HBx Protein of Hepatitis B Virus Activates Jak1-STAT Signaling*

(Received for publication, May 8, 1998)

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The X-gene product (HBx) of the hepatitis B virus plays essential roles in viral replication and the generation of hepatocellular carcinoma. Although the mechanism for HBx action is unclear, HBx may exert its pleiotropic functions through the stimulation of signal transduction pathways including the Ras/mitogen-activated protein kinase cascade and/or inactivation of the p53 function. Here, we investigated whether HBx has the ability to activate the Jak-STAT signaling pathway. As a first step, we established stable cell lines constitutively expressing HBx. In these HBx-expressing stable cells, the tyrosine phosphorylation of various STATs, including STAT3 and -5, was constitutively enhanced by HBx, and the concomitant increase in STAT-dependent DNA binding and transcriptional activation was observed. Furthermore, HBx specifically elevated tyrosine phosphorylation and in vitro kinase activity of Jak1, but not Jak2 or Tyk2, through protein to protein interaction with Jak1. These results clearly establish HBx as the inducer of the Jak-STAT signaling pathway, and at the same time, HBx-mediated Jak-STAT activation may provide a novel mechanism for the pleiotropic functions of HBx, including transformation and promiscuous transcriptional activation.

The hepatitis B virus is known as a causative agent of hepatitis, cirrhosis, and hepatocellular carcinoma (1). Among the four proteins originated from the hepatitis B virus genome, the X-gene product (HBx) has drawn much attention for its pleiotropic functions as a viral transactivator. HBx plays essential roles in viral replication as shown in an in vitro animal study (2) and in a transfection-based replication assay (3). Moreover, HBx induces hepatocellular carcinoma in transgenic mice (4) and deregulates cell cycle checkpoint controls (5). A possible mechanism of HBx-mediated cell cycle activation is the disruption of p53 function as a tumor suppressor through the direct protein to protein interaction (3, 6). Alternatively, HBx may promote cell proliferation by activating the signal transduction cascades including the Ras/mitogen-activated protein kinase (7), c-Jun N-terminal kinase (8), nuclear factor-κB (9), and Src-dependent (10) pathways. Stimulation of these signaling pathways leads to the activation of Activating Protein-1 and nuclear factor-κB-dependent transcriptional activation (7–10).

Supposedly, the combined actions of these mechanisms may be responsible for the HBx-mediated tumorigenesis. On the other hand, HBx induces apoptosis in a p53-dependent manner and sensitizes cells to apoptosis by tumor necrosis factor (11–13), even though the role of HBx-mediated apoptosis in Hepatitis B virus pathogenesis is still not clear.

Many cytokine receptors lack intrinsic tyrosine kinase domains. Instead, they are associated with the Janus kinase (Jak) family of protein-tyrosine kinases to couple ligand binding to tyrosine phosphorylation of intracellular signaling molecules (14, 15). Up to now, four members of the Jak family, including Jak1, Jak2, Jak3, and Tyk2, have been characterized. STAT proteins are a family of transcription factors of 90–110 kDa that are activated upon tyrosine phosphorylation by Jak kinase (14, 15). Growth factor or cytokine treatment leads to the oligomerization of receptor subunits, and receptor-associated Jaks phosphorylate STATs recruited to the receptors by binding to the phosphotyrosine residues through their SH2 domains. In turn, activated STATs are dimerized or multimerized through their SH2 domains, transported into the nucleus, and then bind to the specific STAT-binding DNA sequences leading to the activation of various genes.

Regulation of Jak-STAT is linked to various biological aspects like cell transformation, development, differentiation, apoptosis, etc. Previous reports have suggested that transformation by several oncogenes like src and abl accompanies the activation of the Jak-STAT signaling pathway (16–18), and especially in the liver, the constitutive elevation of Jak-STAT signaling is associated with hepatocyte proliferation in response to growth factor/cytokine or partial hepatectomy (20–22).

Inasmuch as HBx is known to stimulate cell proliferation, and Jak-STAT is associated with the proliferation signal of liver cells, we tested the effect of HBx on Jak-STAT signaling pathway in liver cells. We demonstrate that HBx activates various STATs through the specific stimulation of Jak1 tyrosine kinase. Combined with the fact that HBx activates several growth factor-dependent signaling, including the Ras/mitogen-activated protein kinase, c-Jun N-terminal kinase, and nuclear factor-κB pathways (7–10), the observed Jak-STAT activation establishes HBx as an “internal ligand” of the growth factor/cytokine-dependent signal transduction.

EXPERIMENTAL PROCEDURES

Plasmids—The HBx expression plasmid, pcDNA-X, has been described (3). pMFG-HBx, a retroviral expression plasmid, was constructed by subcloning the Ncol/BamHI fragment of pdcDNA-X into the Ncol/BamHI site of MFG-puro. Eight deletion mutants of HBx (XDIR–XDI8) were constructed by site-directed mutagenesis using pdcDNA-X as the template. The mutant clones were selected by DNA sequencing, and the size of the translated product was confirmed by in vitro translation in the presence of [35S]methionine.

