The Hepatitis B Virus X-associated Protein, XAP3, Is a Protein Kinase C-binding Protein*

(Received for publication, November 14, 1996, and in revised form, March 31, 1997)

Yu-Sheng Cong, Ya-Li Yao, Wen-Ming Yang, Nadarajan Kuzhandaivelu, and Edward Seto†‡

From the Moffitt Cancer Center & Research Institute, Department of Medical Microbiology/Immunology, College of Medicine, University of South Florida, Tampa, Florida 33612

The hepatitis B virus X protein induces transcriptional activation of a wide variety of viral and cellular genes. In addition to its ability to interact directly with many nuclear transcription factors, several reports indicate that the X protein stimulates different cytoplasmic kinase signal cascades. Using the yeast two-hybrid screen, we have isolated a clone designated X-associated protein 3 (XAP3) that encodes a human homolog of the rat protein kinase C-binding protein. One of the activation domains of X (amino acids 90–122) is required for binding to XAP3, while the NH2-terminal part of XAP3 is necessary for binding to X. Both X and XAP3 bound specifically to the γPKC isoenzyme synthesized in rabbit reticulocyte lysates. Overexpression of XAP3 enhanced X transactivation activity. These results support earlier findings that one of the mechanisms of transactivation by X is through involvement with the cellular protein kinase C pathway.

Transcriptional activation is a widespread phenomenon among mammalian viral systems. Mammalian viral proteins that increase the rate of transcription can be divided into two groups based on whether they exhibit sequence-specific DNA binding. For example, the herpes simplex virus 1 (HSV-1) Vmw175 (1, 2), the Epstein-Barr virus BZLF1 (3), the papilloma virus E2 (4), and the simian virus 40 and polyoma virus large T antigens (5) bind specific DNA sequence motifs, whereas HSV-1 VP16 (6, 7), the pseudorabies virus immediate early protein (8, 9), and the adenovirus E1A protein (10, 11) do not. During the last decade, many studies have shown that non-DNA binding viral transactivators achieve their task by direct interaction with different cellular sequence-specific DNA binding transcription factors. For example, VP16 interacts with the Oct-1 protein, thereby positioning the VP16 activating domain at a promoter to enhance transcription (7, 12–17). Similarly, E1A interacts with a number of cellular proteins including ATF-2 (18).

The hepatitis B virus (HBV) X protein is a promiscuous transcriptional transactivator (reviewed in Ref. 19). This conclusion is derived from a large number of studies using mostly transient cotransfection of the bacterial chloramphenicol acetyltransferase (CAT) gene under control of a potential target promoter/enhancer and the X gene under the control of a heterologous promoter in mammalian cells. Induction of transcription by X usually ranges from 2- to 20-fold depending on the target promoter and cell type; whether this transactivation activity contributes to viral function, however, remains to be determined. Fusion of the X protein to the DNA-binding domain of the bacterial LexA repressor resulted in a protein that can activate transcription from a reporter plasmid bearing lexA operator sequences fused to a minimal promoter (20). Similarly, fusion of the X protein to the DNA-binding domain of transcription factor C/EBP increased the ability of X to activate a reporter containing C/EBP binding sites (21). Attempts to demonstrate sequence-specific DNA binding by the X protein so far have not been successful, and, therefore, it is believed that the X protein belongs to the non-DNA binding viral transactivator family, which is brought into a transcription complex by association with cellular DNA-binding factors. So far, a number of transcription factors, including ATF-2 (22), CREB (22, 23), RPB5 (24), TATA-binding protein (25), and p53 (26), have been shown to interact with the X protein. However, unlike most viral activators, X appears to operate through additional mechanisms. For example, X appears to function as a serine protease inhibitor and regulates the turnover of different cellular transcription factors (27). Additionally, increasing evidence suggests that X may use signal transduction pathways to activate transcription. In this regard, both the protein kinase C (PKC) signaling pathway and mitogen-activated kinases have been shown to be involved in X-mediated transactivation (28–34).

To understand the mechanism of X transactivation, we and others have previously used the yeast two-hybrid screen to identify cellular proteins that interact with X (35, 36). Among the clones analyzed were genes encoding a human homolog of a DNA repair protein (XAP1) (35) and a cellular inhibitor of X (XAP2) (36). We have now sequenced and characterized an additional clone from our two-hybrid screen for encoded proteins capable of binding X. One protein, term XAP3, appears to have strong homology to a rat PKC-binding protein.

