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Functional and structural similarity between the X protein of hepatitis B virus and nucleoside diphosphate kinases

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Abstract One of the four genes encoded by hepatitis B virus (HBV) is the regulatory 17 kDa protein called HBx (or pX). HBx is a transcription transactivator of many cellular and viral regulatory elements. We report here that recombinant HBx supports transcription in vitro and has phosphotransfer enzymatic activity. In the presence of EDTA, a phosphoryl-HBx is formed that releases the phosphate residue upon the addition of Mg^{2+}. This two-step NTP hydrolysis reaction is characteristic of a group of enzymes termed nucleoside diphosphate kinases (NDPKs). Remarkably, structural similarity between HBx and NDPKs is also evident. Our findings suggest that HBx has evolved from this group of enzymes but acquired additional activities that satisfy the viral needs.

Key words: Hepatitis B virus; X protein; NDPK; Transcription; Protein kinase

1. Introduction

HBV is the causative agent of a wide-spread viral infection which is associated with the development of hepatocellular carcinoma [1]. One of the four genes encoded by the virus is the X protein which has been recently shown to induce liver cancer in transgenic mice [2]. It was previously shown that X is a transcriptional transactivator of a variety of viral and cellular promoter-enhancer units [3,4]. X does not bind directly to DNA but its transactivation was shown to be mediated through the binding sites of different transcriptional regulatory elements like API, AP2 and NFKB binding sites. Furthermore, X was shown to participate in a protein–protein complex with the cellular transcriptional factors CREB and ATF-2 directing them to bind the HBV enhancer element [5]. Our previous biochemical analysis of bacterially expressed HBx have revealed that the protein possesses hydrolytic activity specific for adenine nucleotides with K_m of ~95 mM. This ATPase (dATPase) activity is not DNA-dependent [6]. Here we further characterized the biochemical properties of this protein and found that recombinant HBx has phosphotransfer enzymatic activity in a manner characteristic of a group of enzymes termed nucleoside diphosphate kinases (NDPKs). We show that HBx and NDPKs are partially similar in function and structure.

2. Materials and methods

2.1. Preparation of recombinant proteins

Protein purification and renaturation was done essentially as described [6]. Proteins of *E. coli* expressing rHBx were extracted by lysis of the bacteria followed by DNase I treatment. The recombinant proteins, concentrated in inclusion bodies, were washed three times with 3 M urea and dissolved in 7 M urea and 0.1 M DTT. After dialysis against 0.2 M acetic acid, the proteins were separated by using reverse phase HPLC (C18). GST-X (constructed by using pGEX vector, Pharmacia) was purified from lysed cells under non-denaturing conditions by absorption to glutathione–agarose beads, followed by elution in the presence of free glutathione.

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2.2. In vitro run-on reaction

For in vitro transcription reaction, MLP containing plasmid, linearized with *Sma*I, was incubated for 45 min at 30°C together with creatine phosphate, 4 mM $MgCl_2$, 0.6 mM of each ATP, CTP, and GTP, 10 μM [α-^{32}P]UTP (50 Ci/mmol), and HeLa whole cell extract.

2.3. NTP hydrolysis and NDPK reaction

For nucleotide hydrolysis and NDPK assays, 10 μl reaction mixture consisted of 50 mM Na/HEPES (pH 8.0), 100 mM NaCl, 10 mM DTT, either 10 mM $MgCl_2$ or 5 mM EDTA, and 10 μM of the corresponding non radioactive nucleotide were incubation at 30°C for 30 min. The reaction was stopped by diluting 1 μl of the reaction mixture into 10 μl of 5 mM EDTA, and 1 μl of this final mixture was loaded onto a PEI–cellulose plate. Thin layer chromatography was carried out with 0.75 M KH_2PO_4 (pH 3.65). The plate was autoradiographed.

3. Results

To study the bichemical properties of HBx, efficient expression systems for the synthesis of recombinant X (rHBx) protein were established in *E. coli*. rHBx was prepared and purified (Fig. 1A). We first assayed in vitro the effect of rHBx on transcription of the adenovirus major late promoter (MLP) in a HeLa whole-cell extract. A specific RNA polymerase II transcript (Fig. 1B, lane 1), which is α-amanitin sensitive (lane 2), is detected. The synthesis of this transcript is enhanced by rHBx (lane 3), suggesting that rHBx is functional. A similar effect was obtained with GST-X chimera recombinant protein (data not shown).

