Hepatitis B Virus X Protein Stimulates the Mitochondrial Translocation of Raf-1 via Oxidative Stress

Jun Chen1,2 and Aleem Siddiqui1*

Department of Medicine, Division of Infectious Diseases, University of California—San Diego, SCRB, MC0711, 9500 Gilman Dr., La Jolla, California 92093,1 and Liver Disease Research Center, Second Xiangya Hospital, Central South University, Changsha 410011, China2

Received 25 January 2007/Accepted 30 March 2007

The human hepatitis B virus (HBV) X protein (HBx) plays a crucial role(s) in the viral life cycle and contributes to the onset of hepatocellular carcinoma (HCC). HBx caused the mitochondrial translocation of Raf-1 kinase either alone or in the context of whole-viral-genome transfections. Mitochondrial translocation of Raf-1 is mediated by HBx-induced oxidative stress and was dependent upon the phosphorylation of Raf-1 at the serine338/339 and Y340/341 residues by p21-activated protein kinase 1 and Src kinase, respectively. These studies provide an insight into the mechanisms by which HBV induces intracellular events relevant to liver disease pathogenesis, including HCC.

Hepatitis B virus (HBV) infection results in a broad range of clinical symptoms, from mild, inapparent disease to fulminant hepatitis. Infection with this virus remains a major worldwide public health problem. It is estimated that there are about 500 million chronic carriers worldwide. Although the sequence of events remains poorly defined, a significant correlation has been made between long-term carriage of the virus and the development of hepatocellular carcinoma (4, 6). Among the HBV proteins encoded by the four open reading frames (S, C, p, and X), the X protein (HBx) plays a crucial role in the cellular signal transduction pathway, and in apoptosis (2, 6, 17). HBx regulates a series of cell-signaling cascades involving most notably the Ras- and Raf-induced mitogen-activated protein kinase pathways (reviewed in reference 2).

The Raf serine/threonine kinases are involved in the Ras- and Raf-induced mitogen-activated protein kinase pathway (1, 15). They act downstream of Ras and are activated in a significant number of human malignancies (1, 15). There are three isoforms of Raf, A-Raf, B-Raf, and C-Raf, each displaying distinct expression profiles (1, 13, 15). C-Raf is ubiquitously expressed in many tissues, whereas A-Raf and B-Raf display tissue-specific expression (1, 15). Only A-Raf and C-Raf have been shown to translocate to mitochondria and regulate apoptosis (1). C-Raf, also known as Raf-1, exists in the cytoplasm as a multiprotein complex of 300 to 500 kDa consisting of heat shock protein 90 and dimeric protein 14-3-3. Binding of Ras to Raf displaces the 14-3-3 complex and unmasks amino acid residues critical for its activation. Mitochondrially localized Raf-1 protects cells from stress-mediated apoptosis. Raf-1 contains a central activation domain whose phosphorylation is activated by p21-activated protein kinase (PAK) at amino acids Ser338 and Ser339 or Src kinase at amino acid residues Y340 and Y341 (5, 8). B-Raf does not contain these tyrosine residues.

The results of this study demonstrate that HBx can stimulate the mitochondrial translocation of Raf-1 via oxidative stress. We previously showed that HBx itself targets to mitochondria and directly interacts with voltage-dependent anion channel 3 (VDAC3) (7, 10, 11, 12). HBx expression induces oxidative stress via calcium signaling and activates cellular kinases, which leads to the activation of transcription factors NF-kB and STAT-3 and others via phosphorylation (2, 3, 14). We observed that HBV-induced oxidative stress also stimulated the translocation of Raf-1 to mitochondria. This activation involves both the Src- and the PAK-mediated phosphorylation of the activation domain of Raf-1. Src inhibitors and dominant-negative PAK mutants abolished HBx-mediated Raf-1 mitochondrial translocation.

To demonstrate the role of HBx protein in regulating Raf-1 translocation, we first examined Raf-1 expression in Huh-7 cell lysates transfected with pCMVXF, which encodes the X gene placed under the transcriptional control of a cytomegalovirus (CMV) promoter containing a Flag sequence. The Western blot results show similar levels of Raf-1 expression in both untransfected and pCMVXF-transfected Huh-7 cellular lysates. (Fig. 1A). We next examined the association of Raf-1 with mitochondria. The results of Western blot analysis of mitochondria prepared according to a detailed procedure (9) from Huh-7 and pCMVXF cells (presented in Fig. 1B) demonstrate that both HBx protein and Raf-1 are associated with mitochondria. VDAC is used as a mitochondrial marker. To ensure that the Raf-1 and HBx signals were not due to cytoplasmic contamination, the lysates were also blotted for a cytoplasmic marker, lactate dehydrogenase (LDH). LDH expression was not observed in these mitochondrial preparations.

