Cooperative repression of cyclin-dependent kinase inhibitor p21 gene expression by hepatitis B virus X protein and hepatitis C virus core protein

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Abstract Co-infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) is common and is associated with a more severe liver disease and increased frequency in the development of hepatocellular carcinoma (HCC). Here, we demonstrated that HBV X protein (HBx) and HCV core protein additively repress the universal cyclin-dependent kinase inhibitor p21 gene at the transcription level. The transforming growth factor-β responsive element and Sp1 site of the p21 promoter were responsible for the effect of HCV core and HBx, respectively. Furthermore, cell growth was additively stimulated by them, suggesting that additive repression of the p21 might be important to understand the cooperative development of HCC by HBV and HCV. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Core; Hepatitis B virus; Hepatitis B virus X protein; Hepatocellular carcinoma; Hepatitis C virus; p21

1. Introduction

Human hepatitis B virus (HBV) and hepatitis C virus (HCV) are two major etiologic agents of chronic hepatitis, which is closely related to the development of hepatocellular carcinoma (HCC). Co-infection with HBV and HCV is common and is associated with a more severe liver disease, increased frequency in the development of HCC (approximately five-fold increase in risk), and resistance to interferon therapy compared to those with single HBsAg or anti-HCV positivity [1–4]. Despite of several epidermiological evidences, the mechanism by which HBV and HCV cooperatively cause HCC is not understood.

Cell cycle progression is driven by the sequential activation of cyclin-dependent kinases (CDKs), which are subject to regulation by positive (cyclins) and negative (CDK-inhibitory proteins) effectors [5]. One such negative effector is the universal CDK inhibitor p21 [6]. While p21 can be transcriptionally regulated by the p53 tumor suppressor protein [7] and is thus believed to participate in the execution of p53 effects, its induction can also be activated by a rapidly growing list of physiological and pathological factors, such as differentiation factors, growth factors, cytokines, and stress factors [8–11]. Through binding to cyclin/CDK complexes, p21 prevents CDK-dependent phosphorylation, and subsequent inactivation of the retinoblastoma protein, which negatively regulates cell cycle progression. p21 also interacts with proliferating cell nuclear antigen (PCNA) to inhibit PCNA-dependent DNA replication [12]. Thus, p21 exerts anti-proliferation activity through inhibition of cyclin/CDK complexes and/or PCNA functions [13,14].

HBV X protein (HBx) and HCV core protein are considered to play important roles during development of HCC. Both proteins are known to stimulate cell proliferation by repressing transcription of the p21 gene [15–18]. The initial aim of this study was to investigate whether there is an additive effect between HBx and HCV core on the repression of p21 gene. Next, we tried to determine the mechanism by which the two proteins additively repress the transcription of p21 gene. Finally, we investigated whether the effect is connected to the additive cell growth stimulation.

2. Materials and methods

2.1. Plasmids

Plasmid pCI-neo-core K which encodes HCV core protein under the human cytomegalovirus immediate-early promoter was described previously [19]. Plasmid pCI-neo-IRES-HBx which encodes X region (nt 1374–1839, accession number D23677) of the HBV DNA was constructed by subcloning the PCR-amplified product into the *Xba*I and *Not*I sites of pCI-neo (Promega) and inserting the encephalomyocarditis virus internal ribosome entry site (IRES) [20] in front of HBx. The bicistronic expression vector pCI-neo-core-IRES-HBx was constructed by subcloning the HCV core gene in front of IRES in pCI-neo-IRES-HBx, thus to translate HCV core protein cap-dependently whereas HBx in an IRES-dependent manner. p21P, p21PΔ1.9, and other mutants of the p21 promoter were described by Datto et al. [8]. pGL2-4×TβRE which contains four copies of transforming growth factor (TGF)-β responsive element (TβRE) in pGL2-basic (Promega) was described previously [8]. pGL2-2×Sp1 which contains two copies of Sp1 binding site in the p21 promoter between −63 and −51 was described previously [15].