Generation of Stable Cell Lines Expressing HBx—Stable cell lines were obtained by Lipofectin-mediated transfection of a mouse hepatoma cell line, Hepa 1–6 with pMFG-HBx plasmid. Twenty-four hours after transfection, the medium was replaced with medium containing...
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RESULTS

Establishment of HBx-stable Cell Line—Here we investigated the effect of HBx on the Jak-STAT signaling pathway. As a first step, we established stable cell lines expressing HBx in a constitutive manner. The expression construct pMFG-HBx is based on the retroviral vector pMFG (23) and depicted in Fig. 1A. pMFG-HBx was transfected into Hepa 1–6 mouse hepatoma cell line, and stable clones were selected in the presence of puromycin. As a negative control, parental pMFG-puro vector was transfected to generate Hepa-puro cell line. The expression of puromycin was confirmed by Western blot analysis using antibodies against the synthetic peptides corresponding to residues 145–154 of HBx. Five μg of crude cell extracts of two stable cell lines, Hepa-X1 and Hepa-X2 were employed. Purified HBx protein is from the baculovirus system and was used as a control. The 17-kDa HBx band is marked by an arrow.

HBx Activates Jak1 Tyrosine Kinase—In the liver, three STATs tested by HBx. The band shift of the STAT5 case was probably resulting from the tyrosine phosphorylation of all 5 STATs in Hepa-X cells reflecting the post-translational modification, which may explain part of the pleiotropic functions of HBx in liver cell failed because of apparent cell death (data not shown). This result probably reflects the previously reported apoptosis phenomena of HBx (11–13).
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HBx specifically activates Jak1 tyrosine kinase. A, HBx induces the tyrosine phosphorylation of Jak1. Left panel, employing the control Hepa-puro (−) and HBX-expressing Hepa-X (+) cells, tyrosine phosphorylation of Jak1 and Jak2 was analyzed by immunoprecipitation with anti-Jak antibody followed by Western blot with anti-phosphotyrosine antibody. The tyrosine phosphorylation of Jak1 but not Jak2 is enhanced in Hepa-X cells compared with Hepa-puro cells (left panel). To control the level of Jak1, the same immunoprecipitants were subjected to Western analysis with anti-Jak1 antibody (right panel). B, HBX induces the kinase activity of Jak1. The effects of HBX on the kinase activity of Jak1, Jak2, and Tyk2 were analyzed by the in vitro kinase assay of the immunoprecipitated proteins. The immunoprecipitates were washed with a kinase buffer and incubated with γ[32P]ATP.

HBx Enhances STAT-dependent Transcription Activation and DNA Binding—The observed tyrosine phosphorylation of STATs led us to test the effect of HBX on STAT-dependent transcriptional activation and DNA binding. The transcriptional activation was assayed in heterologous, human hepatoma cell line HepG2 to test whether the observed STAT activation by HBX is extended to liver cell lines other than Hepa 1–6 (Fig. 5A). The reporter SIE-Luc and its mutant version, mSIE-Luc, were described previously (27) and contain 3 repeats of SIE or mutant SIE, respectively. When SIE-Luc was transiently cotransfected with the HBX expression plasmid pDNA-X, approximately a 4-fold activation of luciferase activity was observed compared with the negative control. The epidermal growth factor treatment of Hepa-puro cells used as a positive control resulted in 7-fold activation. On the other hand, mutant SIE (mSIE)-driven reporter activity was not lowered by the coexpression of HBX, indicating that HBX spe-
HBx specifically activates STAT-dependent transcription activation. In addition, these results suggest that STAT activation is not limited to Hepa 1–6 cells and is not caused by the secondary effect introduced during the establishment of Hepa-X stable cells.

Inasmuch as STAT is known as a DNA-binding transcription factor, we tested the effect of HBx on the DNA-binding activity of STATs by electrophoretic mobility shift assay (Fig. 5B). Nuclear extracts were prepared from the control Hepa-puro and Hepa-X cells and incubated with a labeled APRE probe, which has been shown to bind STAT3 (28). As shown in Fig. 5B, the APRE-binding activity was enhanced by approximately 4-fold in Hepa-X cells. The observed DNA-binding complex was specifically competed by the 100-fold excess of unlabeled APRE but not by an unrelated Fos intragenic regulatory element. Moreover, the addition of anti-STAT3 antibody led to the appearance of a supershifted band indicating that the constitutive enhancement of DNA-binding activity in Hepa-X cells is attributable to STAT proteins.

The Central Region of HBx Is Responsible for STAT Activation—Next, to control the effect of wild type HBx and also to map the domain responsible for the STAT activation, we generated a series of HBx deletion mutants (Fig. 6A) and tested the effects of these mutants on SIE-Luc reporter activity (Fig. 6B). Except for the deletions in the N- (XD1) or C-terminal (XD8) region, all of the internal deletions of HBx resulted in the reduced activation of STAT-dependent transcription, suggesting that a relatively broad, central region of HBx is responsible for STAT activation. Perhaps these internal deletions of HBx alter the conformation of HBx necessary for the interaction with Jak1. The domain necessary for STAT-dependent transcriptional activation is overlapped with the previously identified transcriptional activation domains of HBx (29, 30) except for the difference in the XD2 case. These domain mapping results suggest that a certain cross-talk may exist in the activation mechanism of HBx.

