Interaction of the hepatitis B virus X protein with the lysine methyltransferase SET and MYND domain-containing 3 induces activator protein 1 activation

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ABSTRACT

Hepatitis B virus (HBV) is a widespread human pathogen that often causes chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. The detailed mechanisms underlying HBV pathogenesis remain poorly understood. The HBV X protein (HBx) is a multifunctional regulator that modulates viral replication and host cell functions, such as cell cycle progression, apoptosis and protein degradation through interaction with a variety of host factors. Recently, the nonstructural protein 5A (NS5A) of hepatitis C virus has been reported to interact with methyltransferase SET and MYND domain-containing 3 (SMYD3), which is implicated in chromatin modification and development of cancer. Because HBx shares fundamental regulatory functions concerning viral replication and pathogenesis with NS5A, it was decided to examine whether HBx interacts with SMYD3. In the present study, it was demonstrated by co-immunoprecipitation analysis that HBx interacts with SMYD3. In the present study, it was demonstrated by co-immunoprecipitation analysis that HBx interacts with both ectopically and endogenously expressed SMYD3 in Huh-7.5 cells. Deletion mutation analysis revealed that the C-terminal region of HBx (amino acids [aa] 131–154) and an internal region of SMYD3 (aa 269–288) are responsible for their interaction. Immunofluorescence and proximity ligation assays showed that HBx and SMYD3 co-localize predominantly in the cytoplasm. Luciferase reporter assay demonstrated that the interaction between HBx and SMYD3 activates activator protein 1 (AP-1) signaling, but not that of nuclear factor-kappa B (NF-κB). On the other hand, neither overexpression nor knockdown of SMYD3 altered production of HBV transcripts and HBV surface antigen (HBsAg). In conclusion, a novel HBx-interacting protein, SMYD3, was identified, leading to proposal of a novel mechanism of AP-1 activation in HBV-infected cells.

Key words activator protein 1, hepatitis B virus, HBV X protein, SMYD3.

Hepatitis B virus infection is a global public health problem, more than 350 million people being chronically infected and at risk of developing serious liver disease, including chronic hepatitis, liver fibrosis, cirrhosis and HCC (1–3). HCC is one of the most common cancers in the world, having an annual incidence of more than 500,000 (3, 4). HBV is an enveloped, partially double-stranded DNA virus with a genome size of 3.2 kb. The HBV genome contains four overlapping open reading frames that encode for seven viral proteins; three...
envelope proteins (large [L], middle [M] and small [S] proteins) that share HBsAg, HBCAg and its related secreted protein (HBeAg), polymerase/reverse transcriptase and the HBx (4–7). HBx, a small protein of 17 kDa, is a multifunctional regulator that modulates viral replication and host cell functions, such as cell cycle progression, apoptosis and protein degradation (7, 8). HBx interacts with a variety of cellular proteins and plays important roles in both virus replication and pathogenesis. However, the detailed molecular mechanisms remain incompletely understood (4, 9, 10).

It was recently reported that the methyltransferase SMYD3 is a binding partner of the NS5A of HCV, which is a multifunctional protein that interacts with various host factors and plays pivotal roles in viral replication and assembly (11). Because HBx shares fundamental regulatory functions concerning viral replication and pathogenesis with NS5A, we became interested in examining whether HBx interacts with SMYD3.

SMYD3 has been reported to di- and tri-methylate the lysine-4 of histone H3 and lysine-5 and lysine-20 of histone H4 and regulate gene transcription through its histone methyltransferase activity (12, 13). In addition, SMYD3 was recently shown to methylate non-histone proteins, such as MAP3K2, to promote the development of Ras-driven cancer (14). SMYD3 is reportedly over-expressed in colorectal carcinoma, breast carcinoma and HCC (13, 15). We report here that HBx interacts with SMYD3 and facilitates SMYD3-mediated AP-1 activation.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line Huh-7.5 (16) was kindly provided by Dr. C. M. Rice (Rockefeller University, New York, NY, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium (high glucose) with 50 IU/mL penicillin, 50 μg/mL streptomycin, 10% heat-inactivated FBS and 0.1 mM nonessential amino acids at 37°C in a 5% CO2 incubator. The cells were transfected with plasmid DNA using X-tremeGENE 9 transfection reagents (Roche, Indianapolis, IN, USA).

