The hepatitis B virus X protein enhances the DNA binding potential and transcription efficacy of bZip transcription factors

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The hepatitis B virus X protein interacts with the basic-region, leucine zipper protein (bZip) domain of cAMP response element-binding protein increasing its affinity for the cAMP response element site in vitro and its transcriptional efficacy in vivo (Williams, J. S., and Andrisani, O. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3819–3823). Here we examine pX interactions with bZip transcription factors ATFγ, gadd153/Chop10, ICER IIγ, and NF-IL6. We demonstrate direct interactions in vitro between pX and the bZip proteins tested. In contrast MyoD and Gal4 fail to interact with pX. We also demonstrate by the mammalian two-hybrid assay the direct interaction of pX with cAMP response element-binding protein, ICER IIγ, ATFγ, and NF-IL6 in hepatocytes. In addition, pX increases the DNA binding potential of bZip proteins for their cognate DNA-binding site in vitro. In transient transfections in hepatocytes (AML12 cell line), pX increases the transcriptional efficacy of the bZip transcription factors. NF-IL6-mediated transcriptional activation is enhanced 3-fold by pX. Most interestingly, pX augments the repression mediated by bZip repressors ATFγ and ICER IIγ by 6- and 7-fold, respectively, demonstrating for the first time the involvement of pX in gene repression. We conclude that pX is an enhancer of the DNA binding potential of bZip transcription factors, thereby increasing the transactivation or repression efficacy of bZip-responsive genes.

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The abbreviations used are: HBV, hepatitis B virus; bZip, basic-region, leucine zipper proteins; HBV pX, hepatitis B virus X protein; GST, glutathione S-transferase; CRE, cAMP-response element; CREB, CRE-binding protein; ICER, inducible cAMP early repressor; HIV-LTR, human immunodeficiency virus-long terminal repeat; PAGE, polyacrylamide gel electrophoresis; CMV, cytomegalovirus; IL, interleukin; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus.
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(51), and exposure to toxins (52, 53). While gadd153/Chop10 has strong sequence similarity to CREB-like proteins within the bZip region (43), it contains substitutions of three conserved residues in the basic region critical for DNA binding. Therefore, heterodimers of gadd153/Chop10 and CREB-like proteins are unable to bind to their cognate DNA binding site (43). Accordingly, it has been proposed that gadd153/Chop10 functions as a stress-inducible transcriptional inhibitor (43). However, recent studies indicate that in certain cases gadd153/Chop10 may function as a direct transcriptional activator (54). ATPγs is also of interest in this study since it is a member of the CREB/ATF family of transcription factors, sharing sequence similarity with the bZip domain of CREB and binding to the CRE site (44). However, while CREB is a positive regulator of transcription, ATPγs is a transcriptional repressor (56). Recent studies indicate that ATPγs is induced by a variety of physiological stress conditions, such as mechanically injured and toxin-injured liver (55, 57). ATPγs and gadd153/Chop10 form non-DNA-binding heterodimers (55). Interestingly, during carbon tetrachloride injury of liver, gadd153/Chop10 and ATPγs mRNAs are inversely induced but in an overlapping manner; gadd153/Chop10 mRNA, high in normal liver, decreases upon CCl4 exposure; ATPγs mRNA, low in normal liver, increases upon CCl4 exposure (55).

ICER IIγ (inducible cAMP early repressor) (45), a member of the CREB/ATF family of transcription factors, is of interest since it is an inducible repressor, displaying high degree of amino acid sequence identity with the bZip of CREB and is devoid of a transactivation domain. Thus, ICER IIγ provides an ideal model system to demonstrate the effect of pX in repression of bZip transcription. Recent studies demonstrated ICER IIγ expression in liver regeneration (80).

In this study, we employ in vitro and in vivo (cellular) assays to examine the interaction of pX with the aforementioned bZip transcription factors.

