The Transactivation and p53-interacting Functions of Hepatitis B Virus X Protein Are Mutually Interfering but Distinct

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ABSTRACT

Transactivation of viral and host genes expression by hepatitis B virus X protein (HBx) is believed to be involved in hepatocarcinogenesis. The interaction of HBx with the tumor suppressor p53 and its inhibitory effect on p53 functions have been reported recently. However, the question of whether p53 is directly involved in HBx transactivation has not yet been addressed. In this study, we delineated the interaction sites of HBx and p53 using far-Western blotting and glutathione S-transferase-resin pull-down assays. The results indicate that the HBx-binding sites are located within the oligomerization and specific DNA-binding domains of p53 and that the p53-binding site was confined to a small region in the HBx transactivation domain. Mutual interference of the transactivations by HBx and p53 was detected by CAT assays in a transient transfection system. Strikingly, transactivation by HBx was observed in the p53-negative cells, Saos-2 and Hep3B, indicating that the transactivation and the p53-inhibiting functions of HBx are mutually interfering but distinct.

INTRODUCTION

There is a great deal of epidemiological evidence to suggest that chronic HBV infection is a major risk factor associated with hepatocarcinogenesis (1). However, the mechanisms by which HBV contributes to liver cell transformation remain elusive. No HBV gene acts as a dominant oncogene or acutely transforms liver cells. The woodchuck and ground squirrel hepatitis viruses, the genomes of which harbor the X gene, have been associated with the development of HCC, whereas the oncogenic potential of avian hepadnaviruses, which are devoid of the X gene, has not been established (2, 3). Importantly, the HBV X gene has been shown to have the ability to induce transformation in rodent cells (4–6) and HCC in some transgenic mice lines or to potentiate oncogenesis by oncogenic or chemical carcinogens (7–10). The X gene is frequently integrated into the host genome and expressed in HCC (11–13). HBx, a 154-aa protein encoded by the HBV X gene, has been shown to deregulate cell cycle checkpoint controls (14). On the other hand, HBx is a viral transactivator that can activate the expression of many viral and cellular genes through a wide variety of cis-elements (15–19). Although the mechanism has not been definitely elucidated, HBx transactivation seems to be important for its oncogenic role, because HBx transactivates not only HBV genes but also various host genes that engage in cell proliferation and acute inflammatory responses (15, 20–23).

The tumor suppressor p53 is involved in maintenance of the genomic integrity of cells (24). p53 has multiple functions, including transcriptional activation and repression, modulation of factors engaged in DNA repair, cell cycle control, and apoptosis regulation (25–27). Various DNA tumor viruses encode transforming oncoproteins that interact with p53. These include the SV40 large T antigen, adenovirus E1B, EBV EBNA-5, human cytomegalovirus IE84, and the human papillomavirus E6 proteins (28–30). Disruption of the p53 functions, such as transcriptional activation and p53-dependent apoptosis by viral oncoproteins, is not only beneficial to virus infection but also important in virally induced carcinogenesis.

The direct interaction of HBx with p53 both in vitro and in vivo has been reported recently (31–35). HBx was shown to inhibit p53 transactivation and p53-dependent apoptosis (32, 33, 36). However, there have been no reports showing that HBx transactivation is affected by p53. The relationship between transactivation and the p53-binding functions of HBx has not yet been addressed. In this report, we mapped the interaction sites of both p53 and HBx and investigated whether p53 binding is involved in HBx transactivation. The results show that the p53-binding site is within the transactivation domain of HBx, and p53 has two independent HBx-binding sites located in the specific DNA-binding domain and oligomerization domain, respectively. p53 and HBx interfere with each other's transactivation function. However, because HBx transactivation was clearly detected in p53-negative cells (Saos-2 and Hep3B), HBx transactivation is distinct from its p53-binding function.

