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HBV X protein interacts with cytoskeletal signaling proteins through SH3 binding

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1. ABSTRACT

The aim of this study was to investigate interactions between cellular SH3-containing proteins and the proline-rich domain in Hepatitis B Virus (HBV) X protein (HBx) The proline-rich domain of HBx (amino acids 19-58) as well as the relevant site-directed mutagenesis (proline to alanine residues) were cloned into pGEX-5X-1 and expressed as GST-PXXP and GST-AXXA probes. Panomics SH3 domain arrays were probed using both GST-PXXP and GST-AXXA to identify potential interacting SH3 domain containing proteins. The specific interactions were confirmed by the immunoprecipitation of the full-length SH3 domain-containing protein. We report here the binding assay which demonstrated interaction between PXXP domain in HBx and the SH3-domain containing proteins, in particular various signaling proteins involved in cytoskeletal reorganization. Our findings were consistent with similar virus-host interactions via SH3 binding for other viruses such as hepatitis C virus (HCV) and human immunodeficiency virus (HIV) Further characterization of the proline-rich binding to SH3 domains could yield important information for the design of novel therapeutic measures against downstream disease causative effects of HBx in the liver cells.

2. INTRODUCTION

HBV affects approximately 4 million individuals worldwide (1) and is studied extensively due to its association with severe liver diseases such as liver cirrhosis and hepatocellular carcinoma (2) HBV viral protein HBx's interaction with various cellular proteins holds the key to understanding how HBV carriers are predisposed to liver diseases. Since the cellular receptor for HBV remains elusive, increased understanding of the viral interactions upon entry and replication serves to broaden our knowledge and allow new therapeutic designs.

HBx, a non-DNA binding protein, has been shown to bind various cellular proteins and implicated in the onset of hepatocellular carcinoma (3, 4) In particular, it has been shown that HBx is responsible for the activation of Src family kinases (5, 6) and prolonged activation, with coupled signaling to Ras, could result in detrimental effects. In addition, HBx have been thoroughly studied for its interaction with p53 and it has been shown that HBx binding can result in an inhibition of p53's downstream cellular processes, such as apoptosis and transcriptional activation (7) HBx plays important role in the regulation of transcription pathways and cellular signaling. Although the

exact mechanism of interaction between HBx and cellular protein remain unclear, we know that HBx may bind cellular proteins and potentially deregulate normal signaling pathways leading to disease.

Amongst the many modular domains found in signaling molecules, Src homology 3 (SH3) domains are widely made use of by the eukaryotic cells for signal transduction. Found in many signal adaptor proteins including Grb2, they function in cellular signaling through their binding of proline-rich (PXXP) motifs of their ligands (8) Although they may show cross-interaction due to the similarity in polyproline sequences found, some degrees of specificity are still displayed (9) Such interactions between viral proteins containing proline rich motifs and cellular proteins with SH3 domains have been previously documented and are critical for increased viral survival and replication (10, 11) Perturbations of such signaling pathways by viral intervention might lead to downstream deleterious effects.

It has been known that non-structural proteins, NS5A and NEF of hepatitis C virus (HCV) and human immunodeficiency virus (HIV) respectively, binds to differential cellular proteins containing SH3 domains through their proline-rich motif (10, 11) We seek to understand if HBx, the non-structural protein of HBV involved in various cellular protein binding, could possess similar characteristic as a SH3 domain ligand. To date, we have identified several proline-rich (PXXP) motifs in the non-structural viral protein, HBx and have identified various interacting partners of cellular proteins containing SH3 domains. Our previous findings have confirmed that these interactions could result in both filopodia formation/ membrane ruffling in transfected cells as well as the delayed cellular adhesion (12, 13) Other SH3 domain-containing cellular proteins which have been identified in our reported binding assay (12, 13) have yet to be characterized and a comprehensive understanding of HBV-host interactions via SH3 binding may serve as important impetus for future therapeutic directions. Here, we report the interaction profiles of various HBx interacting cellular proteins and demonstrated the specificity in binding of HBx to cortactin, a critical actin-binding protein in the cell controlling cellular cytoskeleton network.