2.2. Transfection and luciferase assay

NIH 3T3 cells (2×10^5 cells per 60-mm diameter plate) were transfected with a calcium phosphate-DNA precipitate containing 5 μg each of target and effector plasmid DNAs as previously described [21]. To control for variation in transfection efficiency, 1 μg of plasmid pCH110 (Pharmacia) containing the *Escherichia coli lacZ* gene under control of the SV40 promoter was co-transfected. Forty-eight hours after transfection, the level of expression from the target gene (luciferase activity) was analyzed and values obtained were normalized

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Abbreviations: CDK, cyclin-dependent kinase; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; TβRE, transforming growth factor-β responsive element

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to the β-galactosidase activity measured in the corresponding cell extracts. Each experiment was repeated at least three times.

2.3. Semi-quantitative reverse transcription (RT)-PCR and Western blotting analysis

Total cellular RNA was extracted from cells 48 h after transfection using Trizol (Gibco). For RT-PCR, 3 μg of RNA was reverse-transcribed with the corresponding antisense primer. One quarter of the reverse-transcribed RNA was amplified with *Taq* polymerase (95°C, 5 min; 30 cycles of 95°C, 1 min–56°C, 1 min–72°C, 30 s; 72°C, 5 min) using the appropriate primer pairs. The primers for p21, p53, HBx, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were described previously [15]. For the detection of HCV core transcripts, sense primer 5′-TCC GGA TCC CTG TCA TCT TCT GTC CCT-3′ and antisense primer 5′-TCG CTT AGT GGA TCC TGG GGG CAG-3′ were used.

For Western blotting analysis, cells were lysed in buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 0.1% SDS, 1% NP-40) supplemented with protease inhibitors. 10 μg of cell extracts was separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF; Amersham). Western blotting was performed with either anti-p53 monoclonal antibody (Santa Cruz), anti-p21 rabbit polyclonal IgG (Santa Cruz), or anti-actin monoclonal IgG (Santa Cruz), and subsequently detected by chemiluminescent ECL kit (Amersham) as recommended by the manufacturer.

2.4. Generation of stable cell lines and determination of cell growth rate

NIH 3T3 cells transfected with HBV core and/or HBx-expressing plasmids were selected and amplified to obtain the stable cell lines as described previously [19]. The expression level of transfected genes was checked by either RT-PCR or Western blotting analysis. For the determination of cell growth rate, 5×10^4 cells were plated in six-well plates (Nunc) and the total cell number in each well was counted after incubation for the indicated period.

3. Results and discussion

To examine whether HBx and HCV core additively repress transcription of p21, we transiently transfected HBx and/or HCV core-expressing plasmids into NIH 3T3 cells and performed RT-PCR on the level of the endogenous p21 transcripts (Fig. 1a). Consistently to the previous reports [15,16], the NIH 3T3 cells expressing either HCV core (lane 2) or HBx protein (lane 3) clearly showed the decrease of p21 transcription compared to the control NIH 3T3 cells (lane 1) whereas transcription of the housekeeping gene G3PDH was not affected by the viral proteins. Interestingly, the transcription level of p21 was much more repressed, almost undetectable in HBx and HCV core co-expressing cells (lane 4). Similarly to the RNA level, the endogenous p21 protein was also additively decreased by HBx and HCV core protein (Fig. 1b). On the other hand, the p53 was not significantly affected by them at both RNA and protein levels (Fig. 1), suggesting that the effect is p53-independent.

Next, to examine whether HCV core and HBx additively repress p21 at the transcription initiation level, we investigated the effect of them upon the p21 promoter using the p21-luciferase reporter (p21P) which contains the luciferase gene under the control of full-length p21 promoter. Initially, p21P and viral protein-expressing constructs were co-transfected into NIH 3T3 cells and luciferase assay was performed. Consistently to the previous reports [15,16], HBx and HCV core could separately repress the promoter activity of p21 approximately three- and seven-fold, respectively (Fig. 2a). Co-expression of HBx and HCV core much more strongly represses transcription of the p21-luciferase reporter construct, up to 15.9-fold, suggesting that the repression effect of HBx and HCV core on the p21 promoter-luciferase construct is additive.

