells (CCL-2; American Type Culture collection) derived from human cervix epithelioid carcinoma were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Celbio, Milan, Italy). HepG2 constructs encoding native or GFP-fused protein used in this study are cloned in pcDNA3.1 (Invitrogen) under cytomegalovirus promoter. Human PMCA4b wild type (hPMCA4b-wt) and mutated (hPMCA4b-mut) are cloned into PGSV vectors upon SV40 promoter. In hPMCA4b-mut construct the caspase-3 consensus sequence (1077DEID1080) was mutated to (1077DEIA1080) (29). For calcium analyses, we used aequorin probes targeted to the ER (erAEQ) (30), mitochondria (mtAEQ) (31), the subplasmalemmal space (pmAEQ) (32), and the cytosolic aequorin probe (cytAEQ) (33). For subcellular structure analyses, we used mitochondrial and endoplasmic reticulum-targeted GFP probes.

**Transient Transfection**—Transient transfection of the HepG2 cell line was carried out using ExGen solution as already described (34). Transient transfection of HeLa cells was carried out using the “calcium phosphate” coprecipitation method. For aequorin measurements, the cells were seeded into 13-mm coverslips and cotransfected with the HBx expression plasmid and the various recombinant aequorin probes in a 3:1 ratio (1.5 \(\mu\text{g}\) versus 0.5 \(\mu\text{g}\)), thus favoring the expression of HBx protein in the same subset of cells expressing recombinant aequorin. For mitochondrial and ER morphological analysis, cotransfection was performed on 24-mm glass coverslips with 2 \(\mu\text{g}\) of mitochondrial targeted GFP (mtGFP) or ER-targeted GFP (erGFP) and 6 \(\mu\text{g}\) of HBx construct. In the experiments of Fig. 5, a triple transfection was performed to analyze cytosolic [Ca\(^{2+}\)] in cells expressing both HBx and wild type or mutated hPMCA4b. For this purpose, 0.5 \(\mu\text{g}\) of cytAEQ probe, 1.5 \(\mu\text{g}\) of HBx construct, and 1.5 \(\mu\text{g}\) of hPMCA4b-wt or hPMCA4b-mut constructs were used.

**Measurement of Caspase-3 Like Activity**—Caspase-3-like activity was evaluated by using EnzChek caspase-3 assay kit 2 (Molecular Probes). Enzymatic activity was determined spectrophotometrically (L550B Perkin Elmer spectrometer) by measuring the kinetics of fluorescence increase at excitation/emission wavelengths of 496/520 nm.

**Aequorin Measurements**—The aequorin chimeras were cotransfected with a GFP expression plasmid (controls) or the HBx expression plasmid (HBx-overexpressing cells) as described above. For mtAEQ and cytAEQ, 44–48 h post-transfection, the coverslips were incubated with 5 \(\mu\text{M}\) coelenterazine for 2 h in Krebs-Ringer modified buffer (KRB) (125 mM NaCl, 5 mM KCl, 1 mM MgSO\(_4\), 1 mM Na\(_2\)PO\(_4\), 5.5 mM glucose, and 20 mM Hepes, pH 7.4) at 37°C. In the experiments shown in Fig. 5 (C and D), the cells were treated with 100 \(\mu\text{M}\) zVAD-fmk, 20 h post-transfection, and the cytosolic measurements of Ca\(^{2+}\) were performed as described above. For reconstituting with high efficiency erAEQ, the luminal [Ca\(^{2+}\)] of this compartment was first reduced. This was obtained by incubating the cells for 1 h at +4°C in KRB supplemented with 5 \(\mu\text{M}\) n-coelenterazine, the calcium isophorone ionomycin, and 600
**Fig. 2.** Ca\(^{2+}\) homeostasis in the lumen of the ER in control and HBx-transfected cells. [Ca\(^{2+}\)\(_{\text{ER}}\)] measurements were performed in HeLa cells (A) and HepG2 cells (B) transfected with eA quantum (Control, black traces) or cotransfected with eA quantum and HBx (HBx, gray traces). The cells were first perfused with KRB/100 μM EGTA. Where indicated, EGTA was replaced with 1 mM CaCl\(_2\) that was maintained until the steady state [Ca\(^{2+}\)\(_{\text{ER}}\)] was reached. C, effect of agonist stimulation. When the plateau [Ca\(^{2+}\)\(_{\text{ER}}\)] was reached, HeLa cells were stimulated with 100 μM histamine added to KRB/1 mM Ca\(^{2+}\).
Larger Ca\(^{2+}\) Responses in HBx-expressing Cells