Previously we have shown that rHBx has a ribo/deoxy ATPase activity [6]. Here we show that rHBx is weakly phosphorylated in the presence of [γ-^{32}P]ATP and EDTA (Fig. 2A, lanes 1 and 2) but not Mg^{2+}. A shorter mutant of HBx ($rpX^{\Delta 89-117}$), 15 kDa in size (Fig. 2, lanes 7 and 8) behaves in a similar way (lanes 3 and 4), ruling out the possibility that the detected signal is a bacterial contaminant. This was unexpected since protein kinases need Mg^{2+} (or other cations) for their activity and the ion chelator EDTA, inhibits the reaction. Incubation of rHBx with [α-^{32}P]ATP in the presence of Mg^{2+} or EDTA resulted in no ^{32}P incorporation in both cases (data not shown), indicating that the detected signal is not of bound nucleotides. Thus, the observed phosphorylation reaction in the presence of EDTA is distinct from common S/T protein

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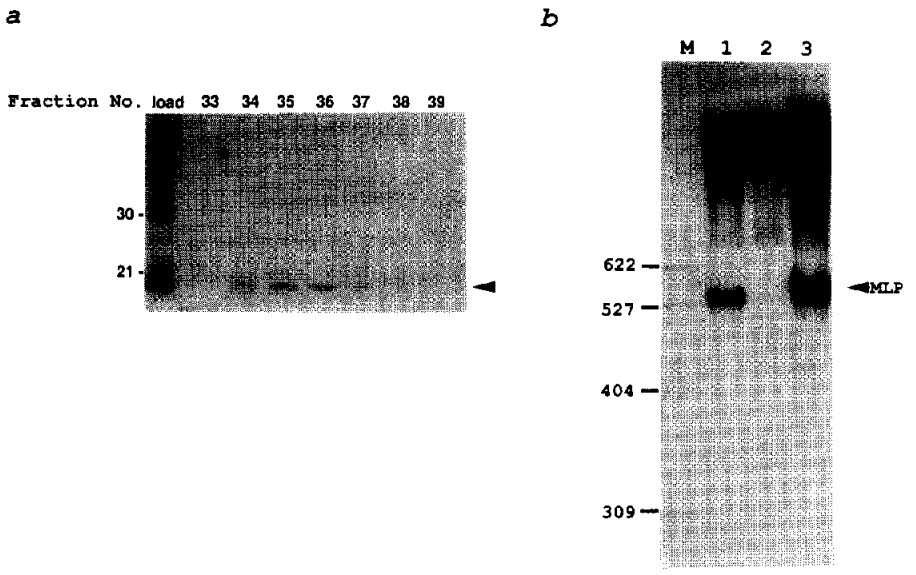

Fig. 1. (A) Purification of rHBx. rHBx was expressed in *E. coli* as described [6] and the relevant HPLC fractions were separated by SDS-PAGE (11.5%) and visualized by silver staining (arrowhead indicates position of rHBx). The molecular weights of standard proteins are indicated on the left. (B) In vitro transcription transactivation by rHBx. In vitro transcription 'run off' analysis was performed using adenovirus major late promoter (MLP). [^{32}P]UTP labeled RNA was separated on a 5% polyacrylamide urea denaturing gel. The reaction mixtures included either 50 ng rHBx protein (lane 3) or α-amanitin (lane 2). The molecular weight sizes of standard single stranded DNA marker (M) are indicated on the left.

kinases but is characteristic of a certain group of enzymes called nucleoside diphosphate kinases (NDPKs [7]). These enzymes transfer the γ-phosphate of a nucleoside triphosphate (NTP) to a nucleoside diphosphate (NDP), and can use all ribo- and deoxy-NTPs as substrates. The NDPK reaction is reversible and is carried out by a ping-pong mechanism with formation of a phosphoryl-enzyme intermediate, which is stabilized in the presence of EDTA [8].

This similarity between the two proteins prompted us to check whether HBx has an NDPK activity. In an NDPK reaction, done in the presence of Mg^{2+}, [γ-^{32}P]ATP was used as a γ-phosphate donor and several NDPs as acceptors. GST-X (Fig. 3A, lane 1) protein, but not the control GST (lane 2), directed a limited production of [γ-^{32}P]GTP, with concomitant appearance of [^{32}P]P_i. This suggests that rHBx has a poor NDPK activity but displays a potent nucleotide hydrolytic activity, as was previously reported [6]. Clearly, when GST-X fusion protein was incubated with either [γ-^{32}P]ATP or [α-^{32}P]ATP either [^{32}P]P_i or [^{32}P]ADP were produced, respectively (Fig. 3B, lanes 3 and 6). Similar data was obtained using rHBx ([6], and Fig. 3C). Nucleotide hydrolysis is an intrinsic HBx activity as the control GST protein was inactive (lanes 2 and 5). Hydrolysis was inhibited under the condition that a phosphorylated HBx is formed, i.e. inclusion of EDTA (Fig 3A, lane 10). This raised the possibility that HBx , in similar to NDPK, catalyses the hydrolysis reaction in two steps. In the first step, HBx is phosphorylated to generate the phosphoryl-enzyme intermediate. In the second, the phosphate is transferred from HBx to an acceptor molecule, either NDP or water. This possibility was checked by generating first phosphoryl-HBx in the presence of EDTA (Fig. 3, lanes 1 in C and D), and than adding Mg^{2+} (lanes 2). The results clearly indicate that the phosphate residue was released from HBx by this treatment, suggesting that phosphoryl-HBx was an intermediary product of nucleotide hydrolysis reaction.