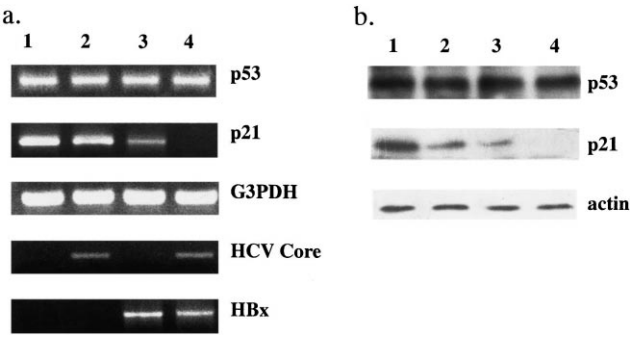

Fig. 1. Additive repression of the p21 gene by HBx and core protein. a: NIH 3T3 cells were transfected with an empty vector (lane 1), HCV core (lane 2), HBx (lane 3), or HCV core and HBx (lane 4) expression vector and the RNA level were measured by semi-quantitative RT-PCR. b: The protein level of p53, p21, and actin in NIH 3T3 cells transfected as above was measured by Western blotting.

We previously determined the region of the p21 promoter responsible for the repression by HBx to a 61-bp region from the transcriptional initiation site [15]. In this study, we tried to determine the HCV core responsive element and the region responsible for the additive effect by HBx and HCV core. To this end, a series of progressive 5′ promoter deletion mutants of the p21 promoter were tested (Fig. 2a). A deletion mutant of p21 promoter, p21PΔ1.9 which does not contain p53 binding sites (Fig. 2a), was additively repressed by HBx and HCV core protein although the basal level of p21 promoter was greatly decreased by the removal of p53 binding sites. This result suggests that the additive repression of the p21 promoter by HBx and HCV core is p53-independent. Further deletion up to −93 did not affect the additive effect of HBx and HCV core. However, the minimal promoter construct, p21P smaΔ1, which contains only 61 bp proximal to the transcriptional initiation site was repressed by only HBx and thus the effect was not additive. Results from these experiments suggest that HCV core represses p21 promoter through a 33-bp region between −93 and −61 whereas HBx through a 61-bp region from the transcriptional initiation site.

To precisely define the regions of the p21 promoter necessary for the additive repression by HBx and HCV core, a panel of the point-mutated p21 promoter between bases −93 and −44 [8] was examined. According to our previous report [15], the sequence of the p21 promoter between −63 and −51 was essential for repression by HBx. In this study we obtained a similar result with p21P 93-S mut#4 and mut#5 (Fig. 2b). p21P 93-S mut#1 and mut#3 were additively repressed by HBx and HCV core in a similar manner to p21P 93-S. However, p21P 93-S mut#2 had almost completely lost the ability to be repressed by HCV core (Fig. 2b). Considering that p21P 93-S mut#2 has a mutated Sp1 site, the Sp1 site might be responsible for the effect of HCV core. However, this hypothesis cannot explain why mutations in other Sp1 sites, for example Sp1-2, were not effective. Therefore, the complete loss of HCV core responsiveness in 93-S mut#2 might be due to another mutation which actually abolishes the effect.

Interestingly, the major HCV core responsive element in the p21 promoter defined above was exactly overlapped with the

a.

		Basal activity (%)	Repression fold Core	HBx	Core/HBx
p21P	-2360 luciferase	100	3.3±1.1	6.9±1.5	15.9±3.5
p21P Δ 1.9	-460	51.1	3.8±0.3	5.7±0.7	13.8±2.2
p21P Sma	-113	44.5	2.8±0.5	4.7±1.3	8.5±1.1
p21P 93-S	-93	41.7	3.2±0.7	5.2±1.1	14.2±2.2
p21P Sma Δ 1	-61	24.2	1.1±0.2	4.4±0.5	4.1±0.7

b.