PKC is a large family of phospholipid-dependent kinases involved in cell growth, differentiation, and carcinogenesis (37–41). The mammalian PKC enzyme family consists of at least 10 members that are divided into three groups based on enzymatic properties and common structural features (reviewed in Refs. 42 and 43). The group A (cPKC) (α, βI, βII, and γ) are calcium-dependent kinases whose activities are stimulated by diacylglycerol (DAG) or phorbol esters. The group B (nPKC) (δ, ε, η, and θ) and group C (aPKC) (ζ and η) are different from those of

* This work was supported by National Cancer Institute Grant R01-CA61257, National Science Foundation Grant MCB-9631067, and American Cancer Society Grant F97USF-1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Molecular Oncology Program, Moffitt Cancer Center & Research Institute, University of South Florida, 12902 Magnolia Dr., Tampa, FL 33612. Tel.: 813-979-6754; Fax: 813-979-6700 or 813-979-3893; E-mail: setoe@moffitt.usf.edu.

‡ The abbreviations used are: HSV-1, herpes simplex virus 1; HBV, hepatitis B virus; CAT, chloramphenicol acetyltransferase; PKC, protein kinase C; HTLV-1, human T-cell lymphotropic virus type 1; DBD, DNA-binding domain; ONPG, o-nitrophenyl-β-D-galactoside; DAG, diacylglycerol; GST, glutathione S-transferase.
group A in their regulatory domains in that a putative Ca$^{2+}$-binding region is absent. The group C PKCs are different from groups A and B in that they possess a single cysteine-rich Zn$^{2+}$ finger motif in the conserved C1 region. The group B PKCs are calcium-independent but can be stimulated by DAG, whereas the group C PKCs are neither calcium- nor DAG-dependent. A number of proteins have previously been identified that associate with PKC (44–48). Some of these PKC-binding proteins serve as substrates for PKC, while interaction of the various PKC isoforms with other cellular proteins may confer the unique localization of each of the PKC enzymes. Recently, it was demonstrated that the human T-cell lymphotropic virus type 1 (HTLV-1) transactivator Tax stimulates PKC translocation to the particulate cellular membrane fraction, suggesting that Tax activates PKC in vivo (49). Further, Tax bound specifically to the α, δ, and η PKC isoenzymes, and the addition of Tax to in vitro kinase reaction mixtures led to the phosphorylation of Tax and an increase in the autophosphorylation of PKC.

In this report, we demonstrate that X binds XAP3 in vivo and in vitro. Second, we found that X and XAP3 can both interact with PKCγ. Finally, we show that an overexpression of XAP3 enhances X transactivation. These results strongly suggest the involvement of PKC as one of the mechanisms in X transactivation.

**MATERIALS AND METHODS**

**Plasmids**—The following plasmids have been described previously. pGSTM-X (36) contains the entire HBV-X ORF subcloned into the pG-Tag vector (50) in-frame with the glutathione S-transferase (GST) polypeptide. pAS-X (36) contains the HBV (subtype adw2; Ref. 51) fragment from nucleotide 1375 to 1853 (Neo/I-α/III) in the pGущDNA-binding domain (DBD)-tagged plasmid, pASI (52). This plasmid expresses a fusion protein containing the Gal4DBD and the full-length wild type X protein, pSP72-PKCa, pSP72-PKγ, and pSP72-PKδ (49) contain the different PKC isomor cDNAs under the control of T7 or SPF phage promoters. pECE-X (53) contains the HBV fragment from nucleotide 1355 to 1897 in the SV40-derived expression vector pCE (54). Reporter plasmids pRVSCT (55) and psv2CAT (56) contain the Rous sarcoma virus and simian virus 40 promoter/enhancers upstream of the CAT gene.