MATERIALS AND METHODS

Plasmid Constructions. The full-length and truncated HBx-D19, -D2, -D3, -D39, -D6, and -D26 expression plasmids have been described previously (37–39). Construction of the truncated HBx mutant HBx-D19 (51–99 aa) was carried out by using PCR cloning. p53 and TBP cDNA derived from pSGH53 and H10 (40), which were kindly provided by Drs. M. Fujii (Tokyo Medical and Dental University, Tokyo, Japan) and M. Hirokoshi (Tokyo University, Tokyo, Japan), respectively, were inserted into the the EcoRI and BamHI sites of pSGUTPL and pGENKI (37). The resultant plasmid, pSGUTPL-p53, was used to construct p53-d1 (1–100 aa), p53-d5 (101–314 aa), and p53-d3 (315–393 aa) using PCR cloning. The p53 mutant R273H coding sequence was constructed by PCR-directed mutagenesis. The histidine-tagged protein expression plasmid pLHis was derived from pETT11d by replacing the Ndel–BamHI fragment with the annealed complementary oligonucleotides, TATGAATTCCCATGAAGCTTGGATC, so as to create the EcoRI, HindIII, and BamHI digestion sites. Plasmids pLHis-HBX and pLHis-p53 were constructed by inserting the HBx and p53 coding sequence into the EcoRI and BamHI sites of pLHis. All of the constructs were sequenced using Taq sequencing kits and a DNA sequencing (370A; Applied Biosystems).

Preparation of Recombinant Proteins. GST-fused proteins were expressed in Escherichia coli with induction by 0.4 mM isopropyl β-D-thiogalactopyranoside for 3 h at 30°C. Cells were harvested and sonicated in PBST buffer (PBS containing 1% Triton X-100). After centrifugation, the extract (supernatant) was collected and stored at −80°C. For purification, the extracts were incubated with glutathione-Sepharose 4B resin (Pharmacia) for 1 h at room temperature. The beads were collected and washed four times with PBST buffer and then eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). The eluted proteins were divided into aliquots and stored at −80°C.

Histidine-tagged HBx and p53 were expressed in the BL21 (DE3 pLysS) transfectants of pLHis-HBX and pLHis-p53 by 0.4 mM isopropyl β-D-thiogalactopyranoside induction at 30°C. Cells were harvested 3 h postinduction and sonicated in denaturing binding buffer (6 mM guanidine chloride, 20 mM sodium phosphate, and 500 mM NaCl, pH 7.8). Histidine-tagged proteins were purified by incubating the sonication supernatant with nickel resin and following extensive washing with denature binding buffer and then denaturing washing.
buffer (6 M guanidine chloride, 20 mM sodium phosphate, and 500 mM NaCl, pH 6.0) and were eluted with denaturing elution buffer (20 mM sodium phosphate and 500 mM NaCl, pH 4). The eluted proteins were renatured by dialysis against a buffer with a sequentially reducing concentration of urea (20 mM sodium phosphate and 500 mM NaCl, pH 7.8). After the final dialysis in PBS, the proteins were divided into aliquots and stored at ~80°C.

**Far-Western Blotting.** The far-Western blotting was performed as previously described (39). The target proteins (200 ng each) were fractionated on SDS-PAGE gels and then electrotransferred onto nitrocellulose membranes, which were denatured, renatured, and blocked with 5% skim milk in modified GBT buffer (10% glycerol, 50 mM Hepes-NaOH, pH 7.5, 170 mM KCl, 7.5 mM MgCl2, 0.1 mM EDTA, 0.1 mM DTT, and 1% Triton X-100) before subjected to far-Western blot (38). The binding reaction proceeded in modified GBT buffer containing 32P-labeled probe (40–100 ng/ml of protein with 2 x 10⁶ cpm/μg of protein), 1% BSA, 2 mM of unlabeled ATP, and the sonicated supernatant of E. coli JM109 transformed by pGENK1, which contained a final GST protein concentration of 1 mg/ml. The membranes were washed five times with modified GBT buffer and exposed to X-ray films (XAR Omat, Kodak) or imaging plates (Fuji).

**GST Resin Pull-down Assay.** Equal amounts (approximately 1 μg) of GST and GST-fused proteins immobilized on 10 μl of glutathione resin were incubated with 100 ng of target proteins (p53-His6 in Figs. 1C and 3C, HBx-His6 in Fig. 2C) in modified GBT buffer containing 1% BSA for 2 h at 4°C. After four washes with modified GBT buffer, the bound proteins were eluted with elution buffer (10 mM Tris-HCl, pH 7.4, 2% SDS, 0.3 mM NaCl, and 1 mM EDTA), separated by SDS-PAGE, and then transferred onto nitrocellulose membranes for Western blotting to detect HBx or p53. The proteins were visualized by ECL (Amersham) according to the manufacturer’s instructions. Preparation of anti-HBx was described previously (39). The anti-p53 monoclonal antibody (Ab-2) was purchased from Oncogene Science.