An additional characteristic of NDPKs is their ability to use both ribo and deoxy nucleotides as substrates [7,8]. Fig. 3B (lane 9), shows that rHBx has a similar activity and incubation of GST-X with deoxy-ATP, [α-^{32}P]dATP, resulted in hydrolysis and the production of [α-^{32}P]dADP. The nucleotide recognition range of rHBx was assayed. We have found that dCTP, UTP, and GTP, poorly generated the phosphoryl-HBx, and were inefficiently hydrolyzed ([6], and data not shown).

Remarkably, X protein also shares sequence similarity with NDPK and these proteins show up to 24% identity and up to 37.7% similarity (Fig. 4). X-ray analysis of *Dictyostelium discoideum* NDPK revealed that NDPK contains four-stranded

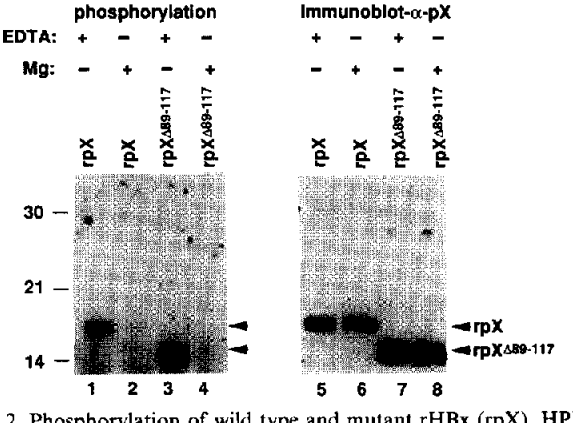

Fig. 2. Phosphorylation of wild type and mutant rHBx (rpX). HPLC purified fractions containing 1 μg rpX (lanes 1–2), or 1.5 μg rpX$^{\Delta 89-117}$ (lanes 3–4) were incubated with 10 μCi of [γ-^{32}P]ATP in a reaction mixture containing 10 μM ATP, 10 mM DTT, 100 mM NaCl, in the presence of either 5 mM EDTA (lanes 1,3) or 10 mM Mg^{2+} (lanes 2,4), at 30°C for 45 min. Then, protein dye was added to the reaction mixtures which were subjected to SDS-PAGE followed by transfer to nitrocellulose filter and autoradiography (lanes 1–4). Next, the filter was hybridized with anti-HBx antibodies (lanes 5–8). The antibodies were raised against a synthetic peptide (amino acids 10 to 31). Note that a small fraction of rpX (34 kDa) and rpX$^{\Delta 89-117}$ (28 kDa), appears as homodimer (marked by asterisks).