Fig. 3. Caspase-3 activity and cleavage of PMCA in HBx-expressing cells. A, caspase-3-like activity was determined in control (Control) and HBx-transfected HepG2 cells (HBx) as detailed under “Experimental Procedures” and expressed as arbitrary units. The caspase-2 inhibitor Ac-DEVD-CHO (Inh) was also used to show the specificity of the activation. B, Western blot analysis of PMCA cleavage was carried out in HeLa cells transfected with GFP plasmid (Control) or with HBx (HBx). Analyses were performed 60 h post-transfection. HeLa cells treated with staurosporine (2.5 \(\mu\)M for 3 h, Stauro) were used as positive controls (almost 80% of cells were undergoing apoptosis). The black arrow points to uncleaved PMCA, and the white arrow points to caspase-cleaved PMCA (120 kDa). Pretreatment with caspase inhibitor zVAD-fmk (100 \(\mu\)M) abolished PMCA cleavage. Densitometric quantification of caspase-cleaved PMCA versus uncleaved PMCA is represented below the blots.

Inc.) (diluted 1:2000), and a secondary goat anti-mouse horseradish peroxidase-conjugated antibody (diluted 1:5000) were used. The signal was revealed using ECL Plus Western blot detection reagent (Amersham Biosciences).

Statistical Analyses—The statistical analyses were performed using a Student’s \(t\) test. \(p\) value \(\leq 0.05\) was considered to be significant. All of the data are reported as the means \(\pm S.E.\)

RESULTS

HBx Expression Enhances Cytosolic Agonist Evoked Ca\(^{2+}\) Signal—In this study, we took advantage of the high subcellular specificity of aequorin probes (35) to determine the direct effects of HBx expression on the dynamics of agonist-dependent [Ca\(^{2+}\)]\(_e\) changes occurring in the cytoplasm and in organelles acting as source (ER) or target (mitochondria) of the Ca\(^{2+}\) signals.

We first investigated in a hepatocellular carcinoma-derived cell line (HepG2) and in an epithelial carcinoma-derived cell line (HeLa) the cytosolic Ca\(^{2+}\) signal elicited by agonists, acting on receptors coupled through G proteins to the production of inositol 1,4,5-triphosphate. In the experiments shown in Fig. 1, HeLa and HepG2 cells either coexpressing HBx and cytAEQ (HBx) or expressing only cytAEQ (Control) were challenged with histamine or ATP. Both in control and HBx-transfected cells, this stimulation caused a rapid rise in cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)) followed by a gradually declining plateau sustained by Ca\(^{2+}\) influx through plasma membrane channels.

In HBx-expressing cells, the [Ca\(^{2+}\)]\(_c\) increases evoked by stimulation with histamine (HeLa) or with ATP (HepG2) were significantly higher than in controls (peak amplitude = 2.9 \(\pm\) 0.09 \(\mu\)M (n = 10) versus 2.2 \(\pm\) 0.04 \(\mu\)M (n = 13) in HeLa cells, \(p\) value \(\leq 0.001\); peak amplitude = 1.2 \(\pm\) 0.04 \(\mu\)M (n = 8) versus 0.5 \(\pm\) 0.03 \(\mu\)M (n = 13) in HepG2 cells, \(p\) value \(\leq 0.002\)) (Fig. 1).

Taking the [Ca\(^{2+}\)]\(_c\) of control cells as 100%, the agonist-evoked [Ca\(^{2+}\)]\(_e\) peak was elevated in HBx-transfected cells to 131% and to 147% in HeLa and HepG2 cells, respectively.