Plasmid construction

Plasmids carrying a greater-than-unit-length (129%) HBV genome that mediate autonomous replication of the viral genome (pUC19-HBV-Bj_JPN56 and pUC19-HBV-C-AT_JPN) were kind gifts from Dr. M. Mizokami (Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Chiba, Japan). To express full-length HBx as Myc/6xHis- and Flag-tagged proteins, the corresponding regions were amplified from pUC19-HBV-C-AT_JPN by RT-PCR and cloned into the KpnI and XbaI sites of pEF1/Myc-His-A (Invitrogen, Carlsbad, CA, USA) and the EcoRI and XbaI sites of pcDNA3-Myc-TEV-Flag (17), respectively. To express a series of HBx deletion mutants, each fragment was cloned into pEF1/Myc-His-A. pEF1A/Flag was constructed by replacing the Myc-His tag of pEF1/Myc- His-A with Flag tag. A full-length SMYD3 cDNA amplified from mRNAs of HEK293T cells by RT-PCR was cloned into pEF1/Myc-His-A and pEF1A/Flag. To express a series of SMYD3 deletion mutants as Myc/6xHis-tagged proteins and GFP-tagged protein, each fragment was cloned into pEF1/Myc-His-A and pAcGFP1-C1 (Clontech, Palo Alto, CA, USA), respectively. To express Myc/6xHis-tagged HBcAg, the corresponding cDNA fragment was amplified from pUC19-HBV-C-AT_JPN and cloned into pEF1/Myc-His-A. A full-length ERK2 cDNA amplified from mRNAs of Huh-7.5 cells by RT-PCR was cloned into pEF1A/Flag. The correct sequences of all the cloned cDNAs were verified by DNA sequencing.

siRNA transfection

To knockdown SMYD3 expression, RNA interference was employed by using Lipofectamine RNAiMAX (Invitrogen). The target sequences for SMYD3 siRNA-1 and siRNA-2 were 5’-AAGTATGGAAGGAAGTTTCAAG-3’ and 5’-AGCCTGATTGAAGATTATT3’, respectively.

Immunoprecipitation

Cultured cells were lysed with a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100 and a protease inhibitor cocktail (Roche). The lysate was centrifuged at 12,000 g for 30 min at 4°C, and the supernatant incubated with anti-c-Myc tag beads (MBL, Nagoya, Japan) or anti-Flag M2 affinity gel (Sigma, St Louis, MO, USA) to immunoprecipitate the corresponding proteins. Alternatively, the supernatant was incubated with anti-HBx rabbit PAb (Abcam, Cambridge, UK), anti-SMYD3 rabbit PAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-GFP rabbit PAb (Molecular Probes, Eugene, OR, USA) and subsequently precipitated with protein A-Sepharose beads (GE Healthcare, Piscataway, NJ, USA). After being washed three times, the immunoprecipitates were analyzed by immunoblotting.

Immunoblotting

The cell lysates were separated by 10 or 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were
incubated with the corresponding primary antibody, followed by incubation with a HRP-conjugated secondary antibody. The primary antibodies used were anti-Myc mouse MAb (Santa Cruz Biotechnology), anti-Flag (M2) rabbit PAb (Sigma), anti-HBx rabbit PAb, anti-SMYD3 rabbit PAb, anti-GAPDH mouse MAb (Millipore) and anti-GFP rabbit PAb. HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Molecular Probes) were used as secondary antibodies. The positive bands were visualized using ECL western blotting detection reagents (GE Healthcare).

**Immunofluorescence**

Cells seeded on glass coverslips in 24-well plates at a density of $6 \times 10^4$ cells/well were transfected with Myc/6xHis-tagged HBx and Flag-tagged SMYD3. After 48 hr, the cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. After being washed with PBS three times, the cells were consecutively stained with primary and secondary antibodies. The primary antibodies used were anti-Myc mouse MAb and anti-Flag rabbit PAb. Secondary antibodies used were Alexa Fluor 594-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes). The cells were washed with PBS, counterstained with Hoechst 33342 solution (Molecular Probes) at room temperature for 5 min, mounted on glass slides, and observed under a confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

**Proximity ligation assay**

Cells seeded on glass coverslips in 24-well plates ($6 \times 10^4$ cells/well) were transfected with pUC19-HBV-Bj_JPN56. After 72 hr, the cells were fixed in 4% paraformaldehyde for 10 min and washed with PBS. In situ proximity ligation assay was performed on the fixed cells using the Duolink II fluorescence kit (Olink Bioscience, Uppsala, Sweden) according to the manufacturer’s instruction. Anti-HBx mouse MAb (Millipore) and anti-SMYD3 rabbit PAb were diluted in an antibody diluent supplied with the kit. Positive interactions were detected with the probes for proximity ligation assay in combination with anti-rabbit PLUS and anti-mouse MINUS. The cells were counterstained with Hoechst 33342 solution, mounted on glass slides, and observed under a confocal laser scanning microscope.

