Interaction of the Hepatitis B Virus X Protein with the Crm1-dependent Nuclear Export Pathway*

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The leucine-rich nuclear export signal (NES) is used to shuttle large cellular proteins from the nucleus to the cytoplasm. The nuclear export receptor Crm1 is essential in this process by recognizing the NES motif. Here, we show that the oncogenic hepatitis B virus (HBV) X protein (HBx) contains a functional NES motif. We found that the predominant cytoplasmic localization of HBx is sensitive to the drug leptomycin B (LMB), which specifically inactivates Crm1. Mutations at the two conserved leucine residues to alanine at the NES motif (L98A,L100A) resulted in a nuclear redistribution of HBx. A recombinant HBx protein binds to Crm1 in vitro. In addition, ectopic expression of HBx sequesters Crm1 in the cytoplasm. Furthermore, HBx activates NFkB by inducing its nuclear translocation in a NES-dependent manner. Abnormal cytoplasmic sequestration of Crm1, accompanied by a nuclear localization of NFkB, was also observed in hepatocytes from HBV-positive liver samples with chronic active hepatitis. We suggest that Crm1 may play a role in HBx-mediated liver carcinogenesis.

Hepatocellular carcinoma (HCC)1 is one of the most prevalent malignant diseases worldwide and hepatitis B virus (HBV) is the major etiologic factor for HCC (1–3). HBV is a DNA tumor virus, which encodes four open reading frames, S/preS, C/preC, P, and HBx (4). The oncogenicity of HBV is largely the result of HBx, the smallest gene encoding a 17-kDa protein (3, 5–8). One major cellular function of HBx is its promiscuous transcriptional activation activity, a property that is believed to contribute to its oncogenicity (9). A wide range of cellular genes can be up-regulated or down-regulated by HBx (9). However, HBx is localized in the cytoplasm mostly and does not bind to double-stranded DNA. A "universal" effect of HBx, on otherwise totally different types of promoters with no obvious consensus sequence, has led to the hypothesis that HBx may regulate gene expression by interacting directly with host general transcription factors (10–12), or indirectly via the activation of protein kinase C and RAS-RAF mitogen-activated protein kinase (MAPK) signaling pathways (13–15). Although HBx can induce neoplastic transformation, presumably by preventing p53-mediated apoptosis (16–18), it also can induce apoptosis in a p53-dependent or -independent manner (19, 20), or sensitize cells to tumor necrosis factor α (TNFα)-induced apoptosis (21). Therefore, the precise mechanism related to its effect is unknown and none of these studies satisfactorily explain the pleiotropic effects associated with HBx.

Close inspection of the HBx sequence revealed a short, hydrophobic, leucine-rich nuclear export signal motif (NES) (Fig. 1A). An NES is located in the center region of HBx (residues 89–100). The center region of HBx is retained in HCC frequently and is essential for its transactivation (22–24). This region also is conserved among HBx from different subtypes (Fig. 1A). Several viral proteins including HIV-1 Rev, HTLV-1 Rex, and adenovirus E4 34-kDa proteins contain functional NESs (Fig. 1A). Similar to HBx, Rev and Rex also are potent viral and cellular transactivators with no apparent DNA binding property (25, 26). In addition, NESs also have been identified in cellular proteins, many of which are involved in transcription, cell signaling cascade, oncogenic transformation, and cell cycle regulators. Examples include protein kinase inhibitor, MAP kinase kinase (MAPKK), TFIIB, Mdm2, p53, IκBα, NF-AT, cyclin B1, c-Abl, and 14-3-3 (reviewed in Ref. 27). The activities of these proteins are tightly regulated by their NESs. The nuclear export receptor Crm1 and its cofactor Ran GTPase are essential in this process by recognizing NESs and mediating nuclear protein export (27, 28). In addition, previous results indicate that Crm1 may be involved in maintaining chromosomal integrity (29) and Ran may play a key role in regulating mitosis initiation by stimulating spindle formation (30, 31). Mutation of the hydrophobic leucine residues to alanines have been shown to disrupt NES function in a number of proteins, including HIV-rev, E4 34-kDa, p53, Mdm2, and cyclin B1 (25, 26, 32, 33).