The cytoplasmic fractions were also prepared and analyzed by a Western blot assay using anti-Raf, anti-Flag (which detects HBx), and anti-LDH (Fig. 1C). Raf-1 kinase levels were again demonstrated that both HBx protein and Raf-1 are associated with mitochondria. VDAC is used as a mitochondrial marker. To ensure that the Raf-1 and HBx signals were not due to cytoplasmic contamination, the lysates were also blotted for a cytoplasmic marker, lactate dehydrogenase (LDH). LDH expression was not observed in these mitochondrial preparations.

To demonstrate the role of HBx protein in regulating Raf-1 translocation, we first examined Raf-1 expression in Huh-7 cell lysates transfected with pCMVXF, which encodes the X gene placed under the transcriptional control of a cytomegalovirus (CMV) promoter containing a Flag sequence. The Western blot results show similar levels of Raf-1 expression in both untransfected and pCMVXF-transfected Huh-7 cellular lysates. (Fig. 1A). We next examined the association of Raf-1 with mitochondria. The results of Western blot analysis of mitochondria prepared according to a detailed procedure (9) from Huh-7 and pCMVXF cells (presented in Fig. 1B) demonstrate that both HBx protein and Raf-1 are associated with mitochondria. VDAC is used as a mitochondrial marker. To ensure that the Raf-1 and HBx signals were not due to cytoplasmic contamination, the lysates were also blotted for a cytoplasmic marker, lactate dehydrogenase (LDH). LDH expression was not observed in these mitochondrial preparations.

The cytoplasmic fractions were also prepared and analyzed by a Western blot assay using anti-Raf, anti-Flag (which detects HBx), and anti-LDH (Fig. 1C). Raf-1 kinase levels were again demonstrated that both HBx protein and Raf-1 are associated with mitochondria. VDAC is used as a mitochondrial marker. To ensure that the Raf-1 and HBx signals were not due to cytoplasmic contamination, the lysates were also blotted for a cytoplasmic marker, lactate dehydrogenase (LDH). LDH expression was not observed in these mitochondrial preparations.

The cytoplasmic fractions were also prepared and analyzed by a Western blot assay using anti-Raf, anti-Flag (which detects HBx), and anti-LDH (Fig. 1C). Raf-1 kinase levels were again demonstrated that both HBx protein and Raf-1 are associated with mitochondria. VDAC is used as a mitochondrial marker. To ensure that the Raf-1 and HBx signals were not due to cytoplasmic contamination, the lysates were also blotted for a cytoplasmic marker, lactate dehydrogenase (LDH). LDH expression was not observed in these mitochondrial preparations. The cytoplasmic fractions were also prepared and analyzed by a Western blot assay using anti-Raf, anti-Flag (which detects HBx), and anti-LDH (Fig. 1C). Raf-1 kinase levels were again similar to those described for Fig. 1A, indicating that not all
FIG. 1. HBx protein induces Raf-1 mitochondrial translocation. (A) Raf-1 levels in untransfected and pCMVXF-transfected Huh-7 cellular lysates. Western blot analysis was carried out using anti-Raf-1 kinase antibody. Anti-actin was used as a protein loading control. (B) Mitochondrial preparations from untransfected and pCMVXF-transfected Huh-7 cells were used for Western blot analysis using anti-Raf-1 kinase antibody. VDAC serves as a mitochondrial marker. Anti-Flag was used to monitor HBx expression, and anti-LDH was used to monitor for cytoplasmic contamination. (C) Cytoplasmic fractions from untransfected and pCMVXF-transfected Huh-7 cells were analyzed by Western blot assays using anti-Raf-1, anti-Flag (which detects HBx), and anti-LDH. LDH is used here as a cytoplasmic marker. (D) Mitochondria were fractionated (9) from untransfected Huh-7 cells and cells transfected with whole-HBV-genome plasmids (HBV1.3L) and an X-defective mutant plasmid of the HBV genome [HBV1.3L (AX)] (gift from J. Ou, USC). Western blot analysis was carried out using anti-Raf-1 kinase antibody and anti-electron transport factor (anti-ETF; a mitochondrial marker) antibody. HBx protein expression was monitored by first immunoprecipitating with anti-HBx antibody (16), followed by immunoblotting with the same antisera (16). (E) Raf-1 mitochondrial translocation (9) in the HBx-transgenic mouse. Mitochondria were fractionated from normal and HBx-transgenic mice (gift from James Ou). HBx was expressed under its native promoter/enhancer (16). Western blot analysis was performed on the mitochondrial preparation. Lane 1, normal mouse liver tissue; lane 2, HBx-transgenic mouse liver Western blots using anti-Raf-1. ETF is used as a mitochondrial marker. HBx expression in the HBx-transgenic mice was determined using anti-HBx antibody by immunoprecipitation (IP), followed by immunoblot analysis with the same antibody (gift from Betty Slagle) (16).