The Larger [Ca\(^{2+}\)]\(_e\) Peak in HBx-expressing Cells Does Not Depend on a Modification of ER Ca\(^{2+}\) Signaling—Given that the [Ca\(^{2+}\)]\(_e\) peak is mostly contributed by ER Ca\(^{2+}\) release and that we previously observed that the anti-apoptotic protein Bcl-2 reduces cellular Ca\(^{2+}\) signals by partially depleting the ER Ca\(^{2+}\) stores (26), a simple explanation of these results was that HBx directly affected ER Ca\(^{2+}\) homeostasis. To verify this possibility, we measured the ER Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_{er}\) with erAEQmut, a low affinity, specifically ER-targeted aequorin chimera (30). As described under “Experimental Procedures,” to maximize reconstitution of the photoprotein, the ER Ca\(^{2+}\) content was reduced during the phase of erAEQ reconstitution with coelenterazine and the subsequent initial phase of perfusion. Under these conditions, [Ca\(^{2+}\)]\(_{er}\) was <10 \(\mu\)M. Then, when Ca\(^{2+}\)\(_{er}\) in the perfusion medium was switched to 1 \(\mu\)M, [Ca\(^{2+}\)]\(_{er}\) rose gradually to reach a steady state plateau value. Surprisingly, in both HepG2 and HeLa cells, the plateau value was similar in cells expressing HBx protein as compared with control cells (steady state value = 426 \(\pm\) 12 \(\mu\)M (n = 11) versus 391 \(\pm\) 11 \(\mu\)M (n = 13) in HeLa cells, \(p\) value = 0.05 (Fig. 2A); steady state value = 333 \(\pm\) 11 \(\mu\)M (n = 8) versus 325 \(\pm\) 11 \(\mu\)M (n = 7) in HepG2 cells, \(p\) value = 0.6 (Fig. 2B)), ruling out an effect of HBx on Ca\(^{2+}\) accumulation by SERCA or on the passive ER Ca\(^{2+}\) leak. Another explanation of the elevation of cytosolic Ca\(^{2+}\) responses could be an effect on the kinetics of Ca\(^{2+}\) release through the inositol 1,4,5-triphosphate receptors. However, when HeLa cells were treated with histamine, the maximal rate of Ca\(^{2+}\) release was similar in HBx-transfected cells (19.6 \(\pm\) 3 \(\mu\)S, n = 8) as compared with control cells (19 \(\pm\) 3.2 \(\mu\)S, n = 9) (Fig. 2C).

Caspase-3-related PMCA Cleavage in HBx-expressing Cells—The mechanisms which maintain [Ca\(^{2+}\)]\(_e\), low in resting condi-
Expression of HBx Is Associated with Inactivation of PMCA and Increase in Ca\(^{2+}\) Content in the Cytosol Rim beneath the Plasma Membrane—To demonstrate that PMCA inactivation was involved in the elevation of cytosolic [Ca\(^{2+}\)], we first measured the [Ca\(^{2+}\)] in the subplasmalemmal space using the aerugin chimera targeted to this domain (32). As shown in Fig. 4A, stimulation of HepG2 cells with ATP in Ca\(^{2+}\)-free (KRB/EGTA) medium evoked a very modest [Ca\(^{2+}\)] rise (amplitude < 1 \(\mu\)M) almost undetected by the low affinity probe, whereas a large and rapid rise was observed upon readdition of Ca\(^{2+}\) (KRB/CaCl\(_2\)). This analysis revealed that the Ca\(^{2+}\) level in these microdomains was significantly higher in HepG2 cells expressing HBx protein (42 ± 3 \(\mu\)M (\(n = 7\)) as compared with control cells (21 ± 2 \(\mu\)M (\(n = 8\)); \(p < 0.0001\)) (Fig. 4A). This result could be due to different and complementary mechanisms: (i) an elevation of Ca\(^{2+}\) influx in HBx-expressing cells and (ii) impairment of Ca\(^{2+}\) extrusion through PMCA. To investigate the first possibility, we measured the capacitative Ca\(^{2+}\) influx in HBx-transfected and control HepG2 cells expressing cytAEQ (Fig. 4B and C). The cells were perfused with KRB/EGTA to buffer extracellular [Ca\(^{2+}\)] and were then stimulated with ATP prepared in the same solution to release Ca\(^{2+}\) from intracellular stores. This maneuver caused a first [Ca\(^{2+}\)] rise that was significantly higher in HBx-transfected versus control cells (peak value 1.06 ± 0.03 \(\mu\)M (\(n = 6\)) versus 0.83 ± 0.03 \(\mu\)M (\(n = 4\))). The perfusion with Ca\(^{2+}\) containing solution (KRB/CaCl\(_2\)) evoked a second [Ca\(^{2+}\)] rise, which corresponds to capacitative calcium influx that was not significantly increased in HBx-expressing cells as compared to control cells (1.04 ± 0.06 \(\mu\)M (\(n = 6\)) versus 1 ± 0.08 \(\mu\)M (\(n = 4\)), respectively) (Fig. 4, B, representative curves, and C). Average of [Ca\(^{2+}\)] peak. Because analyses of Ca\(^{2+}\) influx did not show any significant difference between HBx and control cells and terminate Ca\(^{2+}\) responses to agonists include: (i) active Ca\(^{2+}\) reuptake into the ER through the SERCAs and (ii) Ca\(^{2+}\) extrusion from the cytoplasm to the extracellular space through activation of the PMCA. Because ER Ca\(^{2+}\) signaling was not modified in HBx-expressing cells, an alternative source of the elevated [Ca\(^{2+}\)] in stimulated HBx-expressing cells could be a reduction in the PMCA pumping activity. In support of this possibility, it was recently demonstrated that the widely distributed human PMCA4b isoform contains a caspase-3 consensus sequence (1077DEID1080) that is cleaved by caspase-3 during apoptosis, generating a 120-kDa PMCA fragment with resident activity. As shown in Fig. 4B, Western blot analyses revealed the presence of the 120-kDa PMCA fragment in nontransfected HepG2 cells and nontransfected control cells transfected with pmAEQ alone (Control), or cotransfected with pmAEQ and HBx (HBx). The cells were first perfused with KRB/EGTA 100 \(\mu\)M and challenged with ATP 100 \(\mu\)M (added to the same medium). Then in the continuous presence of the agonist, EGTA was replaced with 1 \(\mu\)M CaCl\(_2\) (KRB/Ca\(^{2+}\)). B and C, capacitative Ca\(^{2+}\) influx in control and HBx-transfected cells. B, representative traces; C, average [Ca\(^{2+}\)] peak. HepG2 cells transfected with cytAEQ (Control) or cotransfected with cytAEQ and HBx (HBx) perfused with KRB/1 \(\mu\)M CaCl\(_2\) were challenged with 100 \(\mu\)M ATP added to the same medium. After 1 min, capacitative Ca\(^{2+}\) entry was initiated by changing the medium to KRB/1 \(\mu\)M CaCl\(_2\) + 100 \(\mu\)M ATP. We then directly assessed caspase-dependent cleavage of PMCA by immunoblotting, using a monoclonal antibody (JA9) recognizing the ubiquitous PMCA4b isoform. As shown in Fig. 3B, Western blot analyses revealed the presence of the 120-kDa band (PMCA cleaved form) in HBx-transfected cells as well as in staurosporine-treated cells. In the latter case, the cleaved form was more abundant (44%) than in HBx-expressing cells (27%), but it should be noted that in HBx-transfected cells cleavage occurs only in the subset of transfected cells (~35%). Only the noncleaved PMCA form was observed in nontransfected cells (control). Incubation with the poly-caspase inhibitor zVAD-fmk prevented pump cleavage in HBx-transfected and staurosporine-treated cells.