Plasmid pGEM-XAP3 was constructed by subcloning an Xhol fragment from the X4 clone into the X site of a modified pGEM7Zf plasmid (pGEM7Zf-x-3X; Ref. 57) such that XAP3 mRNA is expressed from a T7 promoter. Different XAP3 mutants were generated by restriction enzyme digestion and religation of the pGEM-XAP3 plasmid. pGSTM-TBP was constructed by inserting the TAT-binding protein 1 cDNA (58) into the pGущDNA-binding domain (DBD) vector (pGущDNA-HIV, A) containing the TAT-binding protein 1 cDNA. Different X deletion mutants in pAS1 vector were constructed by first subcloning the X cDNA fragment from clone X4 into the pGущDNA-BglI site of a modified pGEM7Zf vector (Ref. 57) such that XAP3 mRNA is translated, 35S-labeled, and sequences were determined. The final sequence was determined (56) in 30-min reactions. All transfections were done using 0.5 μg of XAP3 cDNA and 0.1 μg of XAP3 plasmid. After sonication in STE buffer (10 mM Tris-HCl (pH 8.0) and resuspended in lysis buffer (50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% sodium dodecyl sulfate (SDS)). After addition of acid-washed glass beads (Sigma), samples were vortexed and cell extract recovered. Ten-ninth of each sample was resolved on a 12.5% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membrane. After blocking with nonfat dried milk, the membrane was treated with 1:1000 diluted G4X-binding domain polyclonal antiserum (Santa Cruz Biotechnology) followed by 1:750 diluted alkaline phosphatase-conjugated rabbit-anti-mouse IgG. Subsequently, the blots were developed with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside for 4 h after the addition of DNA phosphate-conjugated rabbit anti-mouse IgG. Subsequently, the blots were developed with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside for 4 h after the addition of DNA phosphate-conjugated rabbit anti-mouse IgG. Subsequently, the blots were developed with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside for 4 h after the addition of DNA phosphate-conjugated rabbit anti-mouse IgG. Subsequently, the blots were developed with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside for 4 h after the addition of DNA phosphate-conjugated rabbit anti-mouse IgG. Subsequently, the blots were developed with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside for 4 h after the addition of DNA phosphate-conjugated rabbit anti-mouse IgG. Subsequently, the blots were developed with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside for 4 h after the addition of DNA phosphate-conjugated rabbit anti-mouse IgG. Subsequently, the blots were developed with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside for 4 h after the addition of DNA phosphate-conjugated rabbit anti-mouse IgG. Subsequently, the blots were developed with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside for 4 h after the addition of DNA phosphate-conjugated rabbit anti-mouse IgG.

**RESULTS**

**Sequence Analysis of a cDNA Clone Encoding an X-associated Protein**—Previously, we have used the yeast two-hybrid network service.

**β-Galactosidase Assays**—Filter lift assays were performed essentially as described (62). Briefly, transformants were allowed to grow at 30 °C for 2–4 days, transferred onto nitrocellulose filters, and frozen under liquid nitrogen. Filters were then incubated with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside incubated at 30 °C, and checked periodically for production of blue color.

For quantitation of β-galactosidase activity in yeast, liquid culture assays were done using o-nitrophenyl-β-D-galactoside (ONPG) as described (63). Individual transformants were inoculated into the appropriate medium and incubated at 30 °C until the cultures reached midlog phase (A500 of 1.0). For each culture, 0.1 ml of culture was mixed with 0.9 ml of Z buffer (60 mM NaHPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, and 50 mM β-mercaptoethanol). The cells were permeabilized with 0.05 ml of CHCl3 and 0.05 ml of 0.1% sodium dodecyl sulfate (SDS). After addition of 0.2 ml of ONPG solution (4 mg/ml ONPG in 0.1 M phosphate buffer, pH 7.0), reactions were incubated at 30 °C for 1 h and quenched by the addition of 1 ml of 1 N Na2CO3. β-Galactosidase activities were determined by measuring absorbances at 420 nm.

**Western Blot Analysis**—Standard protocols were followed (64). For each sample, yeast transformants were grown at 30 °C in 1 ml of selective SC medium containing 2% dextrose to an A500 of 1–2. Cells were collected by centrifugation, and lysates were prepared according to a standard protocol (61). Briefly, lysates were washed in ice-cold 50 mM Tris-HCl (pH 8.0) and resuspended in lysis buffer (50 mM Tris-HCl (pH 8.0, 0.1% Triton X-100, and 0.5% SDS) containing 1 mg phenylmethylsulfonyl fluoride, 1 μg/ml of pepstatin A, and 1 μg/ml leupeptin. After addition of acid-washed glass beads (Sigma), samples were vortexed and cell extract recovered. Ten-ninth of each sample was resolved on a 12.5% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membrane. After blocking with nonfat dried milk, the membrane was treated with 1:1000 diluted G4X-binding domain polyclonal antiserum (Santa Cruz Biotechnology) followed by 1:750 diluted alkaline phosphatase-conjugated rabbit anti-mouse IgG. Subsequently, the blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

**Purification of RNA and Northern Blots**—Total RNA was purified using acid guanidinium thiocyanate-phenol-chloroform extraction method (65), separated on a formaldehyde agarose gel, and transferred onto a Hybond membrane (Amersham). Multiple human tissue Northern blots were obtained from CLONTECH. Prehybridization, hybridization, and high stringency washes were performed as described (61). To control for the relative amount of RNA in each lane, after hybridization with XAP3, the blots were stripped by incubation in 0.5% SDS at 95 °C, and reprobed with the human β-actin cDNA.