-93 -84 -74 -64 -54 -44

Sp1-1 TβRE Sp1-2 Sp1-3 Sp1-4 TATAbox

		Basal activity (%)	Repression fold Core	HBx	Core/HBx
93-S	GAGCGCGGGT CCCGCCTCCT TGAGGCGGGC CCGGGCGGGG CGGTTGTATA	100	3.2±0.7	5.2±1.1	14.2±2.2
93-Smut#1	ATCTAGAAAC	74.5	3.6±1.1	4.2±0.7	13.2±1.5
93-Smut#2	TATCTAGAAC	62.9	1.1±0.2	3.5±1.5	3.7±0.5
93-Smut#3	CTCTAGAAAT	57.9	3.9±0.7	4.9±2.5	14.4±3.6
93-Smut#4	ATCTAGACAT	12.9	3.7±0.5	1.2±0.2	3.5±0.4
93-Smut#5	TCTAGACCGT	16.8	3.7±0.7	1.5±0.2	3.9±0.7
93-Smut#23	CCCGCCGGCT	70.3	1.1±0.2	5.5±0.5	5.8±0.6
Vector		1.6	1.1±0.2	1.3±0.3	1.2±0.4

Fig. 2. Identification of HCV core and HBx responsive elements in the p21 promoter. a: Full-length and deletion p21 promoter constructs [8] were co-transfected with HCV core and/or HBx-expressing plasmids into NIH 3T3 cells. Repression fold was calculated by comparing the luciferase activity of HCV core and/or HBx-expressing cells with the basal activity of the control. The basal luciferase activity of the full-length p21 promoter is designated to 100%. The p53 binding sites are indicated by open boxes. b: 93-S mutant constructs, identical to the wild-type p21P 93-S sequence except the nucleotides shown for each mutant construct [8], were tested as above. The positions of transcription factor binding sites are underlined.

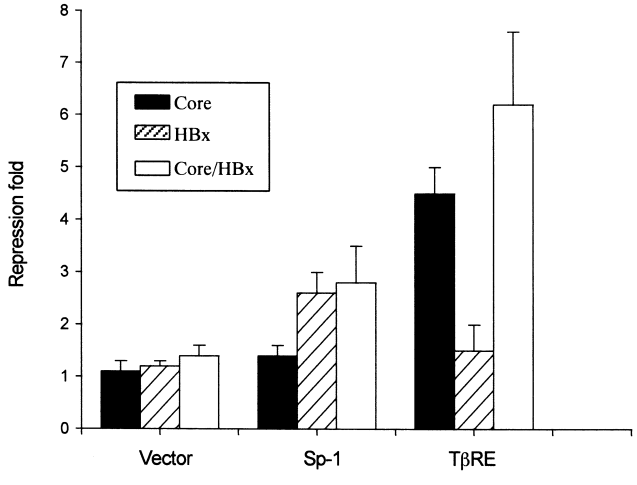

Fig. 3. Repression of TβRE and Sp1 site by HCV core and HBx. pGL2-4×TβRE [8] and pGL2-2×Sp1 [15] were co-transfected with HBx and/or HCV core-expressing plasmids into NIH 3T3 cells. Repression fold was calculated by dividing each luciferase activity by the basal activity obtained with the control vector.

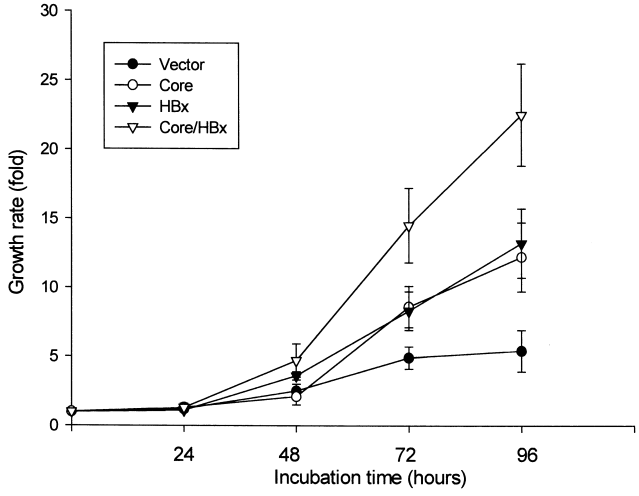

Fig. 4. Additive stimulation of cell growth by HCV core and HBx. The growth fold of either NIH 3T3 cells or HCV core and/or HBx-expressing stable cells for the indicated period was counted through five different experiments.