**qRT-PCR**

Total cellular RNA was isolated using a ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI, USA), and cDNA generated using a GoScript Reverse Transcription System (Promega). qRT-PCR was performed using SYBR Premix Ex Taq (TaKaRa, Kyoto, Japan) with SYBR green chemistry on a 7500 Fast real-Time PCR System (Applied Biosystems, Foster, CA, USA), as described previously (18, 19). GAPDH was used as an internal control. The primers used for qRT-PCR were as follows: HBV, 5'-GCTTTTACTTTTCGCCAAC-3' (nt 1087 to 1106) and 5'-GAGTTCCGCAGTATGGATCG-3' (nt 1262 to 1281); GAPDH, 5'-GCCATCAATGACCCCTTATT-3' (nt 196 to 216) and 5'-TCTG GTCTCTGAGATTTG-3' (nt 1107 to 1129) and 5'-GCTGTTGTTCTCTGCCATGT GT-3' (nt 1207 to 1227).

**Measurement of HBsAg**

HBsAg in the culture medium was measured by using an enzyme immunoassay diagnostic kit (DIAsource, Rue du Bosquet, Belgium) according to the manufacturer’s recommendations. The absorbance was measured using a microplate reader at 450 and 650 nm (Molecular Devices, Sunnyvale, CA, USA).

**Luciferase reporter assay**

Cells seeded in 24-well plates ($6 \times 10^4$ cells/well) were co-transfected with each of the indicated plasmids, a reporter plasmid (pGL4.44[luc2P/AP1 RE/Hygro] for AP-1 or pGL4.32[luc2P/NF-κB-RE/Hygro] for NF-κB) (Promega) and an internal control plasmid (pRL-TK-Renilla) (Promega). After 48 hr, the cells were harvested and assayed for luciferase activity. The luciferase assays were performed using a dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for each sample.

**Statistical analysis**

Results were expressed as means ± SEM. Statistical significance was evaluated by Student’s $t$-test and defined as a $P$ value of <0.05.

**RESULTS**

### Identification of SMYD3 as a novel HBx-interacting protein

We first found in an ectopic expression system that Myc/6xHis-tagged HBx co-immunoprecipitated with Flag-tagged SMYD3 when we used anti-Flag antibody (Fig. 1a). Likewise, Flag-tagged SMYD3
co-immunoprecipitated with Myc/6xHis-tagged HBx when we used anti-Myc antibody (Fig. 1b). Moreover, we confirmed that HBx expressed in the context of HBV replication interacted with Flag-tagged SMYD3 when we used anti-Flag (Fig. 1c) and anti-HBx antibodies (Fig. 1d). We further confirmed that Flag-tagged SMYD3 specifically bound to HBx, but not to HBCAg (Fig. 1e). We also demonstrated that Myc/6xHis-tagged HBx immunoprecipitated with endogenous SMYD3 (Fig. 1f). These results strongly suggest that SMYD3 is a novel HBx-interacting protein.

A C-terminal region of HBx (aa 131 to 154) is involved in the interaction with SMYD3

To map the SMYD3-binding site(s) on HBx, we analyzed co-immunoprecipitation using a panel of deletion mutants of HBx (Fig. 2a) and found that HBx deletion mutants consisting of aa 1 to 130 or smaller failed to co-immunoprecipitate with SMYD3 whereas the full-length HBx (aa 1 to 154) clearly did (Fig. 2b). These results suggest that a C-terminal region of HBx (aa 131 to 154) is important for SMYD3 binding.

An internal region of SMYD3 (aa 269 to 288) is involved in the interaction with HBx

To map the HBx-binding site(s) on SMYD3, we analyzed co-immunoprecipitation using a panel of SMYD3 deletion mutants (Fig. 2c) and found that SMYD3 deletion mutants consisting of aa 1 to 288 or larger clearly co-immunoprecipitated with HBx whereas those consisting of aa 1 to 268 did not (Fig. 2d). These results suggest that an internal region of SMYD3 (aa 269 to 288)
is important for HBx binding. We then tagged this region (SMYD3[269-288]) with GFP and subjected it to co-immunoprecipitation analysis. We found that GFP-tagged SMYD3(269-288) indeed co-immunoprecipitated with Flag-tagged HBx (Fig. 2e), confirming that an internal region of SMYD3 (aa 269 to 288) is important for HBx binding.

**HBx interacts with SMYD3 predominantly in the cytoplasm**

We then examined the intracellular locations of the HBx-SMYD3 interaction. When expressed alone, Myc/6xHis-tagged HBx was localized both in the nucleus and the cytoplasm, predominantly in the former (Fig. 3a, top panel). On the other hand, Flag-tagged SMYD3 was localized predominantly in the cytoplasm, but did show weaker, but significant, staining in the nucleus (Fig. 3a, middle panel). When HBx and SMYD3 were co-expressed, we detected their co-localization, stained in yellow, predominantly in the cytoplasm (Fig. 3a, bottom panel).