3. MATERIALS AND METHODS

3.1. Cell line and plasmids

The human hepatoma cells HepG2 cells (ATCC) were maintained and passaged in Gibco's Minimal Essential Medium Eagle (MEM) (Invitrogen), supplemented with 10% fetal bovine serum (FBS), 1% anti-mycotic (penicillin, streptomycin and amphotericin) at 37^{o}C with controlled CO_2 of 5%. In particular, HepG2 cells were maintained for only 20-30 cycles and passaged at approximately 80% confluency. pGEX-5X-1 was purchased from GE healthcare and used for bacterial expression and fusion protein could be cleaved off from the GST moiety by the use of Factor Xa. Proline-rich region of HBx (a.a. 19 - 58) were amplified from viral HBV DNA (genotype B), extracted from serum samples using the following oligonucleotides: (1) 5'-ACgaattcCGTCCCGTCGGCGCTGAA-3', (2) 5'-CACctcgagTTATTAGTGG TGGTGGTGGTGGTGTCCGCGTAAAGAGAGGTG-3' and cloned in-frame of the GST using restriction enzymes, EcoRI and XhoI.

3.2. Bacterial expression and protein extraction

Constructs of bacterial expression vectors, pGEX were transformed into BL21 (DE3) bacterial strain. Isolated colonies were selected and grown in 5 ml of LB with selective antibiotics overnight at 37^{o}C 250rpm. The cultures were then scaled up 100X accordingly depending on the final volume required. The seeded cultures were then subjected to further growth at 37^{o}C 250 rpm until cell density of OD^{600nm} 0.6 or 1.0 were achieved. 100mM filter-sterilized (0.2µm filter) Isopropyl-beta-D-thiogalactopyranoside (IPTG) (Fermentas) were then added for induction of protein expression (1mM final concentration) The cultures were then incubated at 37^{o}C for a further 4 hours at 250rpm. Protein lysates were then prepared either by Bacterial Protein Extraction Reagent (B-PER) (Pierce) for small scale extraction or by sonication. Basically, large cultures of bacteria (>100ml) were spun down at 5,500rpm for 15min at 4^{o}C, and pellets were resuspended with appropriate amount of lysis buffer (50mM Tris-Cl pH8.0, 150mM NaCl, 1% TritonX-100) supplemented with EDTA-free Complete protease inhibitor (Roche) Cells were then subjected to sonication and clarified at 15,300rpm for 60min at 4^{o}C. The supernatants, containing the soluble protein were then retained for quantification (Bio-Rad's Quick Start Bradford Protein Assay) and use in subsequent experiments.

3.3. GST purification and pull-down

GST-fusion proteins were subjected to purification using Microspin GST-purification modules (GE Healthcare). Essentially, quantified amount of GST-fusion proteins were mixed with the glutathione sepharose 4B beads for 30min with head to end rotation at 4^{o}C. The non-interacting proteins (non-GST fusion) were removed by centrifugation at 3,000rpm for 1min room temperature (R.T.) with 3 washes of ice-cold 1X PBS (600µl) Reduced glutathione were then added and rotated for another 15min 4^{o}C prior to elution of purified sepharose 4B beads. Eventually, the GST-fusion proteins captured by the sepharose 4B beads were then collected by centrifugation at 3,000rpm 1min R.T. The mammalian cell lysates or bacterially expressed proteins for interaction with the bound proteins were then introduced and allowed to interact by subjecting to rotation for 4hours at 4^{o}C. Non-interacting proteins were then removed by centrifugation at 3000rpm 1min. This was then subjected to 3 subsequent washes in 1X PBS/0.005% Tween-20. Finally, elution was performed using reduced glutathione (10mM glutathione in 50mM Tris-Cl pH8.0)

3.4. Panomics SH3 domain arrays

TranSignal SH3 domain arrays were purchased from Panomics Pte Ltd. Essentially, SH3 domains from various known proteins were expressed and spotted in duplicates on the nitrocellulose membrane provided. For