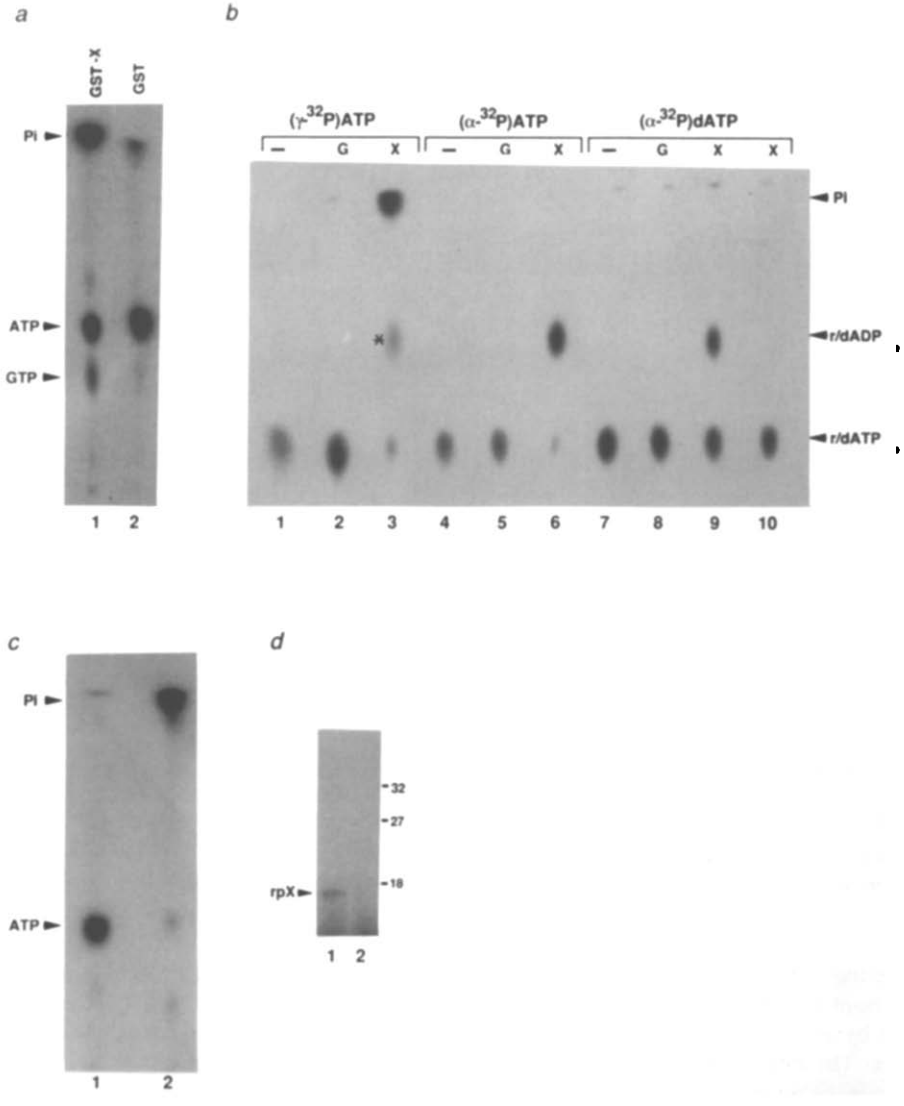

Fig. 3. (A) NDPK activity of GST-X. 100 ng of either GST-X (lane 1) or GST alone (lane 2), were incubated with 1 μCi, [γ-^{32}P]ATP, 10 μM ATP, 2 mM of unlabeled GDP, and 10 mM $MgCl_2$. Reaction products were separated by thin layer chromatography (TLC) and the positions of the products are indicated. (B) ATP/dATP hydrolysis directed by GST-X. 100 ng of GST-X (lanes X), GST alone (lanes G) or no protein (lanes –), were incubated with 1 μCi of radioactive nucleotides as indicated on the top. Reactions were carried out in the presence of either 10 mM $MgCl_2$(lanes 1–9) or 5 mM EDTA (lane 10). (C) Hydrolysis of [γ-^{32}P]ATP in two steps. rHBx (1 μg) was incubated with [γ-^{32}P]ATP in the presence of 5 mM EDTA in a total volume of 20 μl. 1 μl of the mixture was loaded onto a PEI–cellulose plate (lane 1). The sample was dialyzed against the same buffer for 30 min to remove free ATP. Next, Mg^{2+} was added to the dialyzed mixture to a final concentration of 15 mM, and following incubation for additional 30 minutes, 1 μl of the mixture was loaded onto a PEI–cellulose plate (lane 2). (D) Dephosphorylation of phosphoryl-HBx by the addition of Mg^{2+}. rHBx (1μg) was incubated with [γ-^{32}P]ATP in the presence of EDTA as described in C. Following the first incubation, a second incubation was performed with (lane 1) or without (lane 2) the addition of Mg^{2+} (to a final concentration of 15 mM). The products were subjected to SDS-PAGE as described in Fig. 2. Locations of spots corresponding to [^{32}P]P_i, (P_i); [^{32}P]ribo- or deoxy-ADP, (r/d(ADP); [^{32}P]ribo- or deoxy-ATP, (r/d(ATP), and [^{32}P]GTP, (GTP), are indicated. Following the formation of free [^{32}P]P_i, an additional minor product can be detected (B, lane 3, marked by an asterisk). We have preliminary indications that it is [^{32}P]PP_i.

β-sheet structure [9,10]. The three-dimensional structure of HBx is unknown but computer analysis predicted that four β-sheets can be formed at the corresponding regions including the β-4 NDPK protein structural motif that contains the active histidine residue (Fig. 4). Furthermore, the overall architecture of the active site is likely to be preserved. In HBx, the existence of an a helix is predicted for residues 107–126, which corresponds to the α-4 of NDPK, that provides one edge of the cleft [9]. The other edge of the cleft is a loop generated by residues 51–59 (Fig. 4). This region is highly homologous between HBx and NDPK.