In this study, we have investigated the hypothesis that the pleiotropic effects associated with HBx may be contributed by the presence of a NES motif, and HBx may activate cellular gene expression and induce oncogenicity through the modulation of Crm1-mediated functions. We have identified a functional NES motif in HBx. This motif is necessary for HBx-induced cytoplasmic sequestration of Crm1, and subsequently, the nuclear translocation and activation of NFkB. Cytoplasmic retention of Crm1 also is found in liver samples with chronic active hepatitis infected with HBV, a condition that predisposing individuals to the development of HCC. We suggest that

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1 The abbreviations used are: HCC, hepatocellular carcinoma; NES, nuclear export signal; NLS, nuclear localization signal; HBx, hepatitis B viral X protein; HBV, hepatitis B virus; NHE: normal human fibroblasts; LMB, leptomycin B; MAPK, mitogen-activated protein kinase; TNFα, tumor necrosis factor-α; HA, hemagglutinin; PCR, polymerase chain reaction; GFP, green fluorescent protein; GTPγS, guanosine 5′-3-O-(thio)triphosphate.
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the inactivation of the Crm1-mediated pathway may be an early step during viral hepatitis-mediated liver carcinogenesis.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The plasmid pcDNA3-HBXadr-Hatag was constructed by the insertion of a C-terminal hemagglutinin (HA) epitope-tagged full-length HBx into the BamHI and HindIII sites of a pcDNA3 vector (Invitrogen). GFP-HBx and GFP-HBx-NEF were constructed as follows: A 582-base pair fragment released from the digestion of pcDNA3-HBXadr-Hatag with HindIII/NotI and inserted into pEGFP-C2 (CLONTECH) at the HindIII/NotI sites. The resulting GFP-HBx is an N-terminal fusion of HBx with GFP and a C-terminal fusion with HA tag. GFP-HBx-NEF was made by a PCR-based site-directed mutagenesis protocol using GFP-HBx as a template. To generate the HBx-NES mutant (L98A,G99A,L100A), 2 PCR fragments were amplified by PCR using primer pairs (5'-CTGAGACTCCCGAGCTGGAACGTCGCTG-3'/5'-ATCGCTCTAGAGCCGAGAGGTTAAAATCTG-3') and (5'-TGCGAGCGCAGCTCCTTATGAGAAGC-3'/5'-TATAAAGCTTGGTACCGAG-3'). The 2 PCR fragments were denatured and reannealed. Then the HBx-NES mutant was produced by PCR with primers (5'-TATAAAAGCTTGGTACCGAGGATCTTGAGCTC-3'/5'-TATAAAGCTTGGTACCGAGGATCTTGAGCTC-3'). The resulting PCR fragments were sequenced by DNA sequencing. The GFP-NL-NSL-NEF and GFP-NL-NSL-NEF constructs were made by the strategy described previously (34). Briefly, the SV40 NLS motifs (residue 125–133) were fused to the cloning of oligos TNL51 (5'-TCGAGATCCCCCCCCTGCGCCGGCAGGTCGCTG-3'/TNL53-5'-AGCTGCTGGTGGTCTGGAGGCTG-3') into plasmid pEGFP-C1 (CLONTECH) to generate GFP-NLS. GFP-NES and GFP-NEF were constructed by cloning oligos XNESW1 (5'-AAGTTGCATGG-3'), XNESW3 (5'-GATCCATTGCTGAGAGTCCAAGAGTCCTCTTATGTAAGAGCTTGGGCAAGACCTGAG-3'), and XNESM3 (5'-GATCCATTGCTGAGAGTCCAAGAGTCCTCTTATGTAAGAGCTTGGGCAAGACCTGAG-3') into plasmid pEGFP-C1 (CLONTECH) to generate GFP-NLS, GFP-NES, and GFP-NEF. These vectors were confirmed by DNA sequencing. The HBx expression also was verified by an anti-HBx monoclonal antibody (data not shown). NF-κB was detected using an anti-NF-κB antibody (1:100) (Santa Cruz) and Crm1 was detected using an anti-Crm1 polyclonal antibody (1:100). Both antibodies were incubated for 1 h at 37°C. Anti-rabbit IgG conjugated to fluorescein or Texas Red (Vector Laboratories) was used (1:300) for 1 h at room temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole. The following criteria were used to define the subcellular localization, preferentially cytoplasmic localization: protein signals mostly intense in the cytoplasm, preferentially perinuclear localization: protein signals mostly intense in the nucleus. Some of the slides were analyzed with a blind field to avoid any bias. For GFP-NL-NEF reporter expression, cells on the same coverslip were microinjected with GFP-NLS, GFP-NL-NSL-NEF, or GFP-NL-NSL-NEF construct and incubated for 6 h. Cells were monitored under a Zeiss Axioskop fluorescence microscope and representative images were taken using a high-performance laser scanning imaging system (IP Lab Spectrum) with the same exposure time.