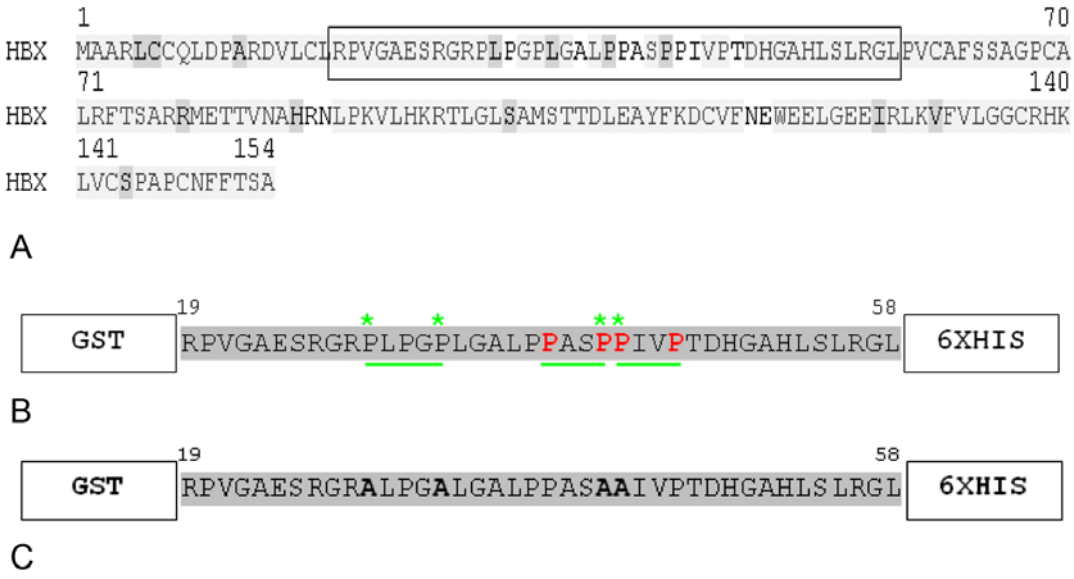

Figure 1. Cloning of proline-rich HBx sequences. (A) The proline-rich hyper variable domain (boxed) of Hepatitis B virus X protein (HBx) Sequences of yellow highlight represent highly conserved amino acids sequences between various genotypes of HBx. (B) The hyper variable proline rich domain (a.a. 19 to a.a. 58) was cloned as fusion protein with GST for use in Panomics SH3 domain array analysis (designated GST-PXXP) Underlined sequences show predicted proline-rich segments of possible multiple PXXP motif sequences. (C) The construct designated GST-AXXA demonstrating point mutations of proline residues (asterisks at positions 29, 33, 42, 43, shown under B) into alanine residues (bold letters)

our probe, GST-fusion proteins were generated as shown in Figure 1B. Essentially, the amino acids sequence of HBx between a.a. 19 and 58 was cloned with an N-terminal GST (pGEX-5X-1) and C-terminal 6X Histidine tag to generate the probe designated GST-PXXP. To explore the potential importance of the proline residues within this region for interaction with cellular proteins, site-directed mutagenesis (QuikChange Site-directed mutagenesis, Stratagene) was carried out as per manufacturer's instructions and specifically, proline residues at position 29, 33, 42 and 43 were mutated to alanine residues to obtain the mutant GST-AXXA probe. Upon bacterial expression, 15µg of the purified GST-fusion proteins (GST-PXXP and GST-AXXA respectively) were incubated with the membrane as per manufacturer's instructions. Non-interacting proteins were removed with the provided wash buffer and interacting proteins were detected by use of anti-Histidine antibodies provided with the membrane. Positive interactors of SH3 domain were identified by the use of SuperSignal West Pico chemiluminescent substrate (Pierce) Controls of the membrane were provided in the form of histidine peptides spotted in duplicates on the right and bottom of the membrane by the manufacturer. Importantly, true positive interaction shows up as duplicated spots.

4. RESULTS

4.1. Proline-rich (PXXP) motif in HBx

In our earlier findings (14), we have identified a variable proline-serine rich region in the non-structural viral HBV protein, HBx. This has provided us with an important clue to understand the differences in proteome profiles generated by transfecting HBx variants. Particularly, the region consists of amino acid residues that resemble that of the PXXP motif, reminiscent of the SH3 domain binding motif. Importantly, the PXXP motifs have been implicated to play a role in viral perturbations of cellular mechanism in the case of closely related hepatitis C virus (HCV) and in addition human immunodeficiency virus (HIV-1) For example, the non-structural HCV NS5A interacts via its C-terminal PXXP motif with Grb2 (11), potentially resulting in attenuation of the mitogenic signaling pathway leading to inhibition of activating protein 1 (AP-1) (15) Likewise, the relatively conserved polyproline sequences in HIV Nef protein was shown to have specific interaction with Hck and Lyn from a tested pool of Src family kinases and these interactions were not observed in protein lacking this PXXP motif (10) The Nef viral protein has in addition been shown to play a role in interaction with Nef-associated kinases (NAK), a serine / threonine kinase. Inoculation of simian immunodeficiency viruses containing specific proline-alanine mutations into *Rhesus Macaques* showed the presence of revertant viruses (16). Hence, the functional importance of the HBx proline-rich region was further investigated in this study.