Expression of HBx Is Associated with Inactivation of PMCA

and Increase in Ca\(^{2+}\) Content in the Cytosol Rim beneath the Plasma Membrane

A

B

C

Fig. 4. Subplasmalemmal Ca\(^{2+}\) homoeostasis and capacitative calcium influx in HBx-expressing cells. A, Ca\(^{2+}\) concentration in the subplasmalemmal space was measured in HepG2 cells transfected with pmAEQ alone (Control) or cotransfected with pmAEQ and HBx (HBx). The cells were first perfused with KRB/EGTA 100 \(\mu\)M and challenged with ATP 100 \(\mu\)M (added to the same medium). Then in the continuous presence of the agonist, EGTA was replaced with 1 \(\mu\)M CaCl\(_2\) (KRB/Ca\(^{2+}\)). B and C, capacitative Ca\(^{2+}\) influx in control and HBx-transfected cells. B, representative traces; C, average [Ca\(^{2+}\)] peak. HepG2 cells transfected with cytAEQ (Control) or cotransfected with cytAEQ and HBx (HBx) perfused with KRB/1 \(\mu\)M CaCl\(_2\) were challenged with 100 \(\mu\)M ATP added to the same medium. After 1 min, capacitative Ca\(^{2+}\) entry was initiated by changing the medium to KRB/1 \(\mu\)M CaCl\(_2\) + 100 \(\mu\)M ATP.
cells, we concluded that PMCA cleavage and thus inactivation could mostly account for the elevation of [Ca$$^{2+}$$].

To support this conclusion, we analyzed the effect of HBx expression on Ca$$^{2+}$$ signaling in cells coexpressing either wild type hPMCA4b (PMCA-wt) or a mutated form in which the caspase-3 consensus sequence was altered (PMCA-mut) (see "Experimental Procedures" for details). For this purpose, in the experiments presented in Fig. 5 (A and B), HepG2 cells were transfected with cytAEQ in combination with HBx and/or the PMCA construct of interest. The cytosolic Ca$$^{2+}$$ signal was measured as described in Fig. 1. The amplitude of [Ca$$^{2+}$$], peak (expressed as a percentage of the [Ca$$^{2+}$$] peak of control cells and averaging all experiments with standard errors) is shown in Fig. 5A. Representative traces are shown in Fig. 5B. In cells overexpressing HBx only, the [Ca$$^{2+}$$] peak was elevated to 131 ± 7% (n = 9) of controls, in agreement with the results of Fig. 1. As previously shown by Brini et al. (36), in cells overexpressing PMCA-wt or PMCA-mut (thus enhancing the Ca$$^{2+}$$ extrusion capacity), the [Ca$$^{2+}$$] peak was lower, and the return to basal level was faster (see representative curves in Fig. 5B).

