Calcium Signaling by HBx Protein in Hepatitis B Virus DNA Replication

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Hepatitis B virus (HBV) infects more than 300 million people and is a leading cause of liver cancer and disease. The HBV HBx protein is essential for infection; HBx activation of Src is important for HBV DNA replication. In our study, HBx activated cytosolic calcium-dependent proline-rich tyrosine kinase–2 (Pyk2), a Src kinase activator. HBx activation of HBV DNA replication was blocked by inhibiting Pyk2 or calcium signaling mediated by mitochondrial calcium channels, which suggests that HBx targets mitochondrial calcium regulation. Reagents that increased cytosolic calcium substituted for HBx protein in HBV DNA replication. Thus, alteration of cytosolic calcium was a fundamental requirement for HBV replication and was mediated by HBx protein.

HBV is a para-retrovirus that replicates in the liver by reverse transcription but encapsidates a partially double-stranded 3-kb circular DNA genome (*1*). HBV replication requires the multifunctional HBx protein (*2*, *3*). In the cytoplasm, HBx stimulates Ras, Src, and c-Jun NH_2-terminal kinase (JNK) signal transduction pathways (*4*–*6*). HBx activation of Src promotes reverse transcription in the virus and DNA replication (*6*–*8*) as well as stimulation of several transcription factors (*1*). HBx might also interact in the nucleus with the transcriptional activator CREB/ATF (cAMP response element–binding protein/activating transcription factor) (*9*–*10*). HBx does not act on Src α-phosphatase or the COOH-terminal Src kinase (Csk) and does not bind Src kinases (*7*, *8*). Pyk2 is a cytoplasmic calcium–activated kinase that activates Src kinases (*11*) and downstream effectors, such as JNKs (*12*). Increased cytosolic calcium activates Pyk2, leading to its autophosphorylation at tyrosine amino acid position 402 (Y402), which creates a binding site for Src kinases and activates them. Because HBx activates the calcium-stimulated transcription factor NFAT (*13*), we investigated whether HBx acts on the calcium-Pyk2 pathway and whether calcium signaling is involved in stimulation of transcription and viral DNA replication.

Hepatic cell lines were transfected with vectors expressing HBx protein, a luciferase reporter dependent on transcription factor AP-1, and a dominant-inhibiting form of Pyk2 known as PKM (*14*). Inhibition of Pyk2 prevented HBx activation of AP-1 (*15*). Transfection of cells with one-eighth the amount of HBx expression plasmid reduced activation of AP-1 by only about one-half (*15*); these data indicated that PKM inhibits HBx activity rather than its expression. To determine whether HBx activates Pyk2, as indicated by Y402 phosphorylation (*14*), we transfected HepG2 cells with vectors expressing HBx or vector alone, with or without PKM expression. HBx induced increased phosphorylation of Pyk2 at Y402 by a factor of 4 without altering Pyk2 abundance (Fig. 1A), comparable to the effect of the phorbol ester tetradecanoyl phorbol acetate (*15*). HBx activation of Pyk2 was likely constitutive, as it was sustained for 48 hours after transfection, the last time point tested. The HBx-induced phosphorylation of Pyk2 correlated with stimulation of Pyk2 kinase activity (*15*).

To determine whether HBx activation of Pyk2 is essential for downstream stimulation of Src kinases, we transfected cells with vectors expressing HBx and PKM or with empty vector; Fyn was immunoprecipitated and tested for autophosphorylation activation by an in vitro kinase assay. HBx induced a factor of 5 increase in Fyn phosphorylation activity, which was prevented by coexpression with PKM (Fig. 1B). Thus, HBx activation of Pyk2 activated downstream Src kinases.