**In Vitro Protein-binding Assay**—In vitro binding between HBx and hCrm1 was determined with the in vitro translated HBx and hCrm1 (16). The HA-tagged HBx and the hCrm1 proteins were made by the one-step TnT (Promega) transcription and translation system (Promega), as described previously (16), from pcDNA-HA-HBx and hCrm1Blue744 vectors (gift of Dr. Gerard Grosfeld), respectively. Aliquots of HBx (25 μl) and hCrm1 (45 μl) were mixed together in CBBL buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Nonidet P-40) in the presence of 0.2 μM GFP-S and 80 μl of anti-HA affinity matrix (Roche Molecular Biochemicals, Indianapolis, IN). The mixture was incubated at 37°C for 60 min. After centrifugation, beads were washed four times with CBBL buffer. The bound proteins were separated on SDS-polyacrylamide gel electrophoresis and Crm1 was detected by Western blotting using anti-Crm1 antibody and followed by the Amersham ECL system. A 10% of in vitro translated hCrm1 was loaded in parallel as the input.

**Immunohistochemistry Analysis**—A total of 12 samples were included in this part of the study. Histopathologically, they included 5 normal liver samples, 7 viral hepatitis cases showing grades 1 to 2 activity according to the criteria of Scheuer (36). In addition to H & E light microscopy, tissue sections were cut from frozen samples with a thickness of 4 μm and mounted on electrically charged glass slides. The sections were fixed in absolute alcohol and then washed with 2 changes of phosphate-buffered saline for 5 min each. They were then quenched with 3% hydrogen peroxide to block the endogenous peroxidase activity for 30 min. Following incubation with 10% horse serum to block the nonspecific binding, the sections were incubated overnight at 4°C with anti-CRM1 antibodies. The sections were prepared in duplicate and two anti-CRM1 antibodies were used, including a goat anti-Crm1 antibody from Santa Cruz at a 1:10 dilution (Santa Cruz) and a rabbit anti-Crm1 antibody (gift from Gerard Grosfeld). The rabbit anti-NF-κB antibody (anti-p65) (Santa Cruz) was used at a 1:100 dilution for staining NF-κB. Biotinylated secondary antibodies and streptavidin peroxidase complex (LSAB) were used. Chromogenic development was obtained by the immersion of sections in 3,3′-diaminobenzidine solution (0.25 mg/ml with 3% hydrogen peroxide). The slides were counterstained with Harris’ hematoxylin and re-hydrated with alcohol and xylene. Slides were evaluated in a blind fashion.

**RESULTS**

**HBx Has a Functional Leucine-rich Nuclear Export Signal**—To test if HBx-NES functions in nuclear protein export, we first investigated whether the subcellular localization of HBx is sensitive to LMB, an antitumor agent that selectively binds to and blocks Crm1-mediated nuclear export (37, 38). Consistent with previous findings (18), HBx was localized in the cytoplasm predominately with a punctate staining pattern...
in appearance when expressed transiently in normal human fibroblasts (NHF) (Fig. 1) and in a HCC-derived cell line (Hep3B) (data not shown). However, LMB treatment causes an increase in nuclear accumulation of HBx (Fig. 1, B and C).

Next, we used a GFP protein export reporter assay described previously (34) to further test if the HBx-NES oligopeptide functions in nuclear export. Several GFP fusion constructs were expressed transiently in normal human fibroblasts. Living cells were monitored under a fluorescence microscope. A nuclear localization signal from SV40 T antigen directs GFP to the nucleus (left panel) while the HBx-NES redistributes it to the cytoplasm (middle panel). Mutations of leucine to alanine in NES abolish this activity (right panel). E, the HBx-NES mutant is localized preferentially in the nucleus. Normal human fibroblasts were microinjected with the GFP-HBx or GFP-HBx-NESM expression vector. Following a 24-h incubation, cells were fixed and stained with 4,6-diamidino-2-phenylindole. The percent of cells in which the GFP signal was found preferentially in the cytoplasm (left panel), in the nucleus (middle panel), or both (evenly distributed; right panel) was determined. Examples of these images are shown. Data are an average of three independent experiments.
ducing nuclear localization of the fusion protein. Consistently, the GFP-NLS reporter was localized in the nucleus of NHF exclusively. However, when an HBx-NES was fused to this reporter, the resulting GFP-NLS-XNES fusion protein can be found in the cytoplasm with a diffused staining pattern in appearance, although no nuclear exclusion of this reporter protein could be observed (Fig. 1D). In contrast, GFP-NLS-XNESM that contains a mutated HBx-NES oligopeptide (L98A, L100A) does not localize in the cytoplasm (Fig. 1D). To further examine if cytoplasmic localization of a full-length HBx is dependent on the presence of an NES, a GFP-HBx fusion gene (HBx) or an NES-mutated GFP-HBx fusion gene (HBx-NESM) was constructed. Again, GFP-HBx was localized in the cytoplasm predominantly with a punctate pattern in appearance, while HBx-NESM was found mainly in the nucleus with a diffused pattern (Fig. 1E). Taken together, these data demonstrate that HBx-NES is functional in Crm1-mediated nuclear export.