4. Discussion

We report here functional and structural similarity between HBx and NDPKs. It is very likely that HBx has evolved from this group of enzymes but has been modified to acquired additional activities that satisfy the viral needs. The human NDPK gene named nm23-H2 is also a candidate suppressor of cancer metastasis [11] and functions in transcriptional regulation of c-*myc* expression [12]. The main biological effect of HBx described so far, is transactivation of transcription. HBx was shown to activate different promoter-enhancer units in vivo

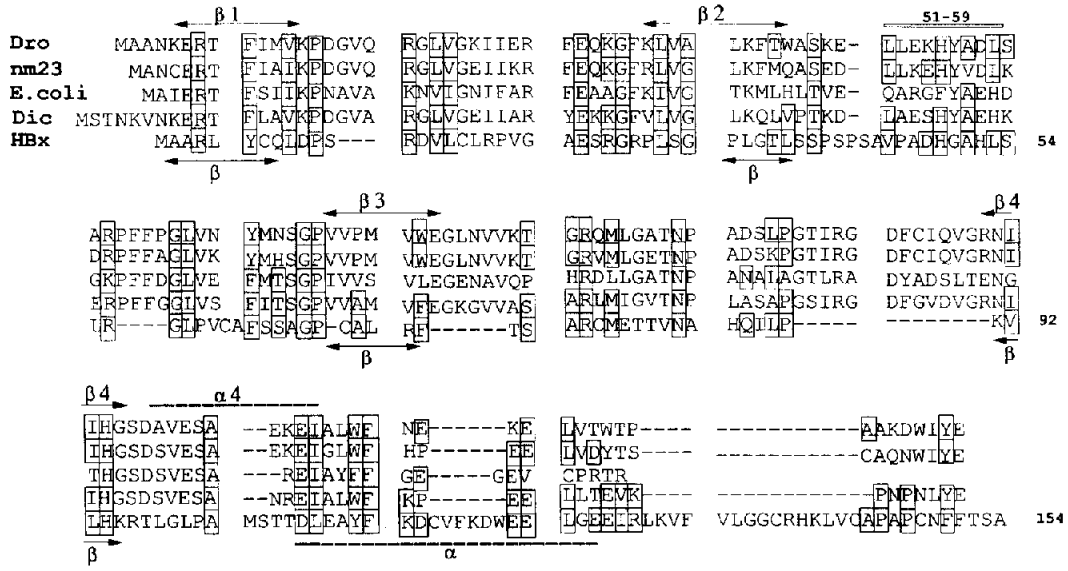

Fig. 4. Comparison of HBx amino acid sequence (HBx) to *Drosophila* NDP kinase awd (Dro), human NDP kinase, nm23 (nm23), *E. coli* NDP kinase (*E. coli*), and *Dictyostelium discoideum* NDP kinase (Dic). Identical and conservative substituted amino acids, with the following grouping: D, E; H; K, R; A, G; S, T; P; I, L, V; F, Y, W; M; C; N; and Q; are boxed. Secondary structure assignment of β-sheets (β1–β4), and α-helix (α 4) of NDPK, detected by X-ray, and the predicted β-sheets of HBx (β) and α-helix (α), are indicated. Numbers of amino acids of HBx, and residues 51–59 of Dic-NDPK forming the loop, are indicated. Single letter amino acid abbreviations was used.

and we show here that bacterially expressed HBx activates in vitro the adenovirus major late promoter. Also, HBx was also implicated in hepatocellular carcinoma formation [2,13]. Thus, these proteins seems also to display similar biological functions.

Previously, the main biochemical activity of HBx detected by us was hydrolysis of ribo and deoxy adenine nucleotides. Here we have extended our study and found that HBx hydrolyses NTPs by two steps one the transfer of γ-P to itself and similar to NDPK, HBx might be phosphorylated at a histidine residue. This step is stabilized by EDTA and the second step is the transfer of P_i to water. The latter is facilitated by Mg^{2+}. In NDPK His-122 residue is the main phosphorylation site, however, three dimensional structure analysis shows that His-55 and His-59 are positioned at the immediate vicinity and might be possible targets [10]. The homologous His residues in HBx are His-94, His-52 and His-49, respectively, however, we do not know which is a preferred residue. We show here that HBx mutant ($rpX^{\Delta 89-117}$) lacking the His-94 is phosphorylated, suggesting that this residue is dispensable. More detailed analysis of HBx mutants is required to better defining the acceptor histidine.

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