Studies were done to determine whether HBx acts on intracellular calcium to activate Pyk2. HBx-transfected cells showed increased phosphorylation of Pyk2 at Y402 by a factor of 5. Phosphorylation was inhibited by the cell-permeable cytosolic calcium chelator BAPTA-AM (Molecular Probes, Eugene, Oregon) at a concentration of 50 μM [twice the median inhibitory concentration (IC_{50})] (*15*). Thus, HBx activation of Pyk2 involves cytosolic calcium action. We therefore determined whether HBx acts on calcium channels in the endoplasmic reticulum, mitochondria, or plasma membrane for its activity. A low (0.5 mM) concentration of EGTA was added to the culture medium for 2 hours to block entrance of extracellular calcium (*16*), or cells were treated with BAPTA-AM (to block cytosolic calcium) or cyclosporin A (CsA). CsA predominantly binds mitochondrial cyclophilins, inhibits the mitochondrial transition pore, and disrupts mitochondrial calcium signaling (*17*, *18*). EGTA had no effect, whereas BAPTA-AM or CsA prevented HBx activation of Pyk2 (Fig. 1C), indicating that HBx likely acts on mitochondrial calcium control. A high concentration of EGTA (3 mM) did not block

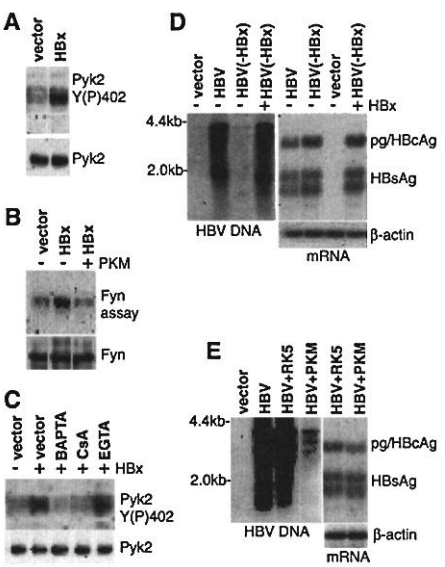

Fig. 1. (**A**) HepG2 cells were transfected with HBx expression plasmid or its empty plasmid (vector) (*7*, *8*, *29*) and with PKM plasmid that expressed a dominant-interfering Pyk2 or its empty plasmid (pRK5) (*14*). Cell lysates were resolved by gel electrophoresis, and immunoblot analyses were performed with antibodies to Pyk2 or Y(P)-402 Pyk2. (**B**) Fyn kinase was immunoprecipitated from cells and autophosphorylation activity (Fyn assay) determined in vitro using [γ-^{32}P]ATP, gel electrophoresis, and autoradiography (*7*, *8*). Fyn protein levels were determined by immunoblot. (**C**) HepG2 cells were treated for 2 hours with 50 μM BAPTA-AM, 3 μM CsA, or 0.5 mM EGTA and analyzed by immunoblot as above. (**D**) HepG2 cells were transfected with replication-competent wild-type genomic HBV DNA, an HBx(–) HBV genomic DNA, or vector, with or without an HBx expression plasmid (*15*). Cytoplasmic HBV core particles were isolated from equal numbers of cells, and viral DNA replication intermediates were detected by Southern blot hybridization (*8*). The smear represents 4-kb mature double-stranded to 2-kb single-stranded immature HBV DNA. Northern blot analysis was carried out using polyadenylated RNA extracted from equal numbers of cells. HBV mRNAs are indicated. (**E**) Southern and Northern blot analyses were performed on HepG2 cells transfected as above, with or without PKM or empty vectors. Quantification was performed by densitometry.

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TPA activation of Pyk2 phosphorylation but partially inhibited activation by HBx (*15*). It is likely that the effect of high levels of EGTA results from a partial requirement for low-level entry of calcium for full HBx-induced activation of Pyk2. Collectively, these data indicate that HBx alters cytosolic calcium regulation, probably by acting on mitochondria.

The requirement for HBx activation of Pyk2 in HBV replication was next examined. HepG2 cells were transfected with a 130% head-to-tail DNA copy of the HBV genome, which replicates in the livers of transgenic mice (*19*), in *Tupaia* hepatocytes in culture (*20*), and in an HBx-dependent manner in HepG2 cells (*21*). Expression of HBx from genomes was abolished by a targeted frameshift mutation (*21*). HepG2 cells were transfected with vector alone, wild-type HBV genomic DNA, or HBx(–) genomic DNA. Cytoplasmic viral core particles, the structures in which viral DNA replication takes place, were isolated, and the level of viral DNA replication was examined (Fig. 1D). HBV DNA replication was reduced by 95% in the absence of HBx expression, but was recovered by cotransfection of an HBx expression plasmid. Northern mRNA analysis showed no reduction in HBV pregenomic (pg)RNA and HBsAg mRNAs in the absence of HBx (Fig. 1D). Cotransfection of wild-type HBV genomic DNA with PKM reduced viral DNA replication by about 93%, similar to HBx(–) HBV samples, without altering viral mRNA levels (Fig. 1E). These results demonstrate that HBx specifically promotes HBV DNA replication in a Pyk2-dependent manner.