MATERIALS AND METHODS

In Vitro Protein-Protein Interaction Assays—Transcription factors ICER IIγ (45), the bZip domain of HIV-1 Nef residues 259–335, and CREB327 (59) were cloned in T7 vector. ATPγs (55) and gadd153/Chop10 (55) were in a derivative of pTFM1 (60) vector. 32P-Labeled proteins were synthesized by the T7/T polynucleotide kinase in vitro transcription/translation system. Plasmids GST-X1–154 and GST-X147–154 were constructed by polymerase chain reaction of pX DNA fragments corresponding either to amino acid residues 1–154 or 147–154 cloned into the EcoRI-HindIII sites of plasmid pGEX-KG (61). cDNA of proteins were expressed in Escherichia coli and purified on glutathione-Sepharose 4B resin (Pharmacia Biotech Inc.), as described previously (62). Bacterial extract obtained from 1 liter of bacterial culture was bound to 500 μl of resin for 30 min and washed (62). Protein concentration of GST and GST-X1–154 bound to the resin was estimated by the Coomassie Blue staining of SDS-PAGE and by comparison to known amounts of bovine serum albumin run on the same gel. In vitro protein-protein interaction assays were carried out as follows: 10 μg of GST and 2 μg of GST-X1–154 proteins immobilized onto 20 μl of glutathione-Sepharose 4B resin were incubated with 4 μl of T7 lysate for 3 h at 4 °C, in buffer containing 25 mM Hepes, pH 7.5, 100 mM KCl, 5 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 40 μg/ml bovine serum albumin, 0.1% Triton X-100, and a protease inhibitor mixture. After incubation, the beads were washed six times in the above buffer. Analysis of the bound protein was by SDS-PAGE and fluorography.

In Vitro DNA-Protein Binding Assays—DNA protein binding assays were carried out as described previously (1). The somatostatin CRE (62, 63) was used as the oligonucleotide probe for CREB, ICER IIγ, ATPγs, and gadd153/Chop10. The nucleotide sequence spanning positions –165 to –173 of the HIV-LTR (58) was used as the DNA-binding probe for NF-IL6. The MyoD/E47 E-box DNA-binding site (64) was kindly provided by Dr. S. Konieczny. Proteins for ICER IIγ, ATPγs, gadd153/Chop10, ATPγs/gadd153/Chop10, and MyoD/E47 were obtained by in vitro translation (TNT, Promega); 1–5 μl of in vitro translation mixture was used for each reaction. Bacterially produced CREB327 (70) and bZip NF-IL6, 15 ng each, were used in the binding reactions. Recombinant GST-X147–154 was added to binding reactions; control lanes contained equal amounts of GST protein. The reaction mixtures were analyzed by native acrylamide gel electrophoresis, as described (62, 63).

Tissue Culture and Transfections—A512 cells were kindly provided by Dr. N. Fausto. A512 cells, maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12, supplemented with 10% fetal calf serum, a mixture of insulin, transferrin, and selenium (Life Technologies, Inc.), 0.1 μM dexamethasone, and gentamicin, 50 μg/ml (65).

In functional assays, A512 cells were transfected by the calcium phosphate coprecipitation method, using the Life Technologies, Inc. transfection kit. Briefly, 10–20% confluent cultures were transfected with 5 μg of CAT reporter plasmid and the indicated amount of expression plasmid. Cells were harvested 48 h after transfection. CAT activity was determined as described (66) using equal amounts of protein extract per assay. Protein concentration of cellular extracts was determined by the Bio-Rad protein assay. Each experiment was repeated a minimum of three times. The CAT reporter vector pC15ΔXE, kindly provided by Dr. M. Bina, contains only the NF-IL6-binding site I, at position –165 to –178 (58).

The mammalian two-hybrid assay was carried out using the 5 × Gal4-Eb’ TATAA-luciferase reporter plasmid (67). The RSV-CREB327–VP16 and RSV-Gal4 vectors were kindly provided by Dr. R. Gaynor (68). The CMV-ICER IIγ (45) was kindly provided by Dr. C. Molina. The ATPγs, gadd153/Chop10, ICER IIγ, and bZip of NF-IL6 (amino acid residues 1–154) were cloned by polymerase chain reaction of the respective fragments into the RSV-VP16 vector (68), at the Nco I site, resulting in the construction of bZip-VP16 fusion proteins, as described by Yin et al. (68). Similarly, RSV-X-Gal4 constructs were prepared by inserting the coding region of pX at the Nco I site, resulting in fusion proteins with the Gal4 DNA-binding domain (amino acids 1–147) at its C terminus. 5 μg of reporter plasmid, 5 × Gal4-Eb’ TATAA-luciferase, were cotransfected with 5–10 μg of each of the four plasmids. Transfections were performed in duplicates (60-mm plates) and repeated a minimum of three times.