**Transfection and CAT Assay**—HepG2 cells were grown in minimal essential medium supplemented with 10% fetal bovine serum. Transfections were done using the calcium phosphate method (66), which included a 1-min glycerol shock 4 h after the addition of DNA phosphate. Forty-eight h after transfection, cells were harvested and CAT activity was determined (56) in 30-min reactions. All transfections were normalized to equal amounts of DNA with pECE or pcDNA/Amp plasmids.
screen to detect human cDNAs coding for products that interact with the X protein (36). Of 5.6 × 10⁶ colonies screened, 12 of the clones were active only when cotransformed with plasmid encoding Gal4DBD fused to X, but not to Rb, SNF1, or several unrelated transcription factors (36). Preliminary DNA sequence analysis revealed seven novel sequences and five that were highly homologous to known sequences. Of the seven novel sequences, six of them were overlapping clones of cDNA encoding XAP2 (36). Of the five known sequences, we have now completely sequenced one cDNA insert designated X4 (we will refer to the product encoded by X4 as X-associated protein-3 (XAP3)).

X Binds XAP3 Specifically in Vitro—To determine whether XAP3 binds X in vitro, we expressed the X protein as a fusion to GST and used it to test for its ability to bind specifically to in vitro 35S-labeled XAP3. As shown in Fig. 1, XAP3 binds to GST-X but not GST (lanes 4 and 6). An unrelated protein, TBP-1, was used as an additional negative control; and, as expected, TBP-1 did not bind GST-X in this assay (lane 7).

Taken together, this suggests that X and XAP3 interact both in vivo and in vitro and the interaction is specific.

Amino Acids 90–122 of X Are Required for Binding to XAP3—The X protein sequences required for binding XAP3 were examined to determine whether they coincided with previously defined activation or regulatory domains. A series of carboxyl-terminal X deletion mutants, as well as internal deletion mutants previously generated and subcloned into pAS1 were tested for their abilities to interact with XAP3 in the two-hybrid system. As shown in Fig. 2A, deletion of the X protein carboxyl-terminal from amino acids 122–154 had no effect on β-galactosidase activities, as measured by filter lift or liquid β-galactosidase assays. However, deletion of amino acids 109–154 eliminated β-galactosidase activities. Further amino-terminal and internal deletions revealed that amino acids 90–122 of X were important for binding XAP3. Western blot analysis indicated that mutants which did not bind XAP3 were expressed efficiently in yeast cells (Fig. 2B), indicating that the loss of binding property of these mutants is not a reflection of loss in protein expressions.

Isolation and Analysis of a Full-length XAP3 cDNA—The predicted amino acid sequence of XAP3 was determined by theoretical translation of the cDNA clone open reading frame.

Since the predicted open reading frame of the X4 cDNA clone isolated from the two-hybrid screen remains open at the 5’ side (beginning at nucleotide 559, Fig. 3A), we initially suspected that this clone represented only a partial XAP3 coding sequence. To obtain a full-length XAP3 cDNA, a λgt11 HeLa cDNA library was screened with a radiolabeled probe corresponding to the 5’ end of clone X4. The complete DNA sequence of the newly isolated clone is illustrated in Fig. 3A. Analysis of the predicted amino acid sequence of XAP3 revealed an open reading frame of 468 amino acids (1.4 kilobases) with an in-frame stop codon upstream of the first methionine at nucleotide 540 (Fig. 3A). This indicated that the original X4 clone did, in fact, contain a full-length XAP3 coding region. Sequence motif searches indicate that XAP3 contains a C3HC4 zinc finger (amino acids 240–284) and three PKC substrate recognition sites (amino acids 32–34, amino acids 41–43, and amino acids 116–119). As shown in Fig. 3B, sequence homology searches revealed that XAP3 shares 76% identity and 85% similarity to a rat PKC-binding protein.²

Northern Blot Analysis of XAP3 mRNA—In an attempt to uncover the functional significance of X and XAP3 interaction,
FIG. 3. DNA and amino acid sequence of XAP3. A, the entire nucleotide sequence and the deduced amino acid sequence of the XAP3 cDNA. The amino acid sequences underlined indicate a C3HC4 RING finger motif; asterisks (*) indicate deduced stop codon for translation. B, sequence similarity between XAP3 and a rat PKC-binding protein (rPKCBP). Similar amino acids (;) and identical amino acids (u) are indicated.
Northern blot analysis was performed to determine the expression pattern of XAP3. We found that a message of approximately 2.5–3.0 kilobases was present in high levels in human brain, placenta, and pancreas; intermediate levels in the heart and skeletal muscle; and low levels in the lung, liver, and kidney (Fig. 4).