Raf-1 kinase translocates to mitochondria, consistent with previous studies (1, 15). pCMVXF-transfected cellular lysates showed HBx expression, and LDH, used here as a cytoplasmic protein marker, was positive for both lysates. The vectors used thus far contained HBx under the transcriptional control of a CMV enhancer/promoter. To verify the role of HBx when expressed under the transcriptional control of the native promoter/enhancer in the context of the whole HBV genome, which recapitulates the viral life cycle, longer-than-genome-length HBV constructs were transfected into Huh-7 cells (6, 16). Analysis of these cells showed Raf-1 mitochondrial translocation, whereas the whole-genome construct, defective in HBx expression, failed to induce mitochondrial translocation of Raf-1, as did the untransfected cells (Fig. 1D). We further

FIG. 2. HBx protein activates both Src-dependent tyrosine and PAK-1-dependent serine phosphorylation of Raf-1 and causes its mitochondrial translocation. (A) HBx activates serine phosphorylation of mitochondrial Raf-1. Western blot analysis was performed on isolated mitochondria fractionated from untransfected Huh-7 cells and those transiently transfected with the pCMV4X plasmid using the anti-Raf-1 Ser338 monoclonal antibody, which recognizes serine338-phosphorylated Raf-1 kinase (Upstate Biotechnologies). Anti-electron transport factor (anti-ETF) was used to detect ETF, a mitochondrial marker. HBx was monitored using anti-Flag antibody. (B) Pak-1 is required for Raf-1 translocation. Results are shown for Western blot analysis of isolated mitochondria prepared from untransfected Huh-7 cells (lane 1), Huh-7 cells transiently transfected with the pCMV4X plasmid (lane 2), Huh-7 cells cotransfected with pCMV4X-Flag (HBx), and dominant-negative Pak-1 protein (the PAK-1 inhibitory domain containing PAK83-149) (lane 2) using anti-Raf-1 antibody. ETF serves as a mitochondrial marker. HBx expression was monitored using anti-Flag antibody (an internal control). (C) Western blot analysis was carried out as described for panel B, except that a Src dominant-negative mutant (gift from R. Jove) was used for cotransfection along with the pCMV4X (HBx) plasmid (lane 2).

examined this phenomenon in transgenic mice harboring the HBx gene expressed under the control of its native promoter/enhancer (16). Mitochondria fractionated from liver tissues of HBx-transgenic mice were examined by a Western blot assay. As shown in Fig. 1D, Raf-1 also translocated to mitochondria in HBx-transgenic mice, as did HBx. This is the first report showing the association of the HBx with mitochondria in vivo using a transgenic-mouse model. We and others have previously shown that HBx targets to mitochondria using a variety of in vitro experimental strategies (7, 11, 12). In normal mouse liver tissue, Raf-1 association with mitochondria was not observed (Fig. 1E). These studies collectively provide evidence that HBx is associated with mitochondria and causes Raf-1 translocation to mitochondria both in cells transfected with HBx alone and in the context of the whole HBV genome. Moreover, a similar association was also seen in transgenic mice expressing HBx under the native promoter/enhancer (16).