RESULTS

Direct Protein-Protein Interactions of HBV x Protein with bZip Proteins—Interactions between pX and CREB were demonstrated by enhanced CREB binding to the CRE site in the presence of pX (1, 29) and by altered methylation interference assays (1). In the present study we employed the CREB/pX-interacting proteins as the model system for developing an assay to detect direct protein-protein interactions. For this analysis the full-length X protein was produced as a fusion with glutathione S-transferase (61). The GST-X1–154 fusion protein is selectively retained by glutathione-Sepharose 4B resin and thus the complex provides a suitable affinity resin.

Recombinant CREB327 (69, 70), 32P-radiolabeled (66), was employed to establish the in vitro conditions for detecting specific binding of CREB327 to the resin-immobilized GST-X1–154. We observe selective binding of 32P-CREB327 to GST-X1–154 but no binding to the control GST-resin (Fig. 1A). Additional control experiments include the following: first, the demonstration that under the conditions detecting CREB/pX interactions (Fig. 1A), transcription factors of a different class, namely Gal41–147 (71) and MyoD (64), did not display detectable binding to GST-X1–154 (Fig. 1, B and C); and second, the demonstration that the interaction of CREB327 with GST-X1–154 involved the bZip domain of CREB327. For this analysis, CREB327 and the N-terminal portion of CREB327, amino acid residues 1–198, were synthesized in vitro (Fig. 2A). In comparative analysis, the N-terminal region of CREB327 did not interact with GST-X1–154 (Fig. 2B), whereas 32P-CREB327 displayed specific binding to GST-X1–154 (Fig. 2C). The results confirm our earlier observations that the bZip of CREB is the interacting target of pX and show that under the established conditions (Fig. 1) we detect specific binding of CREB327 to pX in vitro.

We employed the in vitro protein-protein interaction assay described in Fig. 1A to examine the interaction of bZip transcription factors ATPγs, gadd153/Chop10, NF-IL6, and ICER IIγ with pX. The binding reactions with bZip proteins were carried out exactly as described for CREB327 (Fig. 1A and Fig. 2C).
The Effect of HBV pX on the DNA Binding Potential of bZip Proteins—Gel retardation assays demonstrated that CREB/pX interactions increase the DNA binding affinity of CREB for the CRE site (1). Therefore, we carried out DNA-binding assays to examine the effect of pX on the DNA-binding activity of the bZip proteins (Fig. 3). We incubated the bZip proteins with their cognate radiolabeled DNA-binding site, as a function of the presence or absence of pX. However, pX only potentiates the existing DNA binding activity of bZip proteins. Similarly, the non-bZip transcription factors GAL41–147 (1) and MyoD/E47, which fail to directly interact with pX (Fig. 3), do not acquire enhanced DNA binding by pX. We conclude that pX interacts directly and specifically with bZip transcription factors and increases their DNA binding potential.

Interactions of HBV pX with bZip Transcription Factors in Vivo—To further establish direct interactions between pX and bZip transcription factors in the hepatocyte, we employed the mammalian two-hybrid assay, in the AML12 cell line (65). AML12 cells, derived from transgenic mouse liver, over-express transforming growth factor-α, but otherwise exhibit properties of normal hepatocytes (65). For this analysis we constructed an expression vector containing the activation domain of VP16412–490 in fusion with the C terminus of CREB (68), ICER IIγ, ATF3, and NF-IL6. We also constructed a mammalian expression vector encoding pX in fusion at its C terminus to the DNA-binding domain of Gal41–147. Transient transfections of RSV-X-Gal4 in AML12 cells demonstrated that the pX-Gal4 fusion protein is devoid of detectable transactivation potential (Fig. 5).

Fig. 5 shows the relative amount of reporter expression following cotransfection of both RSV-X-Gal4 and RSV-bZip-VP16 expressing plasmids. With cotransfection of either RSV-CREB-VP16 or RSV-ICER IIγ-VP16 with RSV-X-Gal4 plasmid, we observe a 3- and 6-fold induction of luciferase expression, respectively. Cotransfection of RSV-NF-IL6-VP16 or RSV-ATF3,VP16 with RSV-X-Gal4 induces the expression of luciferase by 4- and 14-fold, respectively. To verify the specificity of our in vivo assay, we employed the pEM-MyoD-VP16 expression construct, kindly provided by Dr. S. Konieczny.3 We observe no detectable interactions occurring in vivo between MyoD and pX by this assay. These observations agree with our in vitro results with MyoD (Figs. 1 and 4). We conclude that in vivo pX interacts directly via protein-protein interactions with the bZip proteins CREB, ICER IIγ, ATF3, and NF-IL6 (Fig. 5).