X-binding Domain in XAP3—To localize a domain within XAP3 that binds the X protein, we in vitro synthesized different 35S-labeled XAP3 mutants and used them to test for binding to GST-X. As shown in Fig. 5, carboxyl-terminal deletion of amino acids 212–468 or internal deletions of amino acids 97–262 or 123–347 had little or no effect on the binding of XAP3 to the X protein (lanes 3–10, 12, and 13). However, deletion of amino acids 13–74 completely eliminated XAP3’s ability to bind X (lane 11). Taken together, these data suggest that the amino-terminal part of the XAP3 protein (amino acids 13–74 in particular) is essential for binding to the X protein. This finding is consistent with the observation that amino acids 13 to 74 is included in the original X4 clone derived from the initial two-hybrid screen.

X and XAP3 Interact with PKC in Vitro—GST binding assays were performed to analyze X protein binding to 35S-labeled PKC isoenzymes translated in vitro with rabbit reticulocyte lysates. Equal quantities of bacterial expressed GST or GST-X fusion protein coupled to glutathione-Sepharose beads were incubated with PKCα, PKCγ, or PKCδ. Interestingly, PKCγ but not PKCα or PKCδ bound specifically to GST-X but not to GST (Fig. 6, lanes 4–9).

To determine which PKC isoenzyme XAP3 binds, similar experiments were performed using GST-XAP3 fusion protein. Intriguingly, like the X protein, XAP3 also bound specifically to PKCγ but not PKCα or PKCδ (lanes 10–12). This result, therefore, suggests a functional significance of the interaction between X and XAP3.

Stimulation of X Transactivation by XAP3—To determine the role of XAP3 in X transactivation, we tested the ability of XAP3 to influence transcription directed by an X-responsive promoter in the presence of the X protein. We constructed a plasmid that expresses XAP3 under the control of the human cytomegalovirus immediate early promoter, cotransfected it into HepG2 cells together with a plasmid that expresses the X protein and a reporter. While the expression of XAP3 had no effect on the reporter plasmid (Fig. 7A, lane 2), the X protein activated the Rous sarcoma virus promoter in HepG2 cells about 10-fold as expected (Fig. 7A, compare lanes 1 and 3). Interestingly, when XAP3 expression plasmid was added to the transfections, transcriptional activation by X was further enhanced 7-fold (Fig. 7A, compare lanes 3 and 4). This enhance-
HBV X Interacts with a PKC-binding Protein

A, representative CAT assays with RSVCAT or SV2CAT reporter plasmids (10 μg each) transfected into HepG2 cells in the presence or absence of plasmids encoding X (3 μg) and XAP3 (10 μg). B, CAT assay results with Rous sarcoma virus promoter CAT construct (10 μg each) transfected into HepG2 cells in the presence of the indicated amount of XAP3 and X expression plasmids. The extent of acetylation in various reactions was determined relative to that for the CAT reporter, and results are presented as the mean of three independent transfections with standard deviations. C, comparison of XAP3 expression in transfected HepG2 cells with Northern blot.

Fig. 7. Enhancement of X transactivation by the XAP3 protein.

A probe to demonstrate that the increase in activity seen in cotransfection of X and XAP3 is due to overexpression of XAP3. As shown in Fig. 7C, XAP3 RNA recovered from HepG2 cells after transfection with X and XAP3 expression plasmids (lane 4) was much higher than from nontransfected cells (lane 1) or cells transfected with plasmids expressing either XAP3 or X alone (lanes 2 and 3, respectively).

DISCUSSION

The identification and characterization of proteins in a cell with which a given protein interacts is often helpful for understanding the function and mechanisms of action of that protein. To gain insight into the function and mechanisms of the X protein, we and others have previously attempted to identify cellular proteins that interact with X. Not surprisingly, many of these X-associated proteins identified were nuclear transcription factors. Therefore, like many viral transcription activators, X may be directed to a promoter to activate transcription by interaction with these nuclear factors. Additionally, X might activate transcription by increasing the activity of these X-associated factors. However, there is ample evidence that the X protein may function by additional mechanisms. In particular, there are well documented reports that the X protein acts through cytoplasmic signaling pathways involving the serine/threonine kinases, PKC, and Raf-1. Our current finding that X interacts with a PKC-binding protein, XAP3, strengthens previous studies suggesting that X is a dual cytoplasmic activator of signal transduction and nuclear activator of transcription factors (32).

X and XAP3 interaction occurred in yeast cells, as well as in a cell-free extract. A detailed analysis of X mutants indicated that the interaction with XAP3 occurred through X residues 90–122. Segments of the X protein required for transactivation have been analyzed previously by many laboratories (67–73). Although there are some disagreements on the exact amino acids essential for X transactivation, most reports suggest that amino acids 105–115 are required for X transactivation function. Therefore, it appears that XAP3 binding correlates with X transactivating activity. Our result is also consistent with an earlier study suggesting that amino acids 105–115 of the X protein interact with cellular factors (74).