As shown in Figure 1A, the relatively short HBx (154 amino acids) showed relatively conserved sequences except at the boxed region comprising of the proline-serine rich domain. To further understand the importance of this region, the amino acids (boxed) were cloned in fusion with GST (pGEX-5X-1) with C-terminal 6X histidine residues (used as tag in the binding assay) The partial sequence was selected as it has been previously shown that an 18 a.a. proline-rich sequence of PAK cloned in fusion with GST showed similar binding kinetics to full length PAK for

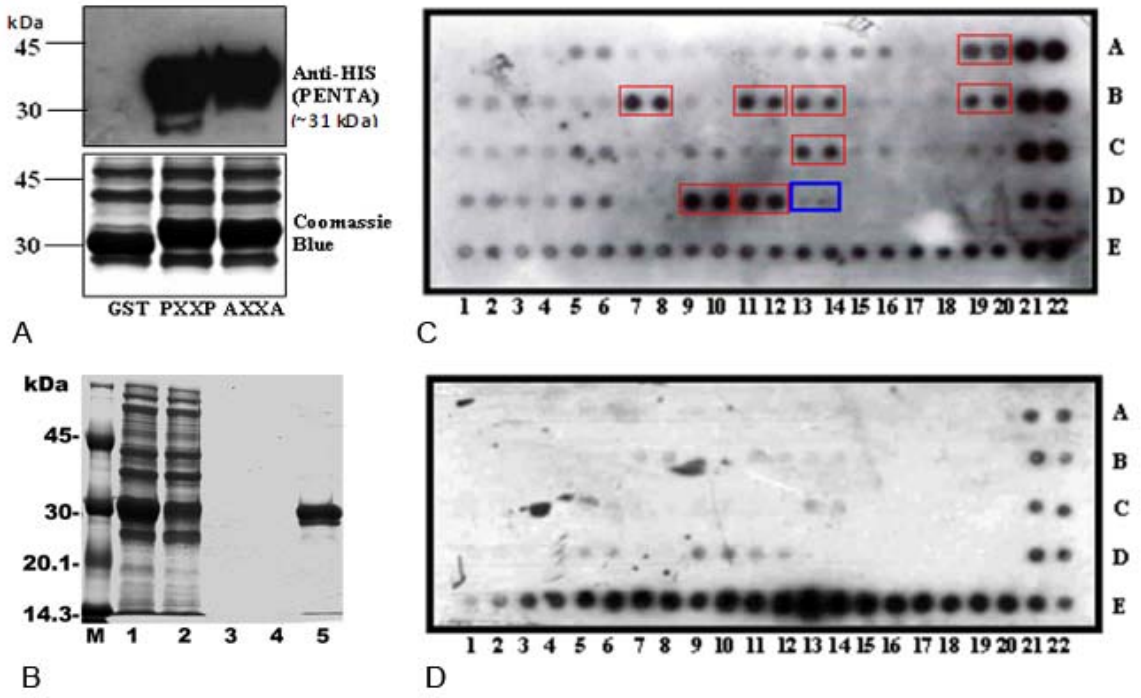

Figure 2. Panomics SH3 domains array interaction with HBx. (A) HBx Proline-rich probe (GST-PXXP) and its alanine mutant (GST-AXXA) were over-expressed and visualized by Coomassie Blue staining (lower panel) The specificity of the expression and the correct size were confirmed by Western analysis using an anti-histidine antiserum (upper panel) (B) Purification of GST-fusion proteins GST-PXXP; GST-AXXA purification was performed accordingly (not shown) Lanes 1, 2, 3, 4 and 5 corresponds to crude lysates, flow through of purification (non GST-fusion proteins), wash 1, wash 2 and purified GST-fusion probes respectively. (C) PXXP showed specific interactive binding to various SH3 domains highlighted in red boxes and showed negative interaction to the GST spots (blue box), a negative control. (D) The specific interaction was verified by the use of the probe with proline-alanine mutant (GST-AXXA)

binding of Nck through the SH3-ligand domain interaction (17) Similar construct was made for the proline rich domain in HBx (Figure 1B), To demonstrate the specificity of these proline residues in HBx interaction with cellular proteins, the proline residues highlighted by asterix show potential proline rich PXXP motifs (GST-PXXP) were mutated to alanine residues (Figure 1C)