HBV pX Enhances the Activity of bZip Transcription Factors in Vivo—Having demonstrated that pX interacts directly with bZip proteins in the hepatocyte (Fig. 5), we performed transient transfection assays to assess the effect of these interactions on the transcriptional activity of the aforementioned bZip proteins. Among the bZip proteins, NF-IL6 is a well documented transcriptional activator (73). Earlier studies by Tesmer et al.
(58) identified the cis-acting element located between nucleotide position −158 to −178 on the LTR of HIV as an NF-IL6 binding site. We employed an HIV-LTR-driven reporter, pC15DXE (58), as the model system for analyzing NF-IL6-mediated transcriptional induction. A representative experiment is shown in Fig. 6A. AML12 cells were transfected under low serum conditions in the presence of 10 nM IL-6 (73). Increasing amounts of CMV4-X expressor were transfected in the presence or absence of CMV4-NF-IL6 vector (58). We observe a 3-fold enhancement in HIV-LTR-CAT transcription by pX only in the presence of the NF-IL6 encoding vector, suggesting that pX action augments the transcriptional efficacy of NF-IL6. In contrast, the pX encoding vector failed to demonstrate enhanced transcriptional induction of a MyoD-dependent transcription system (74), indicating that pX enhances transactivation in a specific manner (Fig. 6B).

HBV pX Enhances the Transrepression Potential of bZip Repressors—We have likewise employed transient transfections in AML12 cells to examine the effect of pX on bZip repressor activity. In Fig. 7, CRE3-CAT reporter activity includes the CRE3-CAT reporter plasmid and the ATF3 (45) and ICER IIg (55) expressor vectors. The pX expression vector encodes the nuclear localization sequence (NLS) in fusion with pX (30) which targets pX exclusively in the nucleus. Titration experiments of increasing amounts of bZip repressor were carried out in the presence or absence of CMV4-NF-IL6 vector (58). We observe a 3-fold enhancement in HIV-LTR-CAT transcription by pX only in the presence of the NF-IL6 encoding vector, suggesting that pX action augments the transcriptional efficacy of NF-IL6. In contrast, the pX encoding vector failed to demonstrate enhanced transcriptional induction of a MyoD-dependent transcription system (74), indicating that pX enhances transactivation in a specific manner (Fig. 6B).

The assay system used to monitor the effect of pX on bZip repressor activity includes the CRE3-CAT reporter plasmid and the ATF3 (45) and ICER IIg (55) expressor plasmids. The pX expression vector encodes the nuclear localization sequence (NLS) in fusion with pX (30) which targets pX exclusively in the nucleus. Titrations of increasing amounts of bZip repressor were carried out in the presence or absence of CMV4-NF-IL6 vector (58). We observe a 3-fold enhancement in HIV-LTR-CAT transcription by pX only in the presence of the NF-IL6 encoding vector, suggesting that pX action augments the transcriptional efficacy of NF-IL6. In contrast, the pX encoding vector failed to demonstrate enhanced transcriptional induction of a MyoD-dependent transcription system (74), indicating that pX enhances transactivation in a specific manner (Fig. 6B).
Establish the hepatocyte function. We employed the CREB/pX system (1) to examine the interactions of pX and several bZip transcription factors, especially those that play a role in hepatic gene expression. The assay monitoring ATF3 repression is carried out in the absence of forskolin stimulation; accordingly, pX expression does not enhance the transcriptional efficacy of endogenous CREB, as shown earlier in Fig. 7. Importantly, all the transfection assays contained equal amounts of total expression plasmid DNA, to account for squelching. We observe at lower levels of transfected ATF3 expression (Fig. 8) increased repression efficacy by ATF3 due to pX action.

We conclude that the viral X protein interacts directly in the hepatocyte with bZip transcription factors and effects increased DNA binding potential to their cognate DNA-binding site and, in doing so, brings about augmented transcription or repression efficacy.

**DISCUSSION**

In this study we employed *in vitro* and *in vivo* (cellular) approaches to examine the interactions of pX and several bZip transcription factors, especially those that play a role in hepatocyte function. We employed the CREB/pX system (1) to establish the *in vitro* conditions to detect direct and specific protein-protein interactions (Figs. 1 and 2). We show (Fig. 3) that the bZip proteins ATF3, ICER IIγ, gadd153/Chop10, and NF-IL6 directly interact with pX. Furthermore, the mammalian two-hybrid assay demonstrated direct interactions between pX and the bZip proteins *in vitro* (Fig. 5), confirming our *in vitro* observations. This is the first demonstration of direct interactions between pX and bZip proteins in the cellular environment of the hepatocyte.