XAP3 contains a C3HC4 (RING finger) zinc-binding domain that is also present in a number of other transcription factors and proto-oncogenes (reviewed in Ref. 75). Currently, the exact function of the RING finger motif is unclear. Although RING finger-containing proteins, such as BRCA-1 (76) and PML (77), have been strongly implicated in cell growth regulation and transcription, extensive efforts to identify sequence-specific DNA binding activity for such domains have been unsuccessful, and it appears most likely that RING finger domains participate in protein-protein interactions. Our data suggest that X and XAP3 interaction occurs through the extreme amino-terminal region of XAP3, outside the RING finger domain. Thus, it is possible that XAP3 uses its RING finger domain to interact with PKC or other as yet unidentified proteins.

Previous studies suggest that the HTLV-1 transactivator Tax protein binds specifically to the α, δ, and η PKC isoenzymes (49). Here, we demonstrate that X and XAP3 interact selectively with PKCζ, suggesting that the three proteins may form a ternary complex in vivo. It is not known at this time whether X can directly bind PKCζ, since the extract used for the binding assay probably contains XAP3. In addition to the three PKC isoenzymes we have tested, there exist at least six other PKC subunits. Currently, we do not know whether these other subunits also bind X and XAP3 or whether they are important for the regulation of transcription by X. Our results showing interaction of one of the PKC isoenzymes with X and...
HBV X Interacts with a PKC-binding Protein

XAP3 do not exclude the possibility that other isoenzymes may also be important for X activation. Further experiments will address these issues.

How might X-XAP3 interaction influence X’s ability to activate transcription? An inspection of the X protein sequence revealed that it possess recognition motifs for PKC (78, 79) (amino acids 54–56 and 75–77), and by metabolic labeling, it has been shown that the X protein is capable of being phosphorylated (80). Therefore, it is tempting to speculate that XAP3 may serve as a bridging protein connecting X and PKC, which then facilitates the phosphorylation of X. The consequence of X phosphorylation could be a redistribution of the X protein to a different cellular compartment, resulting in up-regulation of transcription. This would be similar to the transcription factor NF-kB whose ability to activate transcription is regulated by phosphorylation and association and dissociation with the cytoplasmically located inhibitor IxB (81, 82). Our data showing that overexpression of XAP3 enhances X transactivation fits well with this model. Studies are now under way to determine the ability of PKC to phosphorylate X in the presence of XAP3.

Another possibility is that perhaps by interaction with XAP3, X is drawn to the signaling cascade to activate PKC. This model should be interpreted with caution, however, as it is only one report that X causes membrane translocation of PKC, elevation of the endogenous PKC activator DAG, and subsequent activation of PKC (29). In contrast, more studies have shown that the X protein actually does not directly activate PKC. Therefore, it is more reasonable to speculate that through interaction with XAP3, X might not directly activate PKC but instead acts on a subsequent step after PKC activation. One possibility is that perhaps by association with XAP3, X can then cause an increase of autophosphorylation of different PKC subunits or a decrease in PKC turnover. Additionally, it is conceivable that XAP3 may facilitate X to target PKC to a particular subcellular compartment such as the nucleus, where PKC can phosphorylate and activate other transcription factors that regulate gene expression.

It is worth mentioning that a casual inspection of the XAP3 sequence revealed regions that are characteristic of transcriptional activation domains (reviewed in Ref. 83): proline-rich (amino acids 122–179; 29%) and glutamine-rich (amino acids 183–228; 26%). This raises an additional possibility that an alternative, although not mutually exclusive, model is that X recruits XAP3 to the same promoter to activate transcription. In this scenario, a DNA sequence-specific binding protein recruits X to a promoter, which in turn recruits XAP3 to the same promoter to activate transcription.

Numerous transcriptional regulatory sequences from heterologous viral and cellular promoters have been reported as targets for transactivation by the X protein. However, in many cases conflicting results have been obtained for the capacity of X to transactivate a certain regulatory sequence. It has been suggested that this discrepancy may be due to differences of cell lines used for each assay, and that X might transactivate through cellular proteins that are cell type-specific (84). Our previous finding (36) that a X-associated factor, XAP2, inhibits X activation, coupled with the current results that XAP3 enhances X activation, may partially explain the cell type-specific activation phenomenon by X. Perhaps different cell types contain different levels of XAP2 and XAP3, and the net effect on X’s transactivation ability is dependent on the delicate concentration of these cellular proteins.