4.2. Identification of interacting SH3 domain-containing cellular proteins

To identify cellular proteins that might potentially interact with HBx through the PXXP SH3 domain binding, SH3 domain arrays (Panomics) were incubated with the wild type (Figure 1B) and mutant (Figure 1C) HBx probe. It has previously been used to show the specific binding of Tec family of kinases, Etk, to the proline-rich domain of p53 in response to DNA damage (18) In particular, we subjected our test probes (GST-PXXP) and mutant probes (GST-AXXA) to Panomics SH3 domain arrays I and II as per manufacturer's instructions. The expression of our probes are shown in Figure 2A. Correct in-frame cloning was identified by the detection of the histidine residues using mouse monoclonal anti-HIS antibodies as opposed to that of GST alone without histidines. The protein showed equivalent expression levels in both the GST-PXXP and GST-AXXA clones (Figure 2A top panel) and was subjected to purification using glutathione sepharose 4B as per manufacturer's instructions (Figure 2B) 15 µg of purified probes as observed in Figure 2B lane 5 were then subjected to interactions with the Panomics SH3 domain arrays.

The Panomics nitrocellulose membranes contain immobilized SH3 domains of various identified proteins in duplicates. Positive interactions are indicated by the presence of chemiluminescence in both spots. Negative interactions are shown by no chemiluminescence while one out of the duplicate showing chemiluminescence indicates a false positive interaction. The interaction results of WT GST-PXXP and mutant GST-AXXA probes are demarcated in Figs. 2C and 2D respectively. Interestingly, specific interactions were identified as boxed up in Figure 2C. Negative interaction (GST alone, spots D13, 14) and weak interactors were eliminated in the screen. The positions of the spots D15 to D20 represent unloaded material to show specificity of interactions. Row E and Columns 21, 22 contain histidine peptides spotted for position identity. In the case of binding assay with HBx, the interactions (Figure 2C) were verified by the use of mutant proline-alanine, GST-AXXA on the accompanying membrane of the kit (Figure 2D). Our results therefore suggested that the proline residues of HBx were responsible

Table 1. Interacting SH3 domain-containing proteins

Panomics SH3 domain Array I interacting proteins		
Position	**Domain**	**Full Name of Interacting Proteins**
A (7,8)	Cortactin	Cortactin
A (11,12)	Yes1	Yamaguchi sarcoma virus oncogene homolog 1
A (19,20)	CRK-D2	Avian sarcoma virus CT10 oncogene homolog, domain #2
B (11,12)	*c-Src*	*Cellular Rous Sarcoma viral oncogene homolog*
C (5,6)	Y124	PAK-interacting exchange factor beta *
C (11,12)	RasGAP	Ras GTPase-activating protein 1
D (3,4)	Abl	Abelson tyrosine kinase
D (11,12)	ITSN-D1	Intersectin, SH3 Domain #1
Panomics SH3 domain Array II interacting proteins		
Position	**Domain**	**Full Name of Interacting Proteins**
A (5,6)	Abl2	Abelson-related protein; Arg
A (19,20)	OSF	Osteoclast stimulating factor 1
B (7,8)	Tec	Tyrosine-protein kinase Tec
B (11,12)	PIG2	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma 2
B (13,14)	ARH6	Rho guanine nucleotide exchange factor 6
B (19,20)	EFS	Embryonal Fyn-associated substrate
C (13,14)	RHG4	Rho-GTPase-activating protein 4 *
D (7,8)	VINE-D1	Vinexin, SH3 Domain #1 *
D (9,10)	VINE-D3	Vinexin, SH3 Domain #3
D (11,12)	*c-Src*	*Cellular Rous Sarcoma viral oncogene homolog*

The table summarized the various interacting proteins and their respective positions on the Panomics SH3 domain array. Arrays I and II were used and various interesting cytoskeleton modifying proteins as well as important proteins in signal transduction were shown to interact with the HBx proline-rich region. Importantly, the specificity of the binding was demonstrated by the binding of c-Src by GST-PXXP on both membranes (italic) Asterices show our previously characterized interaction.

for its interaction with SH3 domain-containing cellular proteins. The interacting proteins identified through our screen were summarized in Table 1.