The requirement for cytosolic calcium in HBx-dependent viral replication was examined. Cells transfected with either wild-type or HBx(–) HBV genomic DNA were treated for 4 days with BAPTA-AM, CsA, or CGP37157, an inhibitor of the mitochondrial sodium-calcium pump (*17*). CGP37157 was used to provide an additional independent line of evidence for the importance of mitochondrial calcium in HBx-mediated HBV replication. Inhibitors were used at low levels, with no toxicity evident during treatment. CsA, BAPTA-AM, and CGP37157 all inhibited HBV replication in cytoplasmic core particles by 90 to 93% compared to untreated controls (Fig. 2A), similar in magnitude to inhibition of Pyk2 or the absence of HBx expression. Northern mRNA analysis showed a 50% reduction in pgRNA and HBsAg mRNAs (Fig. 2A). Cytosolic core particles were purified and incubated with [α-^{32}P]deoxynucleotide triphosphates (dNTPs) to examine endogenous HBV polymerase activity (Fig. 2B). In untreated controls, full-length double-stranded DNA products were produced, indicative of pgRNA reverse transcription and DNA-dependent DNA synthesis. PKM inhibition of Pyk2 or treatment of cells with CsA reduced DNA replication by 87 and 92%, respectively. In cells transfected with HBV genomic DNA and treated with low levels of BAPTA-AM for 4 days, viral DNA replication was reduced by 90%, whereas HBV mRNA levels decreased by less than half (Fig. 2C). These data show that HBx activation of HBV reverse transcription and DNA replication involves alteration of cytosolic calcium and coupled activation of Pyk2. The requirement for cytosolic calcium in HBx transcriptional stimulation was investigated in HepG2 cells transfected with luciferase reporters controlled by transcription factor AP-1 or CREB, with or without CsA treatment of cells (Fig. 2D). HBx activation of AP-1–dependent transcription was reduced almost 70% by treatment of cells with CsA (10 μg/ml). HBx stimulation of CREB-dependent transcription was resistant to high-dose CsA treatment, consistent with HBx activation of CREB by direct interaction (*22*). These data indicate that HBx transcriptional activation of AP-1, but not of CREB, requires alteration of cytosolic calcium.

We next investigated whether HBx activity can be replaced by reagents that increase cytosolic calcium. Cells were transfected with plasmids expressing wild-type HBV or HBx(–) DNA genomes and treated for 4 days with low, nontoxic levels of the calcium mobilizing agents valinomycin (1 nM) or thapsigargin (20 nM). Reverse transcription and DNA replication of (HBx)HBV was increased by valinomycin treatment by a factor of 20, to the same level as seen in wild-type (HBx+) virus, and by thapsigargin treatment by a factor of 10 (Fig. 3A). Neither agent increased cytoplasmic levels of HBV pgRNA or HBsAg mRNA. The low levels of thapsigargin or valinomycin induced a stimulation of Pyk2 activity by a factor of 3 or 5, respectively (Fig. 3B). These results demonstrate that HBx activation of viral reverse transcription and DNA replication can be replaced by agents that mobilize cytosolic calcium.

HBx acts on cytosolic stored calcium to stimulate Pyk2–Src kinase signal transduction pathways that activate HBV reverse transcription and DNA replication, and in some instances it functions as a moderate transcriptional activator. Three lines of evidence indicate that HBx stimulation of HBV reverse transcription and DNA replication involves alteration of cytosolic calcium and activation of Pyk2–Src kinase signal transduction: (i)