DISCUSSION

In this report, we have shown that HBx interacts with Jak1-tyrosine kinase leading to the activation of the Jak1-STAT signaling pathway. However, the exact mechanism through which HBx activates Jak1 needs further investigation. Inasmuch as HBx can form a dimer (30), HBx dimerization might bring associated Jak1 molecules into close proximity allowing cross-phosphorylation and autoactivation, which in turn leads to the activation of multiple STATs. The full activation of STATs requires serine phosphorylation in addition to tyrosine phosphorylation (28). Considering that HBx activates serine/threonine kinases like mitogen-activated protein kinase and Jun-N-terminal kinase (7, 8), we do not exclude the possibility that HBx assists the activation of STATs through serine phosphorylation in addition to tyrosine phosphorylation (31).

Recently, Klein and Schneider (10) reported that HBx activates Src kinase, and this event is linked to HBx-mediated Ras pathway activation. Because Src is known to activate Jak1 and STAT3 (17, 19), there is a possibility that HBx-mediated activation of Src and Jak/STAT is closely related. However, in the Hepa-X cell system, we could not detect any enhancement of Src tyrosine phosphorylation by HBx (data not shown). Although this might be a cell type-specific phenomenon, at least we propose that Jak-STAT activation in Hepa-X cells is not a result of Src activation.

Importantly, HBx-mediated Jak-STAT activation may explain the promiscuous actions of HBx as a viral transactivator. The observed Jak1-STAT activation, in combination with the
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fact that HBx also activates the serine/threonine kinase pathway through the activation of Ras and Src (7, 10), establishes that HBx behaves like a typical growth factor and acts at the membrane proximal level. As reported previously, activated Jak1 may recruit a number of molecules, including STATs, She, and Raf (33–36). In addition, activated STAT3 can act as an adaptor protein, recruiting phosphatidylinositol 3-kinase to the receptor complex (37). Therefore, activation of Jak1 thereby mediates multiple impacts of HBx on cytoplasmic signaling pathways as well as on nuclear transcriptional events.

The finding in this report establishes hepatitis B virus as the first example of a transforming DNA virus, the gene product that activates the Jak/STAT signaling pathway. Up to now, Jak/STAT activation was limited for some oncogenic retroviruses like v-src, v-abl, and human T-cell lymphotropic virus (16–19). The activation of the Jak/STAT-signaling pathway may contribute to the transforming potential of HBx, even though the role of Jak/STAT signaling in mitogenesis is still under debate. Cumulating evidence indicates that the Jak/STAT pathway is activated in the transformed cell (16–19), and the genetic studies of the HOP/D-STAT pathway in Droso- sophila are suggestive of a role of Jak/STAT in cell proliferation and oncogenesis (32). Moreover, a cross-talk between Jak/STAT and the oncogenic Ras pathway has been reported extensively (33–36). In cytokine-growth factor signaling, activated Jak's tyrosine phosphorylates she or Raf, which in turn activates the Ras-Raf-mitogen-activated protein kinase pathway. Perhaps previously reported Ras/mitogen-activated protein kinase activation by HBx may be mediated through Jak/STAT. Taking this information together, we propose that the HBx-mediated Jak-STAT activation contributes at least in part to the development of hepatocellular carcinoma.

In addition to the potential role in transformation, HBx-mediated Jak/STAT activation may be linked to a variety of pathogenic phenomena caused by the infection of the hepatitis B virus. For example, constitutive activation of STAT3 leads to the dysregulation of the interleukin-6 gene, the main mediator of the acute phase response and liver cirrhosis (38, 39).

In conclusion, we found that the constitutive expression of HBx results in the activation of the Jak1/STAT signaling pathway, which may potentially provide the mechanism for promiscuous transcription activation by HBx, cirrhosis, and hepatocellular carcinoma. Activation of Jak1/STAT by the viral transactivator may be a common strategy adopted by a number of viruses to exploit the host cell machinery.

Acknowledgments—We thank other members of the laboratory for helpful discussions. We especially appreciate Dr. J. Ihle for the Jak1 and Jak2 baculovirus. We thank J. K. Chung, H. Y. Chung, and J. S. Suh for various materials.

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FIG. 6. Central region of HBx is responsible for STAT activation. A, construction of HBx deletion mutants. Eight different internal deletion mutants of HBx were generated by site-directed mutagenesis and subcloned into the expression vector, pcDNAI/Amp. The domains deleted are indicated at the left. The identity of the HBx mutants was confirmed by DNA sequencing and in vitro translation using reticulocyte lysate as shown in the lower panel. B, the central region of HBx is responsible for STAT activation. The HBx mutants were cotransfected with SIE-Luc reporter into HepG2 cells. The experiments were also performed in duplicate and at least 3 times.
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