**Transfection and CAT Assay.** The p53-responsive CAT reporter pG13CAT was kindly provided by Dr. B. Vogelstein (The Johns Hopkins University, Baltimore, MD) (41). The HBx-responsive CAT reporters, pHECx2CAT and NF-κBx3CAT, and the transient transfection and CAT assay have been described previously (42). Total cell lysates were prepared from cells that were harvested 48 h after transfection. The CAT assay reactions proceeded for 60 min at 37°C using 20 μg of protein from the transfected cell lysates (42). The fractionated TLC plates were exposed to imaging plates, and the CAT activities were measured as the percentage of the conversion to acetylated forms of [14C]chloramphenicol (% acetylation; Amersham) using a Bioimage analyzer (BAS1000; Fuji). Transfection and CAT assays were performed at least three times with each combination of transactivator and CAT reporter constructs. Representative data are shown.

**RESULTS**

**HBx and p53 Directly Interact with Each Other in Vitro.** We at first investigated the direct interaction between HBx and p53. By far-Western blotting, the HBx probe bound to p53, RPBS, and itself but not to TBP or GST (Fig. 1A). The specific binding of p53 to HBx was not also detected by far-Western blotting using the p53 probe, which bound to HBx, TBP, and itself but not to RPBS and GST (Fig. 1B). Similar results were obtained with the GST resin pull-down assay, which showed that histidine-tagged p53 was efficiently retained by GST-HBx but not by GST-RPBS and GST (Fig. 1D). These results suggested a direct interaction between HBx and p53 in vitro and are consistent with the reports of other groups, who published data on physical and functional interactions between HBx and p53 while this study was being conducted (31–35).

**HBx Binding Sites Reside in the Specific DNA-binding and Oligomerization Domains of p53.** The regions of interaction between HBx and p53 were mapped by far-Western blotting. p53 has at least three functional domains, namely, the transactivation domain in the NH2-terminal part, the specific DNA-binding domain in the middle part, and the oligomerization domain in the COOH-terminal part (43, 44). Three truncated mutants of p53 covering each of the three
domains were constructed in GST fused forms and expressed in E. coli. The HBx probe bound to p53-d3 (315–393 aa) and p53-d5 (101–314 aa) but not to p53-d1 (1–100 aa; Fig. 2A). This result was confirmed by the alternative approach of the GST-resin pull-down assay. Histidine-tagged HBx was expressed in and purified from E. coli. GST-fused forms of wild-type and mutated p53 proteins immobilized on glutathione resin were incubated with histidine-tagged HBx. After extensive washing, the bound proteins were eluted and Western blotted. In agreement with the results of far-Western blotting, wild-type p53, p53-d3, and p53-d5 but not p53-d1 bound to HBx (Fig. 2B). The tumor-derived p53 mutant, p53-R273H, also bound HBx (Fig. 2, A, Lane 5, and C, Lane 7). These results indicate that p53 has two independent HBx-binding sites located in either the specific DNA-binding domain or the oligomerization domain and that the transactivation domain is dispensable for HBx-binding. The deletion of either of the binding sites and the single point mutant p53-R273H reduced the HBx-binding ability (Fig. 2, A and C, compare -d3, -d5, and -R273H to p53 full), suggesting that both binding sites contribute to the HBx binding of the full-length p53.

p53-binding Site Resides in the HBx Transactivation Domain.
We previously reported that HBx consists of a functional transactivation domain (51–148 aa) and a regulatory domain (1–50 aa; Ref. 37). The p53 probe bound to HBx-5D1(51–154 aa) but not to HBx-3D5 (1–50 aa), indicating that the p53-binding site is located within the transactivation domain of HBx but not the regulatory domain (Fig. 3A). Further delineation indicated that the p53-binding region is located in a small region of the COOH-terminal portion of HBx (102–136 aa) because the p53 probe bound to the truncated HBx proteins harbor 102–136 aa (HBx full, -5D1, -5D2, -5D4, -5D12,
I-C0) but not to -D19 (51—99 aa; Fig. 3A). A similar requirement for this region of HBx for p53 binding was also detected using the GST-resin pull-down assay (Fig. 3C).

**p53 Interferes with the Transactivation Activity of HBx.** Next, we examined whether p53 has an effect on HBx transactivation by a CAT assay using the reporter pHECx2CAT, which harbors the HBx responsive element derived from the HBV enhancer I core (42). HBx and p53 were transiently coexpressed in HepG2 cells, and the transacting activity of HBx was examined by CAT assay. p53 had no detectable effect on the reporter (Fig. 4, Lanes 2—4). However, the HBx transactivation activity was severely repressed by p53 in a dose-dependent manner (Fig. 4, Lanes 5—8). A similar effect was observed when p53 and HBx-D1, which constitutes the transactivation domain of HBx, were coexpressed (Fig. 4, Lanes 9—12). The repression by p53 could be alleviated by an excess amounts of HBx (Fig. 4, Lanes 13—15). These results indicate that p53 and HBx interact functionally in vivo.