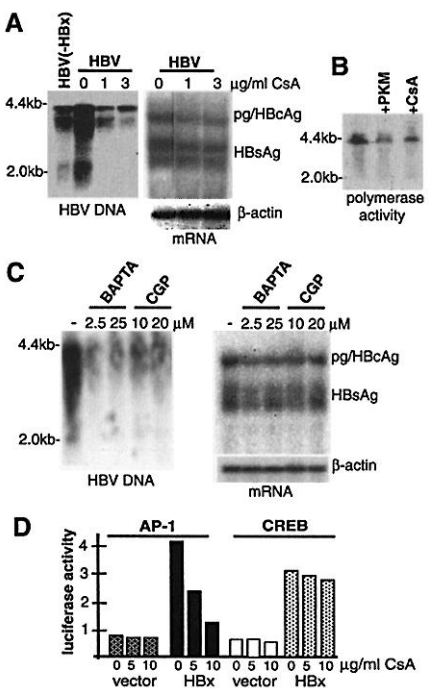

Fig. 2. HepG2 cells were transfected and treated for 4 days with CsA (**A** and **B**), BAPTA-AM, or CGP37157 (CGP) (**C**). Cytoplasmic HBV core particles were isolated, HBV DNA replication detected by Southern blot hybridization, and mRNA levels determined by Northern blot hybridization. In (**B**), endogenous polymerase activity of HBV pol protein was assayed in isolated cytoplasmic core particles obtained from equal numbers of cells using [α-^{32}P]dNTPs in vitro (*30*). Products were resolved by gel electrophoresis and autoradiography. (**D**) Cells were transfected with empty vector or HBx expression plasmid and luciferase reporters containing four binding sites for AP-1 or CREB, linked to a TATA box promoter (*15*). Cells were treated with CsA, as above, at the indicated dose and assayed for luciferase activity. A typical result is shown, which did not vary by more than 10% in three independent trials.

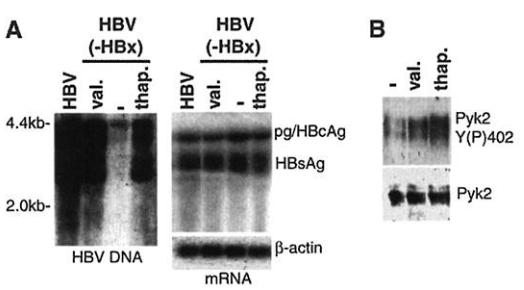

Fig. 3. (**A**) HepG2 cells were transfected with HBV or HBx(–) HBV genomic DNA, then treated with valinomycin (val.) or thapsigargin (thap.) for 4 days. Cytoplasmic core particles were isolated from equal numbers of cells, and HBV replication was examined by Southern blot hybridization. HBV transcription was analyzed by Northern blot hybridization. (**B**) Nontransfected HepG2 cells were treated with valinomycin or thapsigargin, and the abundance of Pyk2 or activated Pyk2 Y402 phosphorylation was determined by immunoblot.

Activation of Pyk2, which is critical for stimulation of HBV DNA replication in tissue culture, is typically mediated by increased levels of cytosolic calcium. Chelation of cytosolic calcium with BAPTA-AM blocked HBx activation of Pyk2 and HBV DNA replication. (ii) Inhibition of mitochondrial channels with CGP37157 or CsA blocked HBx activation of HBV DNA replication. (iii) Reagents that increase the level of cytoplasmic calcium functionally replace HBx in viral DNA replication. Thus, HBx acts on stored cytosolic calcium as a fundamental activity for HBV replication.

The molecular target for HBx-mediated calcium stimulation of HBV replication is not known. However, calcium stimulates a variety of protein kinases, and phosphorylation of the COOH-terminus of the HBV core protein is essential for viral replication (*1*), which suggests a possible link between the two. The regulation of intracellular calcium is central to the control of cellular metabolism, cell cycle, signal transduction, protein synthesis, transcription, and apoptosis (*18*). It is therefore not surprising that a number of viruses, including retroviruses such as HIV, rotovirus, adenovirus, and Rubella virus, encode regulatory proteins that alter normal calcium homeostasis to benefit one or more aspects of viral replication (*23–26*). A potential interaction between HBx and mitochondria has been reported (*27–29*), as well as a possibly modest dissolution of the mitochondrial transition pore potential (*29*). Taken collectively with this work, the alteration of cytosolic calcium is an important and central activity of HBV HBx protein and provides a new target for antiviral intervention.

References and Notes

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