Interaction between Crm1 and HBx—Nuclear export receptor Crm1 is known to interact with proteins containing NESs and to mediate nuclear protein export. The majority of Crm1 protein is found in the nucleus of normal cells (39). Consistently, every NHF cell displays an endogenous nuclear-diffused Crm1 distribution (Fig. 2). When HBx was expressed transiently in NHF, Crm1 was often found in the cytoplasm with a punctate staining pattern. Approximately 39% ± 2% of the HBx-expressing NHF cells displayed co-localization of Crm1, and over 99% of cytoplasmic punctate Crm1 signals were co-localized with HBx (Fig. 2A). The punctate staining patterns containing both Crm1 and HBx signals were not always the same in appearance (Fig. 2A, panels c-l). These results suggest that the cytoplasmic HBx and Crm1 are not necessarily associated with a single subcellular component. In contrast, no cytoplasmic co-localization was found in NHF cells expressing HBx-NESM. These data indicate that HBx was able to partially retain Crm1 in the cytoplasm and suggest that HBx binds to Crm1 in vivo. Consistently, in vitro translated HBx can physically interact with in vitro translated Crm1 (Fig. 2B).

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Enhanced by the treatment of TNF-α, HBx alone increased luciferase activity weakly which could be further amplified by the HBx-mediated transactivation (9). Consistently, HBx alone increased luciferase activity even in the presence of TNF-α (Fig. 3A). The luciferase activity was normalized by an internal Renilla luciferase vector.

Cytoplasmic Localization of Crm1 Associated with HBV-infected Liver Samples—To investigate if Crm1 is sequestrated cytoplasmically in liver samples from chronic active hepatitis patients with HBV infection, we performed immunohistochemistry analysis of Crm1 in frozen tissue sections or in paraffin-embedded sections by anti-Crm1 polyclonal antibody. A total of 12 liver samples with samples from 5 normal individuals were included in this study. The presence of HBV DNA including Core, preS, and HBx genes in these samples was verified by PCR (data not shown). Among 7 HBV positive cases, liver samples show chronic active hepatitis with grades 1 to 2 activity (Table I). While 5/5 normal samples revealed nuclear Crm1 staining exclusively, 6/7 HBV samples showed cytoplasmic Crm1 staining (Fig. 4 and Table I) in hepatocytes. An anti-NF-κB antibody was also used to determine the status of NF-κB in these liver samples. Again, normal liver samples revealed mainly a cytoplasmic localization of NF-κB while HBV positive liver samples showed a nuclear distribution of NF-κB. The above findings provide an in vivo physiological significance for the presence of a cytoplasmic sequestration of Crm1 accompanied by an activation of NF-κB associated with HBV infection.

**Discussion**

We have demonstrated that the oncogenic HBx contains a functional NES, and that this NES is required for the HBx-mediated activation of the NF-κB signaling cascade. We also have shown that HBx is able to bind to and sequester Crm1 in the cytoplasm, which correlates with the nuclear localization and activation of NF-κB. Furthermore, liver samples from HBV-infected patients with chronic active hepatitis, a condition predisposing individuals for the development of liver cancer, also display cytoplasmic sequestration of Crm1 and nuclear localization of NF-κB. It is possible that HBx may use a Crm1-dependent mechanism to modulate cellular gene transcription. Our data provide a plausible model whereby HBx may induce oncogenicity through the modulation of the Crm1-mediated pathway.

The studies described in this report may offer a common mechanism to explain the pleiotropic effects of HBx. First, Crm1 transports and controls many cellular proteins including NF-κB, NF-AT, MAPKK, and p53, whose activities are known to be regulated by HBx (14, 17, 40, 41, 43). Second, Crm1 also regulates cytoplasmic mRNA transport. Inactivation of Crm1 by HBx, in principle, would alter the stability of mRNA thereby influencing gene expression. Consistent with this hypothesis is our findings that HBx is able to sequester Crm1 in the cytoplasm. The HBx-NES is essential for sequestering Crm1 in the cytoplasm and for the activation of NF-κB. Inactivation of Crm1-mediated nuclear protein export is known to activate this target (44). Therefore, the promiscuous transactivation-mediated by HBx may be a secondary effect resulting from alteration of Crm1 function.