Mitochondrial translocation of Raf-1 requires phosphorylation of Ser and Tyr residues in its activation domain (5, 7). To
investigate the role of HBx in phosphorylating Raf-1 at Ser\textsuperscript{338} residues, mitochondrial preparations from untransfected and pCMVXF-transfected Huh-7 cells were used for Western blot analysis using antiserum that recognizes Ser\textsuperscript{338} residues of Raf-1. The results show that indeed HBx mediates the phosphorylation of Raf at Ser\textsuperscript{338} residues (Fig. 2A). Serine phosphorylation of Raf-1 is catalyzed by PAK-1 (8). Infection of untransfected and pCMVXF-transfected Huh-7 cells with a retrovirus encoding the autoinhibitory domain (PAK\textsubscript{S3-149}) of Pak-1 abrogated mitochondrial translocation of Raf-1, whereas in cells expressing HBx alone, Raf-1 migrated to mitochondria (Fig. 2B). Tyrosine\textsuperscript{340,341} phosphorylation of Raf-1 is known to be catalyzed by Src kinase (5). Huh-7 cells coexpressing HBx and dominant-negative Src kinase (kinase-dead pM5Hmet295; gift from Richard Jove [Moffat Cancer Center]) also failed to cause Raf-1 mitochondrial translocation (Fig. 2C). Together, these results indicate that HBx must induce the activation of Src and Pak-1 kinases to phosphorylate Raf-1 at the Tyr and Ser residues, respectively, to trigger its mitochondrial translocation. HBx activates both Tyr and Ser/Thr kinases (reviewed in reference 2).

To examine whether HBx-induced reactive oxygen species play a role in the Raf-1 activation process, HBx (pCMVXF transfected)-expressing cells were treated with antioxidants N-acetyl cysteine and pyrrolidine dithiocarbamate (PDTC; 50 \mu M) (lane 2) and N-acetyl cysteine (NAC; 30 mM) (lane 3) for 6 h each and subjected to Western blot assays using Raf-1 antibody. Electron transport factor (ETF) serves as a mitochondrial marker. Anti-Flag was used to detect HBx (internal control).

The results show that indeed HBx mediates the phosphorylation with anti-Flag antibody (Flag tagged to HBx). The precipitated with anti-Raf-1 antibody, followed by immunoblotting with anti-electron transport factor (anti-ETF) (a mitochondrial marker).

FIG. 3. HBx induces Raf-1 mitochondrial translocation via oxidative stress. Western blot analysis of isolated mitochondria prepared from untransfected Huh-7 cells (lane 4) and Huh-7 cells transiently transfected with pCMV4X (lanes 1 to 3) is shown. Cells were either untreated (lanes 1 and 4) or treated with antioxidants pyrrolidine dithiocarbamate (PDTC; 50 \mu M) (lane 2) and N-acetyl cysteine (NAC; 30 mM) (lane 3) for 6 h each and subjected to Western blot assays using anti-Raf-1 antibody. Electron transport factor (ETF) serves as a mitochondrial marker. Anti-Flag was used to detect HBx (internal control).

FIG. 4. HBx protein directly binds mitochondrial associated Raf-1. (A) Mitochondrial preparations from untransfected and pCMV4X-transfected Huh-7 cells were first immunoprecipitated with anti-Raf-1, followed by immunoblotting with anti-Flag (which detects HBx) (lane 1) antibodies. (B and C) Western blot analysis of the mitochondrial preparations with anti-Flag (HBx) and anti-electron transport factor (anti-ETF) (a mitochondrial marker).

These studies collectively demonstrate the ability of HBx to induce the mitochondrial translocation of cytoplasmic Raf-1, and while resident in mitochondria, HBx forms a protein-protein complex with Raf-1 kinase. The functional significance of this interaction may be that it reinforces the antiapoptotic program in infected hepatocytes. We previously noted the absence of cytochrome c release in HBx-expressing cells (unpublished results). Mitochondrial Raf-1 participates in the survival program (13), but the exact mechanism behind the antiapoptotic role of Raf-1 remains to be investigated. HBx-activated Raf-1 may contribute to HBV-associated liver oncogenesis.

This work was supported by NIH grant CA64415 to A.S. We thank James Ou for transgenic mice and HBV1.3L (ΔHBx) vectors and B. Slagle for anti-HBx sera.

REFERENCES

anion channel, HVDAC3, and alters its transmembrane potential. J. Virol. 74:2840–2846.