Finally, as mentioned before, PKC is involved in regulation of cell growth, differentiation, and carcinogenesis. Overexpression of wild type or mutant PKC may lead to disordered cell growth and transformation. It will be interesting to further dissect the interaction between X, PKC, and XAP3 and deduce the biological significance of these interactions. In addition to its transactivation ability, X has been shown to transform cells (85–87) and to cause liver cancer in some transgenic mice (88, 89). It is, therefore, possible that X targets some particular PKC isoforms and binding their proteins to form a complex that mediates the control of cell growth.

Acknowledgments—We thank John Brady and Paul Lindholm for PKC plasmid and Denise Cooper, Rich Jove, Nancy Oshawa, and Jerry Wu for discussion and critical reading of the manuscript.

REFERENCES

1. Faber, S. W., and Wilecox, K. W. (1986) Nucleic Acids Res. 14, 6067–6083
2. Michael, N., Spector, D., Marramann-Nazoo, P., Kristie, T. M., and Roizman, B. (1988) Science 239, 1531–1534
3. Farrell, P. J., Rowe, D. T., Rooney, C. M., and Kouzarides, T. (1989) EMBO J. 8, 127–132
4. Spadotto, B. A., Byrne, J. C., and Howley, P. M. (1988) J. Virol. 62, 3143–3150
5. Fried, M., and Prives, C. (1986) Cancer Cells 4, 1–16
6. Kristie, T. M., and Roizman, B. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4065–4069
7. McKeown, J. L. C., Kristie, T. M., and Roizman, B. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7061–7065
8. Ahmayer, S. M., Feldman, L. D., and Roeder, R. G. (1985) Cell 43, 821–829
9. Ahmayer, S. M., Workman, J. L., and Roeder, R. G. (1988) Genes Dev. 2, 542–553
10. Ferguson, B., Krippi, B., Andrisani, O., Jones, N., Westphal, H., and Rosenberg, M. (1985) Cell 40, 2853–2861
11. Chatterjee, P. K., Brunner, M., Flint, S. J., and Harter, M. L. (1988) EMBO J. 7, 835–841
12. Gerster, T., and Roeder, R. G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6347–6351
13. O’Hare, P., and Goding, C. R. (1988) Cell 52, 435–445
14. O’Hare, P., Goding, C. R., and Haigh, A. (1988) EMBO J. 7, 4231–4238
15. Preston, C. M., Frume, M. C., and Campbell, M. E. M. (1988) Cell 52, 425–434
16. Kristie, T. M., LeBowitz, J. H., and Sharp, P. A. (1989) EMBO J. 8, 4229–4238
17. Stern, S., Tanaka, M., and Herr, W. (1989) Nature 341, 624–630
18. Liu, F., and Green, M. R. (1994) Nature 368, 520–525
19. Rossner, M. T. (1992) J. Med. Virol. 36, 101–117
20. Forrester, J. E., Mitchell, P. J., and Yen, T. S. B. (1990) Nature 344, 72–74
21. Unger, T., and Shaul, Y. (1989) EMBO J. 8, 1889–1895
22. Maguire, H. F., Holdifer, J. P., and Siddiqui, A. (1991) Science 252, 842–844
23. Williams, J. S., and Andrisani, O. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3819–3823
24. Cheong, J., Yi, M., and Murakami, S. (1995) EMBO J. 14, 143–150
25. Quadri, I., Maguire, H. F., and Siddiqui, A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1003–1007
26. Feitelson, M. A., Zhu, M., Duan, L. X., and London, W. T. (1993) Oncogene 8, 1109–1117
27. Takada, S., Kido, H., Fukutomi, A., Mori, T., and Kuike, K. (1994) Oncogene 9, 341–348
28. Cross, J. C., Wen, P., and Rutter, W. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8078–8082
29. Kekulf, A. S., Lauer, U., Weiss, L., Lubber, B., and Hofschneider, P. H. (1993) Nature 361, 742–745
30. Lucito, R., and Schneider, R. J. (1992) J. Virol. 66, 983–991
31. Benn, J., and Schneider, R. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10350–10354
32. Dein, K. M., Klein, N., Lucito, R., and Schneider, R. J. (1995) EMBO J. 14, 473–475
33. Benn, J., Su, F., Doria, M., and Schneider, R. J. (1996) J. Virol. 70, 4978–4985
34. Wang, H.-D., Yuh, C.-H., Dang, C.-Y., and Johnson, D. (1995) Mol. Cell. Biol. 15, 6720–6728
35. Lee, T. H., Elledge, S. J., and Butel, J. S. (1995) J. Virol. 69, 1107–1114
36. Kishimoto, A., and Nishizuka, Y. (1989) in Protein Kinase C (Kuo, J. F., ed) pp. 3–15, Oxford University Press, New York
37. Kikkawa, U., and Nishizuka, Y. (1989) J. Biol. Chem. 264, 25534–25540
38. Mochly-Rosen, D. (1994) Semin. Cancer Biol. 5, 277–284
39. Mochly-Rosen, D., Kramer, P. A., and Borek, E. (1995) Biotechniques 13, 866–869
51. Valenzuela, P., Quiroga, M., Zaldívar, J., Gray, W., and Rutter, W. J. (1980) in Animal Virus Genetics (Fields, B. N., Jaenisch, R., and Fox, C. F., eds) pp. 57–70, Academic Press, New York.

52. Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, W. H., and Elledge, S. J. (1993) Genes Dev. 7, 555–569.

53. Seto, E., Yen, T. S. B., Peterlin, B. M., and Ou, J. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8286–8290.

54. Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., and Rutter, W. J. (1986) Cell 45, 721–732.

55. Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I., and Howard, B. H. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6777–6781.

56. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051.

57. Yang, W.-M., Inouye, C., Zeng, Y., Bearss, D., and Seto, E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12845–12850.

58. Nelbock, P., Dillon, P. J., Perkins, A., and Rosen, C. A. (1990) Science 248, 1650–1653.

59. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.

60. Frangioni, J. V., and Neel, B. G. (1993) Anal. Biochem. 210, 179–187.

61. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

62. Breeden, L., and Nasmyth, K. (1985) Cold Spring Harbor Symp. Quant. Biol. 50, 643–640.

63. Guarente, L. (1983) Methods Enzymol. 101, 181–191.

64. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

65. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.

66. Graham, F., and van der Eb, A. (1973) Virology 52, 456–457.

67. Faktor, O., and Shaul, Y. (1990) Oncogene 5, 867–872.

68. Ritter, S. E., Whitten, T. M., Quets, A. T., and Schloemer, R. H. (1991) Virology 182, 841–845.

69. Murakami, S., Cheong, J., and Kaneko, S. (1994) J. Biol. Chem. 269, 15118–15123.

70. Levrero, M., Balsano, C., Natoli, G., Avantaggiati, M. L., and Elfassi, E. (1990) J. Virol. 64, 3082–3086.

71. Balsano, C., Avantaggiati, M. L., Natoli, G., DeMarzio, E., Will, H., Perricaudet, M., and Levrero, M. (1991) Biochem. Biophys. Res. Commun. 176, 983–992.

72. Arii, M., Takada, S., and Koike, K. (1992) Oncogene 7, 397–403.

73. Kumar, V., Jayasuryan, N., and Kumar, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5647–5652.

74. Takada, S., and Koike, K. (1994) Virology 205, 503–510.

75. Berg, J., and Shi, Y. (1996) Science 271, 1081–1085.

76. Miki, Y., Swensen, D., Shattuck-Eidens, P. A., et al. (1994) Science 266, 66–71.

77. Kakizuka, A., Miller, W. H., Umesono, K., Warrell, R. P., Frankel, S. R., Murty, V. V., Dimitrovsky, E., and Evans, R. M. (1991) Cell 66, 663–674.

78. Mahoney, C. W., and Huang, K.-P. (1994) in Protein Kinase C, (Kuo, J. F., ed) pp. 16–63, Oxford University Press, New York.

79. Pearson, R. B., and Kemp, B. E. (1991) Methods Enzymol. 200, 62–81.

80. Scheck, N., Bartenschlager, R., Kuhn, C., and Schaller, H. (1991) Oncogene 6, 1735–1744.

81. Baeuerle, P. A., and Baltimore, D. (1988) Cell 53, 211–217.

82. Baeuerle, P. A., and Baltimore, D. (1988) Science 242, 540–546.

83. Mitchell, P. J., and Tjian, R. (1989) Science 245, 371–378.

84. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

85. Schek, N., Bartenschlager, R., Kuhn, C., and Schaller, H. (1991) Oncogene 6, 1735–1744.

86. Mahoney, C. W., and Huang, K.-P. (1994) in Protein Kinase C, (Kuo, J. F., ed) pp. 16–63, Oxford University Press, New York.

87. Pearson, R. B., and Kemp, B. E. (1991) Methods Enzymol. 200, 62–81.

88. Schek, N., Bartenschlager, R., Kuhn, C., and Schaller, H. (1991) Oncogene 6, 1735–1744.

89. Baeuerle, P. A., and Baltimore, D. (1988) Cell 53, 211–217.

90. Baeuerle, P. A., and Baltimore, D. (1988) Science 242, 540–546.

91. Mitchell, P. J., and Tjian, R. (1989) Science 245, 371–378.

92. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

93. Schek, N., Bartenschlager, R., Kuhn, C., and Schaller, H. (1991) Oncogene 6, 1735–1744.