When HBx was coexpressed with PMCA constructs, a striking difference was observed between PMCA-wt and PMCA-mut. In the former case, HBx markedly increased the agonist-dependent [Ca$$^{2+}$$] peak in PMCA-wt + HBx-overexpressing cells: 110 ± 2.5% (n = 10) versus 88 ± 2.5% (n = 13; p value = 0.003) in cells overexpressing only PMCA-wt. Conversely, the increase was marginal in PMCA-mut + HBx-overexpressing cells: 82 ± 2.5% (n = 6) versus 78 ± 4% (n = 6; p value = 0.96) in cells overexpressing only hPMCA4b-mut.

To further demonstrate the impact of caspase-dependent PMCA cleavage on [Ca$$^{2+}$$], in HBx-expressing cells, we measured [Ca$$^{2+}$$] in HBx-transfected cells after caspase inhibition. HeLa cells either coexpressing HBx and cytAEQ (HBx) or expressing only cytAEQ (Control) were treated with zVAD-fmk (100 μM) 20 h post-transfection. As shown in Fig. 5C, representative curves, and D, percentage of [Ca$$^{2+}$$] peak, of control cells, zVAD treatment reduced the agonist-induced [Ca$$^{2+}$$], peak in HBx-expressing cells (2.1 ± 0.09 μM (n = 10) versus 2.6 ± 0.09 μM (n = 10; p value ≤ 0.001) to a level similar to that of control cells (1.9 ± 0.09 μM (n = 7)) (Fig. 5C). No
modification of $[Ca^{2+}]_m$ was observed in control cells treated with zVAD (data not shown). Overall, these results demonstrate that alteration of cytosolic $[Ca^{2+}]$ in HBx-expressing cells is consequent of caspase-dependent PMCA cleavage and inactivation.

Expression of HBx Protein Induces a Reduction of Mitochondrial Ca$^{2+}$ Responses and a Modification of the Organelle Morphology—Finally, we investigated the effect of HBx on mitochondrial Ca$^{2+}$ homeostasis, which could be affected by two mechanisms: (i) HBx protein has been shown to localize to the mitochondria (21, 22) and (ii) the cytosolic Ca$^{2+}$ overload occurring at each cell stimulation could be responsible for the opening of the permeability transition pore (PTP) and thus inducing major morphological alterations of mitochondria.

We measured mitochondrial Ca$^{2+}$ concentration $[Ca^{2+}]_m$ at rest and after agonist stimulation using a specifically targeted aequorin chimera, mtAEQ, cotransfected with HBx. As shown in Fig. 6, HBx expression caused a significant reduction of the $[Ca^{2+}]_m$ rise evoked by histamine stimulation in HeLa cells (peak amplitude 66 ± 5 µM (n = 11) in HBx-expressing cells versus 103 ± 4 µM (n = 12) in control; p value ≤ 0.0001) (Fig. 6A). Only a small reduction of the $[Ca^{2+}]_m$ rise upon ATP stimulation was observed in HBx-expressing HepG2 cells (peak amplitude 21 ± 0.67 µM (n = 8)) versus control (24.5 ± 0.84 µM (n = 9)) (Fig. 6B). This result could be due to the low mitochondrial response to agonist stimulation in HepG2 cells (24.5 µM) as compared with HeLa cells (103 µM).

Reduction of mitochondrial Ca$^{2+}$ uptake in HBx-overexpressing cells could be induced by: (i) a reduction in the efficiency of mitochondria in accumulating Ca$^{2+}$, and (ii) a morphological alteration of mitochondria that could reduce the sites of close contact between ER and mitochondria, allowing rapid mitochondrial Ca$^{2+}$ uptake (37).

To test the first possibility, we measured mitochondrial Ca$^{2+}$ uptake rate in permeabilized control and HBx-overexpressing HeLa cells. As described under "Experimental Procedures," the cells were first permeabilized in a Ca$^{2+}$-free buffer mimicking the cytosolic ionic composition (IB) containing digitonin. After a brief wash with IB/EGTA (1 min), mitochondrial Ca$^{2+}$ uptake was initiated by perfusing the cells with IB containing an EGTA-buffered $[Ca^{2+}]$ (IB/CaCl$_2$) of 1 µM (Fig. 6C) or 5 µM (data not shown). Analyses of the traces did not show a difference in the Ca$^{2+}$ uptake kinetics between the two groups of cells (Fig. 6C), thus indicating that HBx did not reduce the efficiency of mitochondrial Ca$^{2+}$ accumulation.