The bZip proteins, which are shown to directly interact with pX, are induced in response to environmental cues such as growth factors, cellular stress, and cytokines. ATF3 and ICER IIγ are induced as immediate early genes, in response to conditions of cellular stress (55) and cAMP induction (45), respectively. ATF3 mRNA expression is of particular interest for pX interactions due to its transcriptional induction in the hepatocyte following mechanical or toxin-induced liver injury (55). Importantly, the ATF3 homodimer acts as a repressor of CRE-mediated transcription and forms heterodimers with gadd153/Chop10 (55). Similarly, NF-IL6 is rapidly induced in the liver during the acute phase response (42).

We demonstrate here the functional significance of these bZip/pX interactions by *in vitro* (Fig. 4) and *in vivo* assays (Figs. 5–8). As we demonstrated previously with CREB (1), we observe that pX significantly enhances the DNA-binding potential of these bZip proteins, *in vitro*. Importantly, pX enhances the DNA binding potential of bZip proteins that are capable of DNA binding; it does not convert non-DNA-binding bZip proteins, such as gadd153/Chop10 or ATF3/gadd153/Chop10 heterodimer, to DNA binding.

In the hepatocyte, pX expression enhances the transcriptional efficacy of each of the bZip proteins tested. Employing the HIV-LTR as our model system, we show that the transcriptional activation by NF-IL6 in the hepatocyte is enhanced 3-fold by pX. This is in agreement with our earlier observations (1). Based upon the results of Figs. 3, 4, and 6, we conclude that 1) *in vivo*, NF-IL6 and pX interact directly, 2) these interactions increase the DNA binding affinity of NF-IL6 for its binding site, and 3) this increased affinity increases the transcriptional efficacy of NF-IL6 by pX.

Regarding the transactivating effect of pX on the HIV-LTR, it has been demonstrated (18, 21, 75) that pX requires multiple cis-acting elements (18) for full transactivation, including the sequences containing the NF-IL6-binding site I at positions −158 to −178 of the HIV-LTR (18). Although there is no direct evidence demonstrating HIV infection in hepatocytes *in vivo*, HIV DNA has been detected in CD4+ T lymphocytes and monocytes (76). Our results are consistent with the possibility that the NF-IL6/pX interactions in T lymphocytes lead to higher HIV expression, contributing to the observed synergy between HIV and HBV infections (76). Recent studies have explored the mechanisms of NF-IL6 activation of HIV-LTR in monocytic U937 cells (77, 78). However, the effect of NF-IL6/pX interactions in lymphocytic or monocytic cell culture systems is not yet understood.

Importantly, pX also enhances the repression efficacy of bZip repressors ATF3 and ICER IIγ. We interpret the results of the
in vitro (Figs. 3 and 4) and in vivo (Figs. 7 and 8) assays to mean that direct interactions between pX and bZip repressors, ICER II and ATF3, increase their DNA binding affinity, thus resulting in increased repression efficacy by pX.

This is the first report describing the involvement of pX in enhancing transcriptional repression of genes responsive to bZip repressors ATF3 and ICER II. The implication of this observation is that pX, by targeting bZip transcription factors, can potentiate not only specific transcriptional activation but also transcriptional repression of cellular genes. In light of the recent observation that ATF3 is transcriptionally induced in injured liver (55), our observation is of importance and significance for hepatocyte physiology.

It is well established that CREB/ATF and possibly C/EBP proteins are required for the activation of transcription from the viral HBV enhancer I (79). The effect of the aforementioned inducible bZip proteins on the interaction of CREB with pX in the activation of HBV enhancer I-mediated transcription is unknown. Competition of the inducible bZip proteins with the constitutive CREB for interaction with pX may have direct implications in the progression of the viral infection. We are currently investigating the role of pX in the nucleus in mediating CREB/pX and/or bZip/pX interactions during hepatocyte growth and hepatocarcinogenesis.

In conclusion, the results presented in this study support the hypothesis that pX acts as an enhancer of the DNA binding potential of bZip transcription factors, thereby increasing their transcriptional activation or repression of cellular and/or viral...
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HBV pathogenesis and may, in turn, provide new insights...
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