Moreover, proximity ligation assay demonstrated that HBx expressed in the context of HBV replication interacted with endogenous SMYD3 almost exclusively in the cytoplasm (Fig. 3b).

**SMYD3 does not significantly affect HBV transcription**

To determine the possible functional relevance of the HBx-SMYD3 interaction in HBV replication, we examined the effect of SMYD3 overexpression on HBV-Bj_JPN56 transcription. HBx expression (Fig. 4a, upper panel), HBV RNA synthesis (Fig. 4b) and HBsAg production (Fig. 4c) were barely affected by
SMYD3 overexpression. On the other hand, ectopic expression of ERK2, which is involved in the regulation of HBV replication (20), significantly promoted HBV RNA synthesis (Fig. 4b) and HBsAg production (Fig. 4c).

Next, we examined the effect of SMYD3 knockdown on HBV-Bj_JPN56 transcription. As shown in Fig. 4d, two siRNAs against SMYD3 (siSMYD3-1 and siSMYD3-2) reduced the SMYD3 mRNA expression by approximately 35% and 80%, respectively, compared with control siRNA. Knockdown of SMYD3 expression by using siSMYD3-2 did not exert significant effects on HBV RNA synthesis (Fig. 4e) and HBsAg production (Fig. 4f).

**HBx-SMYD3 interaction induces AP-1 activation**

SMYD3 has been reported to methylate MAP3K2 (14), which is linked to activation of ERK and also possibly JNK (21), leading to activation of AP-1 and NF-κB signaling (22, 23). Therefore, we examined the possible effects of the SMYD3-HBx interaction on AP-1 and NF-κB activation. Luciferase reporter assay revealed...
that co-expression of HBx and SMYD3, the former being expressed either ectopically or in the context of HBV replication, significantly enhanced AP-1 signaling compared with SMYD3 expression alone (Fig. 4g). As a control, co-expression of HbcAg and SMYD3 did not affect AP-1 signaling under these experimental conditions. In contrast, NF-κB signaling was not influenced by co-expression of HBx and SMYD3, whereas co-expression of HBx and ERK2 as a positive control significantly augmented NF-κB signaling (Fig. 4h).
These results suggest that HBx-SMYD3 interaction is involved in AP-1 activation but not in NF-kB activation.

DISCUSSION

Many studies have indicated the importance of HBx in the HBV life cycle. HBx exerts its activities by interacting with a variety of cellular partners that are located in either the cytoplasm or nucleus (4, 24). In this study, we identified SMYD3 as a novel HBx-interacting protein (Fig. 1). We also found that a C-terminal region of HBx (aa 131–154) and an internal region of SMYD3 (aa 269–288) are involved in the interaction (Fig. 2). Immunofluorescence and proximity ligation assays revealed that HBx interacts with SMYD3 predominantly in the cytoplasm (Fig. 3).

SMYD3 is involved in regulation of gene activation through its histone methyltransferase activity (13). In the present study, we observed that co-expression of HBx with SMYD3 significantly enhances AP-1 activation (Fig. 4g). This result raises the possibility that HBx-SMYD3 affects signaling cascades, such as the JNK/c-Jun/AP-1 pathway, leading to alterations in the cellular characteristics of HBV-infected cells. Recently, SMYD3 was reported to methylate MAP3K2, which is involved in the regulation of the Ras/JNK (or Ras/ERK) signaling pathway in cancer cells (14). It is reasonable, therefore, to assume that AP-1 activation induced by co-expression of HBx and SMYD3 is mediated through the SMYD3/MAP3K2/Ras/JNK signaling pathway. In this connection, it has been reported that HBx induces AP-1 activation (25, 26), and that SMYD3 is overexpressed in HCC (13). Moreover, expression of AP-1 is reportedly significantly greater in HBV-associated HCC than in HBV-negative HCC (27).

As for the possible effect of SMYD3 on virus replication, it has been reported that SMYD3 is a negative regulator of HCV particle production through the interaction with HCV NS5A (11). Another methyltransferase, protein arginine N-methyltransferase 1, has been reported to interact with HBx to negatively regulate HBV transcription (28). In the present study, we did not observe any significant effects of either overexpression or knockdown of SMYD3 on HBV transcription (Fig. 4a-f). Further studies are needed to elucidate the possible effect of HBx-SMYD3 interaction on the entire life cycle of HBV replication.

In conclusion, our present results suggest that HBx interacts with SMYD3 to cooperatively promote AP-1 activation. The HBx-SMYD3 interaction may provide a novel mechanism of HBV-related pathogenicity, especially regarding HCC.

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DISCLOSURE

The authors declare that they have no conflicting interests.

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