To test the second hypothesis, we analyzed mitochondrial structure by using a mtGFP probe. For this purpose, HeLa and HepG2 cells were cotransfected with mtGFP and HBx or transfected with mtGFP alone, and organelle structure was evaluated 48 h post-transfection using a digital imaging system. Fig. 7A shows representative images of control and HBx-transfected HepG2 (panels a–c) and HeLa (panels d–f) cells. In control HepG2 and HeLa cells (Fig. 7A, panels a and d, respectively), the typical tubular interconnected mitochondrial network was observed. In HBx-transfected HepG2 (Fig. 7A, panels b and c) and HeLa cells (Fig. 7A, panels e and f), a major morphological alteration was evident. In HBx-transfected HepG2 cells, mitochondria were almost round and fragmented (Fig. 7A, panel b), and in some cells they were completely swollen (Fig. 7A, panel c). In HBx-transfected HeLa cells, the alteration of the mitochondrial network was less pronounced than in HepG2 cells (Fig. 7A, panel e); still, we noticed a large number of cells with completely fragmented mitochondria (Fig. 7A, panel f). The effect was specific for mitochondria. Indeed, when the ER structure was investigated in HepG2 (Fig. 7B) and HeLa cells (data not shown), using an eGFP chimera, no major difference was observed between control (Fig. 7B, panel a) and HBX-overexpressing cells (Fig. 7B, panels b and c). In both cases, we observed the typical ER structure with fine reticular staining diffused to the whole cell.

HBx-induced Apoptosis Is Related to the Perturbation of Calcium Homeostasis—Finally, we evaluated the effect of the alteration in Ca$^{2+}$ signaling on HBx-dependent apoptosis. We first determined the percentage of apoptotic cells and the kinetics of the process in HepG2 and HeLa cells expressing HBx using two different methods: (i) morphological analyses based on the evaluation of the percentage of apoptotic nuclei (fragmented nuclei) in GFP-positive cells and (ii) molecular analyses based on the evaluation of cytochrome c release from mitochondria. As shown in Fig. 8A, the percentage of apoptotic cells was markedly higher in HBx-transfected cells than in controls (cells transfected with GFP empty vector alone), an effect that was more pronounced in HepG2 than in HeLa cells. In HepG2 cells, the percentage of apoptotic cells in HBX-expressing cells
as compared with controls was 21% versus 5% (36 h post-transfection) and 35% versus 5% (48 h post-transfection) (Fig. 8A, left panel). In HeLa cells, the percentage of apoptosis in HBx-expressing cells as compared with controls was 12.5% versus 3% (44 h post-transfection) and 22% versus 2% (52 h post-transfection) (Fig. 8A, right panel). In agreement with this finding, Western blot analyses demonstrated that HBx expression caused release of cytochrome c into the cytoplasm; the quantification of cytosolic cytochrome c signal, normalized to that of β-tubulin, revealed a significant cytochrome c release in HBx-expressing HeLa cells 36 h post-transfection (2.4-fold increase versus control cells) that is maximal at 48 h post-transfection (3.2-fold increase versus control cells) (Fig. 8B).

We then evaluated the effect on HBx apoptotic efficiency of experimental protocols that acted on Ca<sup>2+</sup> signaling and/or other cellular processes. In HepG2 cells, both incubation in Ca<sup>2+</sup>-free, EGTA-containing media (that reduced the state of filling of ER Ca<sup>2+</sup> stores) and the loading of an intracellular Ca<sup>2+</sup> chelator (BAPTA-AM) caused in HBx-expressing cells a partial but significant reduction of apoptosis (17.5% in HBx with EGTA and 19% in HBx with BAPTA). A partial reduction of apoptosis was also observed by inhibiting caspase activation with zVAD-fmk or by blocking PTP opening with CsA (23% in HBx + zVAD and 18.5% in HBx + CsA). Combination of these treatments or overexpression of the anti-apoptotic Bcl-2 protein, which acts both on Ca<sup>2+</sup> signaling and on other apoptotic pathways, caused a higher reduction of apoptosis (10.5% in HBx with CsA and BAPTA, 11.5% in HBx with zVAD and BAPTA, and 11.5% in HBx with Bel-2) (Fig. 8C). Similar results were obtained in HeLa cells (Fig. 8C, inset).