**HBx Represses p53 Transactivation Activity.** The effect of HBx on p53 transactivation was investigated by CAT assay. Because the background of the p53-responsive reporter was high in HepG2 cells, perhaps due to the endogenous p53, it was difficult to observe the effect of HBx. Therefore, we choose Saos-2, a p53-negative osteosarcoma cell line. The introduction of p53 transactivated the p53-responsive reporter pG13CAT efficiently in this cell line, whereas HBx had no effect on the reporter (Fig. 5, compare Lanes 5—7 to 2—4). However, the transacting activity of p53 was repressed by HBx when HBx and p53 were coexpressed (Fig. 5, Lanes 9—11). HBx-5D1, which harbors the p53-binding site, exhibited a repression similar to the full-size HBx (Fig. 5, Lanes 12—14). HBx-3D5 and -D19, which could not bind to p53, showed no effect on p53 transactivation (Fig. 5, compare Lanes 15—17 to Lanes 18—20). The transactivation-defective mutant HBx-5D4, which harbors the p53-binding site, exhibited similar repression (Fig. 5, Lanes 21—23). These results indicate that HBx interferes with p53 functionally and that the p53-interacting site in the transactivation domain of HBx is responsible for the inhibitory effect of HBx on p53 transactivation.

**HBx Exerts Transactivating Activity in p53-negative Cells.** Because the p53-binding site was mapped in the HBx transactivation...
of p53 (Fig. 6A). Because different X-responsive elements responded
by HBx, indicating that HBx transactivation does not require the presence
having HBV enhancer I core or NF-κB-binding sites was transactivated
domain, we addressed whether p53 interaction is involved in the HBx
pSG5UTPL-p53-d5 plus 1 μg of pSG5UTPL-HBx (Lanes 14—16, respectively); and 1, 2, and 3 μg of pSG5UTPL-p53 
plus 1 μg of pSG5UTPL-HBx (Lanes 1—3). The amount of pSG5UTPL-HBx is shown in μg at the bottom of the figure. The total amount of DNA added per transfection was adjusted to 10 μg with the control vector, pSG5UTPL. B, introduction of p53 to Saos-2 cells (p53 negative) suppresses HBx transactivation. The transfected plasmid DNA was 5 μg of pHECx2CAT together with 5 μg of pSG5UTPL (Lane 1); 1, 2, and 3 μg of pSG5UTPL-p53 (Lanes 2—4, respectively); 1, 2, and 3 μg of pSG5UTPL-p53 plus 1 μg of pSG5UTPL-HBx (Lanes 5—7, respectively); 1, 2, and 3 μg of pSG5UTPL-p53-d3 plus 1 μg of pSG5UTPL-HBx (Lanes 11—13, respectively); 1, 2, and 3 μg of pSG5UTPL-p53-d5 plus 1 μg of pSG5UTPL-HBx (Lanes 14—16, respectively); and 1, 2, and 3 μg of pSG5UTPL-p53-R273H plus 1 μg of pSG5UTPL-HBx (Lanes 17—19, respectively). The total amount of DNA added per transfection was adjusted to 10 μg with the control vector, pSG5UTPL. The CAT activity was measured 48 h after transfection and measured as percentage of conversion.

Fig. 6. HBx exhibits transactivation activity in p53-negative cells. A, p53-negative hepatoma cells (Hep3B) were transfected with pHECx2CAT or NF-kBx3CAT together with pSG5UTPL-HBx. The amount of pSG5UTPL-HBx is shown in μg at the bottom of the figure. The total amount of DNA added per transfection was adjusted to 10 μg with the control vector, pSG5UTPL. B, introduction of p53 to Saos-2 cells (p53 negative) suppresses HBx transactivation. The transfected plasmid DNA was 5 μg of pHECx2CAT together with 5 μg of pSG5UTPL (Lane 1); 1, 2, and 3 μg of pSG5UTPL-p53 (Lanes 2—4, respectively); 1, 2, and 3 μg of pSG5UTPL-p53 plus 1 μg of pSG5UTPL-HBx (Lanes 5—7, respectively); 1, 2, and 3 μg of pSG5UTPL-p53-d3 plus 1 μg of pSG5UTPL-HBx (Lanes 11—13, respectively); 1, 2, and 3 μg of pSG5UTPL-p53-d5 plus 1 μg of pSG5UTPL-HBx (Lanes 14—16, respectively); and 1, 2, and 3 μg of pSG5UTPL-p53-R273H plus 1 μg of pSG5UTPL-HBx (Lanes 17—19, respectively). The total amount of DNA added per transfection was adjusted to 10 μg with the control vector, pSG5UTPL. The CAT activity was measured 48 h after transfection and measured as percentage of conversion.