TβRE which is known to mediate transcriptional activation of the p21 gene by TGF-β [8]. More direct evidence that the Sp1 binding site is not responsible for the HCV core responsiveness was obtained with 93-S mut#2.3. This construct has a mutation of bases −76 and −77 from CT to GG, thus maintains the consensus Sp1 binding site but showed a significantly reduced ability to be activated by TGF-β [8]. The promoter activity in 93-S mut#2.3 was not repressed by HCV core at all, suggesting that destruction of the TβRE in both 93-S mut#2 and 93-S mut#2.3 was responsible for the loss of HCV core responsiveness. Therefore, we concluded that the TβRE and the Sp1 sites between −63 and −51 of the p21 promoter are responsible for the effect of HCV core and HBx, respectively. Mutation in one of these sequences, which abolishes the response by either HBx or HCV core, eventually results in loss of the additive effect, thereby both sequences are necessary for the additive effect.

To determine if the TβRE between −83 and −74 of p21 promoter defined above is sufficient for the repression by HCV core, this fragment was used in an attempt to confer HCV core response to a heterologous promoter. pGL2 T+I 4×TβRE which has four copies of the TβRE inserted 5′ of the TATA box [8] was repressed by HCV core but not responsive to HBx (Fig. 3). In addition, pGL2-2×Sp1 [15] which contains two copies of Sp1 binding site was responsive to HBx but not to HCV core (Fig. 3). Both luciferase constructs were not additively repressed by HCV core and HBx, as expected.

Because the tumor repressor p21 protein is an universal inhibitor of cyclin–CDK complexes and DNA replication that induces cell cycle arrest at the G1–S checkpoint, the additive repression of p21 by HCV core and HBx may result in additive growth stimulation of the cells. To test this possibility, we prepared several NIH 3T3 cell lines stably expressing HCV core and/or HBx and measured their growth rates. Five different cell lines with each plasmid were selected and tested to show that differences in their growth rates are not just due to the chance selection of cell clones that grow at different rates. In a similar pattern with the transient transfection experiments, these cell lines expressed a decreased level of the p21 protein compared to the parent cells (data not shown). The growth rate of either HCV core- or HBx-expressing cell lines was, as expected, approximately two-fold faster compared to that of the control cells (Fig. 4). In addition, the growth stimulation effect of HCV core and HBx was additive, suggesting that the repression of p21 is properly reflected by the stimulation of cell growth.

In the present study, we provide a clue to understand the mechanism for the synergistic effect of HBV and HCV co-infection on the progression from chronic hepatitis to HCC, demonstrated by several epidermiological studies [1–4]. However, some contradictory data that additional infection of HBV does not play a role in patients with chronic hepatitis C were also reported [22]. Although our present study cannot explain the latter situation, several possibilities can be considered. First, differences in the conformation of HBx or HCV core protein might be involved. Both proteins seem to exert most of their functions through protein–protein interactions and can be regulated by modifications, such as phosphorylation [23,24]. Actually, according to our previous report, the p21 regulatory activity of HCV core protein is affected by the phosphorylation status of the protein [16]. Therefore, differences in the amino acid sequences, especially at the phosphorylation sites of the protein, could result in contradictory outcomes both in vitro and in vivo. Another possibility to be considered is the physiological status of host cells. Considering that both proteins function through cellular factors such as Sp1 and TGF-β signalling molecules, as demonstrated in the present study, the activity of these regulatory factors in the host cell may affect the final outcome of co-infection. Further studies based on these possibilities may elucidate the contradictory data concerning the synergistic effect of dual infection in vivo.

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