4.3. HBx interacts with cytoskeletal and signal transduction proteins

As shown in Table 1, various cytoskeletal modifying and signal transducing related proteins were identified as HBx-interacting proteins. Specific interaction of HBx proline-rich region was observed with the identification of control c-Src, previously shown to be important in HBV infection (5, 6) Negative controls of GST only (D13,14) and unspotted regions (D15 to D20) denoted the specificity of the interaction screen. Other proteins of interests were cortactin (cortical acting-binding protein), beta-PIX (RhoGEF) and vinexin (cytoskeleton protein) In particular, we have previously characterized the binding of beta-PIX (13) and vinexin beta (12) via this proline-rich SH3 domain interaction (Asterix on Table 1) Importantly, the interaction of HBx proline-rich domain to beta-PIX has been shown to be important in upregulating viral replication via the activation of Rac1. Therefore, our findings are the first report on the myriad of interacting SH3-domain containing proteins with HBx.

4.4. HBx interaction with cortactin mediated by the proline residues

To confirm the interaction of HBx with the SH3 domain-containing cytoskeletal proteins, cortactin which is an actin-binding protein in the cell controlling cellular cytoskeleton network (19) was selected in our further analysis. The full length cortactin was expressed as a GST-fusion protein. The GST moiety was subsequently removed through the use of specific protease, factor Xa to yield full length cortactin (Figure 3A) A GST pulldown assay was conducted where we confirmed the immunoprecipitation of HBx GST-PXXP probe of the full length cortactin (Figure 3B) This specific interaction was not detectable in GST only pulldown analysis and was drastically reduced by the use of the GST-AXXA probe. Our data thus supported the specificity of interaction between HBx and cortactin via SH3 binding.

To further characterize the interaction between HBx and cortactin, we made use of cellular lysates from HepG2 cells (containing endogenous cortactin) and subjected them to GST-pulldown as described in Figure 3B. After GST immunoprecipitation, SDS-PAGE was conducted and proteins were transferred to a nitrocellulose membrane. Western analyses were carried out using rabbit polyclonal anti-cortactin antibody (Santa Cruz) on endogenous cellular cortactin.

As observed in Figure 3C, equivalent amount of probe GST, GST-PXXP and GST-AXXA were bound to glutathione sepharose 4B (Figure 3C, panel Bait) Equivalent amount of cell lysates containing relatively similar amount of endogenous cortactin (Figure 3C, panel flow) was subjected to interaction with the bait. After removal of non-specific interactions, the bound proteins were subjected to Western analyses. As observed, GST-PXXP showed specific immunoprecipitation of mammalian endogenous liver cortactin, in contrast to no interaction observed when GST was used as bait. In addition, the proline residues mutation to alanine in the GST-AXXA bait showed a very drastic reduction of the amount of