An alternative mechanism for HBx to activate NF-κB is through the degradation of IκBα in the cytoplasm by inducing its phosphorylation and ubiquitination. However, we did not observe any detectable degradation of IκBα in cells expressing HBx by Western blot analysis with anti-IκBα antibody (data not shown). This also correlates with our results that HBx alone only displays a weak activation of NF-κB that can be enhanced significantly by TNF-α (Fig. 3C). It is possible that a small degree of dissociation of the NF-κB/IκBα complex, followed by the degradation of IκBα induced by HBx, may contribute to the nuclear localization of NF-κB. A recent study by Wei and colleagues (45) indicate that HBx may induce the nuclear import of NF-κB/IκBα by binding to IκBα. Interestingly, the region of IκBα that is critical for HBx-induced nuclear import of IκBα contains a functional NES (45). These findings are consistent with our data that HBx-induced nuclear localization of NF-κB/IκBα is dependent on the IκBα-NES.
HBx is thought to be small enough to diffuse passively through the nuclear pore complex. The fact that this oncogenic viral protein has acquired NES activity, is preferentially localized in the cytoplasm, and modified nuclear export implies that nuclear export may be an important target for viral-mediated oncogenesis. Interestingly, cellular oncoproteins such as c-Abl and Mdm2 are known to be involved in a Crm1-dependent nuclear export. Analogous to viral hepatitis oncoprotein, the cellular oncoproteins, in principle, may induce neoplastic transformation also through the disruption of the Crm1-mediated mechanism. It is known that TPR and CAN are nuclear pore complex-associated proteins that act as the docking sites and are essential for Crm1-mediated nuclear export (27). Earlier findings demonstrated that the TPR and CAN genes are mutated through translocation in thyroid carcinoma, gastric carcinoma, and acute myeloid leukemia (46–52). We also found that cytoplasmic sequestration of Crm1 is associated frequently with HCC (data not shown). In addition, Crm1 and Ran may play a role in mitosis initiation (29–31). It is possible that the inactivation of Crm1 and Ran may induce genomic instability, a predisposing factor for cancer development. Thus, we postulate that the inactivation of the Crm1-mediated nuclear export is a common event during viral and cellular oncogenesis.

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**TABLE I**

| Case | Histology | Grade | Virus Status | Crm1 Localization | NF-κB Localization | Score |
|------|-----------|-------|--------------|-------------------|---------------------|-------|
| 1    | Normal    | 0     | Negative     | Nuclear            | Cytoplasmic         | 4+    |
| 2    | Normal    | 0     | Negative     | Nuclear            | Cytoplasmic         | 3+    |
| 3    | Normal    | 0     | Negative     | Nuclear            | Cytoplasmic         | 2+    |
| 4    | Normal    | 0     | Negative     | Nuclear            | Cytoplasmic         | 1+    |
| 5    | Normal    | 0     | Negative     | Nuclear            | Cytoplasmic         | 1+    |
| 6    | CAH       | 2     | HBV          | Cytoplasmic/nuclear| Cytoplasmic/nuclear | 2+    |
| 7    | CAH       | 2     | HBV          | Cytoplasmic/nuclear| Nuclear             | 2+    |
| 8    | CAH       | 2     | HBV          | Cytoplasmic/nuclear| Nuclear             | 1+    |
| 9    | CAH       | 2     | HBV          | Cytoplasmic/nuclear| Nuclear             | 1+    |
| 10   | CAH       | 2     | HBV          | Cytoplasmic/nuclear| Nuclear             | 2+    |
| 11   | CAH       | 1     | HBV          | Cytoplasmic/nuclear| Nuclear             | 2+    |
| 12   | CAH       | 1     | HBV          | Cytoplasmic/nuclear| Nuclear             | 2+    |

**Fig. 4.** Cytoplasmic sequestration of Crm1 and nuclear localization of NF-κB associated with HBV-infected liver samples. Frozen sections of a normal (A, C, and E) and an HBV positive (B, D, and F) liver samples were analyzed by H & E staining (A and B), anti-Crm1 antibody staining (C and D), or anti-NF-κB antibody staining (E and F). Representative images are shown. Arrows indicate representative cells that are positively stained by anti-Crm1 or anti-NF-κB antibodies. Primary magnifications: H & E images, ×200; IHC images, ×400.