**DISCUSSION**

In this work, we have investigated the molecular mechanisms involved in the HBx-related control of cell death. The concept that a putative oncogene like HBx may modulate both cell proliferation/transformation and apoptosis has now been established for several oncogenic viral proteins, such as E1A of adenovirus 5 (38) and E2 of papillomavirus (39). This suggests a common pathway used by viruses to subvert the control of cell proliferation and cell death. In the past, some evidence has been provided suggesting an involvement of Ca<sup>2+</sup> in the biological effects of HBx: (i) HBx-mediated transactivation involves calcium-dependent transcription factors, i.e. NFAT (40), and calcium-dependent signaling pathways, i.e. the Ras/MAPK and Pyk2 kinase cascades (41), and (ii) HBx has been shown to colocalize with mitochondria (22), an organelle that has been recently shown to play an important role in shaping calcium signals (42, 43). However, no direct measurements of Ca<sup>2+</sup> have ever been performed that accurately assess the HBx-induced modifications of Ca<sup>2+</sup> signaling.

By monitoring Ca<sup>2+</sup> with aequorin probes, we have now shown that the overexpression of HBx in hepatic (HepG2) and nonhepatic (HeLa) cell lines significantly increases the Ca<sup>2+</sup> signals evoked by stimulation with InsP<sub>3</sub>-linked agonists and enhances HBx-induced apoptosis. These results well match those obtained with the anti-apoptotic protein Bcl-2, which exerts the opposite effect, i.e. a reduction in agonist-dependent Ca<sup>2+</sup> signals. What is conceptually different is the mechanism by which the Ca<sup>2+</sup> signaling alteration is achieved; Bcl-2 enhances the passive leak from the ER (thus acting on the avail-
ability of agonist-releasable Ca\(^{2+}\)), and HBx does not affect the Ca\(^{2+}\) stores. Neither the rate of Ca\(^{2+}\) accumulation, nor the leak, nor the rate of release through the InsP\(_3\)-gated channels is different in control and HBx-overexpressing cells. Also considering that no morphological change could be detected in the ER, these data indicate that the ER is not the primary site of action of HBx. This finding is consistent with previous data showing that targeting HBx to the ER membrane through a prolactin cassette abolishes both its apoptotic and its transactivating activity (44).

Rather, HBx acts on the molecular routes that drive the termination of Ca\(^{2+}\) signals, the most effective being the active extrusion of Ca\(^{2+}\) by the plasma membrane Ca\(^{2+}\) ATPase. In HBx-expressing cells we could observe activation of caspase-3, cleavage of PMCA at a caspase-3-sensitive site, and an increase in the [Ca\(^{2+}\)] in the subplasmalemmal space. Overexpression of a PMCA mutant, in which the caspase-sensitive site was mutated, or inhibition of caspase activation prevented HBx from enhancing the cytosolic [Ca\(^{2+}\)] rise, whereas the overexpression of wild type PMCA did not. These data well correlate with those obtained in a radically different model of cell death, that induced in neurons by treatment with staurosporin, in which caspase-dependent PMCA cleavage was shown to cause Ca\(^{2+}\) overload (29). The fact that HBx amplifies the cytosolic Ca\(^{2+}\) signals acting on the Ca\(^{2+}\) pumps is not entirely surprising if one takes into account the functional properties of the Ca\(^{2+}\) release and uptake mechanisms. Indeed, if the state of filling of Ca\(^{2+}\) stores were increased (e.g., by increasing the SERCA activity or reducing the Ca\(^{2+}\) leak), this would not per se determine an enhancement of agonist-dependent \([\text{Ca}^{2+}]_c\) transients. Rather, the higher [Ca\(^{2+}\)]\(_e\) (and ensuing faster flow through inositol 1,4,5-triphosphate receptors) would potentiate the feedback inhibition of the channel and thus decrease, rather than increase, net release to the cytosol, as demonstrated by Brini et al. (36) in experiments in which [Ca\(^{2+}\)]\(_e\) was increased by overexpressing SERCAs. Conversely, PMCA represents a very effective target for enhancing Ca\(^{2+}\) responses, because it represents the most powerful route, allowing the rapid return of [Ca\(^{2+}\)]\(_e\) to basal levels (36, 45).

What are the downstream targets of the HBx-enhanced Ca\(^{2+}\) signal? Mitochondria are obvious candidates, because organelle Ca\(^{2+}\) overload has been shown to promote opening of a large
Fig. 9. Schematic model illustrating the effect of HBx on Ca\(^{2+}\) signaling and its possible significance for HBx-induced apoptosis. At high expression levels (black arrows), HBx protein could induce apoptosis through mitochondrial dysfunction and caspase activation. At moderate or low expression level (gray arrows), HBx could trigger a moderate activation of caspases that is not sufficient to induce apoptosis. However, caspase-dependent PMCA cleavage could enhance Ca\(^{2+}\) signals, alter mitochondrial structure, and cause further release of caspase cofactors, thus allowing the final commitment to cell death.