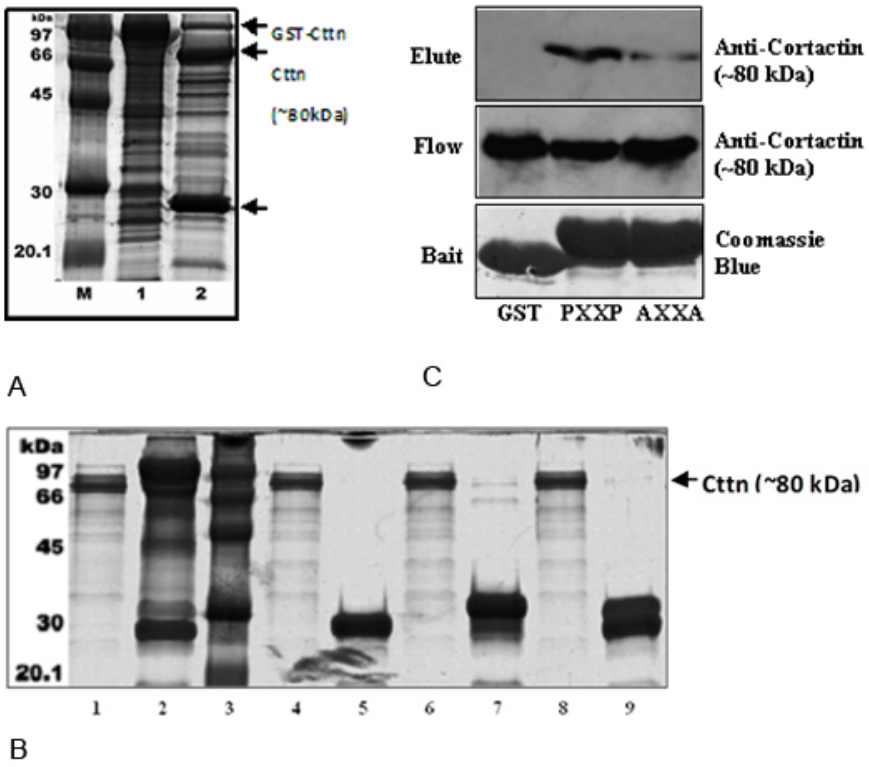

Figure 3. Immunoprecipitation of full-cortactin by HBx. (A) Cortactin was cloned as a GST-fusion protein in pGEX-5X-1 (GST-Cttn) and properly expressed (lane 1) GST moiety was removed (lane 2) to obtain purified cortactin (Cttn) (B) GST and GST-Cttn (undigested) were removed by immunoprecipitation, leaving the lysates with cortactin for use in GST pulldown analysis (lane 1) Lane 2 represents the crude GST-Cttn extract while Lane 2 signifies the protein rainbow marker RPN756 (GE Healthcare) GST (negative control), GST-PXXP and GST-AXXA were first immunoprecipitated. HBx PXXP region binds cortactin (Lane 7) but not GST-AXXA (Lane 9) No binding was observed with GST alone (Lane 5) Lanes 4, 6 and 8 represents the equal loading of Cttn for interaction purposes. (C) GST pulldown system (GST, GST-PXXP and GST-AXXA as bait) for endogenous cortactin using lysates of HepG2 cells were performed. Endogenous cortactin was detected by anti-cortactin antibody.

immunoprecipitated endogenous cortactin, as per that observed in bacterial expressed cortactin (Figure 3B) The interaction of HBx with cortactin may well play a critical role in conjunction with vinexin beta, causing delayed cell adhesion as previously observed by our group (12)

Interestingly, overexpression of cortactin have been identified to be highly associated to HCC formation (20) and was later found to be related to HCC motility and metastasis (21) In addition, cortactin has been proven to be a critical component of the actin cytoskeleton targeted by various bacterial, viral and protozoan pathogens. The SH3 domain of cortactin lies in the C-terminus while the N-terminus acidic domain is important for binding as well as activation of the Arp2/3 complex eventually leading to the assembly of actin networks (22) Therefore, cortactin may act as a mediator of actin cytoskeleton through its interaction with various signaling pathways via its SH3 domain. We propose the potential attenuation of the cytoskeleton of the cell by HBx via SH3-mediated interaction with the myriad of SH3 domain containing proteins. Such proline-rich SH3 domain binding could well be a potential unifying role played by non-structural viral proteins, as shown previously in HIV's Nef protein as well as HCV's NS5A, in conjunction with our findings (12, 13)

5. DISCUSSION

This novel interaction between HBx and cellular proteins demonstrated in our report should shed new insights on the mechanism of HBV-related disease progressions. Varying amino acid sequences in HBx of various genotypes may also play an important role, as intensity of binding to common SH3 domain containing proteins and/or differential myriad of interacting proteins may vary (data not shown) These differences can lead to attenuation of different signaling or cytoskeletal pathways which may explain the clinically observed differential disease outcome associated with viral genotypes (23, 24) Interestingly, it has been recently found that the proline-serine rich domain of HBx binds to N-terminal WW domain (another signaling modular unit) of Pin1, through a C-terminal isomerase domain (25) The binding was found to enhance hepatocarcinogenesis and was suggested to be potentially HBV genotype specific (26)

Artificial SH3 domains have recently been explored as therapeutics against HIV-1 Nef protein. It has been shown to inhibit cellular functions of the Nef protein by interference of Nef binding with their known cellular partners, Pak2 and NFAT (27) Similar strategies could be employed to generate novel therapeutics for the elusive HBV to minimize downstream disease progression in HBV carriers.

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