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domain, we addressed whether p53 interaction is involved in the HBx
transactivation. In Hep3B, a p53-negative hepatoma cell line, the reporter
having HBV enhancer I core or NF-κB-binding sites was transactivated
by HBx, indicating that HBx transactivation does not require the presence
of p53 (Fig. 6A). Because different X-responsive elements responded similarly to HBx in Hep3B cells, the HBx transactivation in a p53-negative background seems to be a general phenomenon. In the p53-negative Saos-2 cells, a similar result was obtained (Fig. 6B, Lanes 5—7). Furthermore, coexpression of p53 with HBx in Saos-2 cells repressed the HBx transactivation (Fig. 6B, Lanes 8—10). The p53 truncated mutants p53-d3 and p53-d5 and the substitution mutant p53-R273H, which bind to HBx, failed to repress HBx transactivation, suggesting that HBx binding alone is not sufficient to repress HBx transactivation (Fig. 6B, Lanes 11—19). These results imply that HBx transactivation is independent of the presence of p53 and that the physical interaction of p53 and HBx is insufficient to repress HBx transactivation.

DISCUSSION

In this report, we delineated the interaction sites of p53 and HBx using far-Western blotting. The results show that the p53-binding region (102—136 aa) is within the transactivation domain of HBx and that the HBx-binding sites of p53 are within the specific DNA-binding and oligomerization domains that are separated from both the activation domain (1—40 aa) and the putative trans-repression domain of p53 (45).

HBx consists of two functional domains, namely, the regulatory domain and the transactivation domain (37). p53 binding does not require the regulatory domain and was mapped within the transactivation domain. We recently found that RPB5 (a common subunit of RNA polymerases I, II, and III) and a general transcription factor (TFIIIB) bind different regions within the HBx transactivation domain and that both RPB5 and TFIIIB bindings are essential for HBx transactivation (38, 39). The p53-interaction site of HBx is different from the RPB5-binding site, but is overlapped with that of TFIIIB. The ability of HBx to interfere with the p53-transactivation was mapped to the p53-binding region of HBx, suggesting that the interference may be the direct result of p53 binding to HBx. The direct interaction of p53 and HBx may prevent p53 from accessing the regulation element of the p53-responsive genes (32, 33), and thus communicating with the regulatory proteins in cell cycle checkpoint controls (13). Another possible mechanism by which HBx inhibits p53 functions is through changing the subcellular localization of p53 (34), although this is still controversial (12, 36).

Because HBx can directly interact with p53 and because p53 acts as a repressor of transcription when the promotor is devoid of a p53-binding cis-element, one may speculate that HBx exerts its transactivation activity through the release of p53 repression. However, our results strongly argue against this possibility. HBx exhibited a transactivating function in the p53-negative background, which was observed in both Saos-2 and Hep3B, using different X-responsive cis-elements. Therefore, it is clear that the HBx transactivation is distinct from the p53-binding function, although the HBx transactivation could be affected by excess p53.

The mechanism of the inhibitory effect of p53 on HBx is not clear at present. It is possible that p53 inhibits HBx transactivation by competing with TFIIIB to bind to HBx because the p53-binding site overlaps with the TFIIIB-binding site in HBx; it is also possible that the TFIIIB binding is essential for HBx transactivation. However, this speculation seems unlikely because the p53 truncated mutants p53-d3 and p53-d5, which have HBx-binding ability in vitro, cannot repress HBx transactivation. Alternatively, the repression of HBx transactivation by p53 may be due to a general p53 repression function because p53 can inhibit the activities of reporters that do not harbor a p53-responsive element, perhaps through interacting with TBP or its associated factors (TAFs; Refs. 46 and 47). The trans-repression by p53 was reported to be independent of the transactivation function, and the oligomerization domain is essential for the trans-repression but not transactivation (45). It remains to be established whether p53 inhibits basal promoter activity and HBx transactivation through the same mechanism.

At present, the biological and pathological significance of the interaction of p53 and HBx has not been well established. The relative amounts of p53 and HBx in cells might be crucial in evaluating the effect of p53 and HBx binding. Due to the high background of the p53-responsive reporter in HepG2, we examined the functional interference of p53 and HBx...


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