HBx

Mitochondria

structure alteration reduced calcium signals cytochrome c release

Caspase activation

PMCA cleavage

[Ca\(^{2+}\)\(_c\)]

zVAD

PMCA-mut

BAPTA-AM

EGTA

Bcl-2

Apoptosis

HBx

direct effect?

Bcl-2

Caspase activation

zVAD

PMCA-mut

BAPTA-AM

EGTA

Bcl-2

Apoptosis

size pore, known as the PTP, with ensuing swelling and release of cytochrome c and other pro-apoptotic factors into the cytosol (46). In turn, cytosolic cytochrome c, by complexing with Apaf-1 and procaspase-9 to form the apoptosome, processes and activates effector caspases, such as caspase-3. Such a scenario appears likely upon HBx expression, given that (i) in HBx-expressing cells, mitochondrial structure was grossly altered (in turn impairing their capacity to accumulate Ca\(^{2+}\)), and (ii) significant cytochrome c release was detected, and (ii) treatment of the cells with the PTP inhibitor cyclosporin A throughout HBx expression reduced the apoptotic efficiency of the viral protein. Interestingly, different mechanisms appear to be responsible for converting the physiological signal for mitochondrial metabolic priming (a rise in matrix Ca\(^{2+}\) concentration) into the trigger of apoptotic cell death. Hajnoczky and coworkers (47) demonstrated that mitochondria can detect the simultaneous presence of lipid mediators of apoptosis (such as ceramide) and a Ca\(^{2+}\) rise evoked by an inositol 1,4,5-triphosphate-generating agonist, thus allowing a Ca\(^{2+}\) signal of normal amplitude to cause organelle swelling and release of cytochrome c. In addition, we showed that the lipid mediator itself can promote Ca\(^{2+}\) release from the ER and sustained Ca\(^{2+}\) loading into mitochondria (48). Now, we show that a pro-apoptotic viral protein acting on the molecular mechanisms that allow the prompt re-extrusion of agonist-released Ca\(^{2+}\) can obtain the same effect.

Overall, these data indicate that an alteration in calcium signaling is an early and important event in HBx-induced apoptosis. All experimental conditions that counteract this effect (overexpression of noncleavable PMCA, cell loading with Ca\(^{2+}\) buffers, and partial depletion of ER Ca\(^{2+}\) stores) or impair the downstream events (opening of the PTP) cause a significant reduction in the apoptotic efficiency of transfected HBx. However, apoptotic efficiency is not entirely abolished, thus suggesting that Ca\(^{2+}\) may act as an important potentiation loop, facilitating the rapid commitment of cells to death. As proposed in the model of Fig. 9, HBx could, possibly through limited perturbation of mitochondrial outer membrane and partial release of cytochrome c, activate caspase-3, initiating the apoptotic cascade. At high and/or prolonged expression this may be sufficient to induce cell death. Caspase-3, however, also cleaves PMCA. As a consequence any Ca\(^{2+}\)-mediated signal that reaches the cell is potentiated and may trigger mitochondrial Ca\(^{2+}\) overload, further cytochrome c release, caspase-3 activation, and thus irreversible commitment to the death program. Thus, moderate expression of HBx protein could also be effective in causing apoptosis through the Ca\(^{2+}\)-mediated amplification loop. At lower levels of expression, triggering of apoptosis would not occur, and the other effects of HBx would become the main effect. Such a possibility appears to correlate well with the pleiotropic functions and the apparent discrepancies in the phenotypic effects of HBx expression. In fact, HBV replication in the liver of transgenic mice that carry the entire HBV genome is associated with a low level of HBx mRNA, without any evidence of cytopathy (49). At increased HBx levels, apoptosis becomes the main effect, and the efficacy is tuned by the Ca\(^{2+}\)-dependent potentiation loop. Much remains to be understood on the intracellular effects of HBx (and on the cross-talk with other signaling routes). The elucidation of the molecular basis of its effect on calcium signaling (and of its role in regulating apoptosis) provides some new clues on therapeutic approaches for addressing chronic HBV infection and its complications.

Acknowledgment—We thank Prof. J. T. Penniston for the human PMCA4 J49 antibody.

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Caspase-dependent Alterations of Ca\textsuperscript{2+} Signaling in the Induction of Apoptosis by Hepatitis B Virus X Protein
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*J. Biol. Chem.* 2003, 278:31745-31755.
doi: 10.1074/jbc.M304202200 originally published online June 10, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304202200

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