Short communication

The hepatitis B virus X protein is a potent AMP kinase

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The hepatitis B virus X-protein (HBx) has been expressed in Escherichia coli both as an unfused protein and with an N-terminal hexaHis-containing fusion sequence. Both forms of HBx, after purification, displayed a potent AMP kinase activity, in which HBx phosphorylates AMP to ADP, using ATP as the exclusive phosphate donor. We also found that HBx has previously unreported GTPase and GTP-ADP nucleoside diphosphate kinase activities.

Hepatitis B is a major world-wide disease; its long term risks include chronicity, which in turn leads to a high incidence of cirrhosis and hepatocellular cancer (Szmunness, 1978). The hepatitis B virus (HBV) genome encodes four proteins, the smallest of which is the 154 amino acid (17.5 kDa) non-structural X-protein (HBx), for which the suspected role as a liver carcinogen has been widely investigated. Many authors have referred to the HBx as a potent transactivator, both of the HBV genes and of a variety of cellular pathways. Kim et al. (1991) reported that direct integration of the X-gene into mouse embryonic cells caused multiple and disseminated tumours. Rossner (1992) reviewed the transactivational role of HBx and concluded that HBx was a promiscuous transcriptional activator of many viral and cellular regulatory sequences. Further support for the transactivational role of HBx came from Kekule et al. (1993) and Murakami et al. (1994) who suggested that the protein kinase C pathway serves as a target for HBx activation. Recently, interaction between HBx and p53 in infected liver tissue was reported by Feitelson et al. (1993), and Wang et al. (1994) proposed that formation of a complex between HBx and p53 inhibits the sequence-specific binding of p53 to DNA. The activation of Ras-GTP complex formation, with a consequent activation of the Ras-Raf MAP kinase signalling cascade by HBx has also been reported (Benn & Schneider, 1994).

HBx also supports a number of enzymic activities, which have only recently come to light. The auto-phosphorylation of HBx was described by Wu et al. (1990). ATPase and dATPase activities, and also GTP-ADP phosphodiester kinase (NDPK) activity have been reported by De-Medina et al. (1994) and De-Medina & Shaul (1994). We now add to the already described enzymic activities and describe a previously unreported kinase activity associated with HBx; we also report and describe an as yet unknown GTPase activity and GTP–ADP NDPK activities as being part of the HBx repertoire.

The hepatitis B (strain AYW) X-gene was expressed as an unfused protein from the vector pET3d in BL21-DE3 cells (Studier et al., 1990); translation was from the initial methionine of the HBx sequence. HBx was obtained in the insoluble fraction and was purified after solubilization in 8 M-urea, ion-exchange chromatography on CM-cellulose and subsequent gel-filtration, and then renatured by stepwise dialysis at pH 5.0. The purity of this material is shown in Fig. 1 (a). The X-gene was also inserted into the pTrcHis B expression vector (Invitrogen). The hexaHis HBx was expressed in Tgl cells, from which soluble protein was obtained and purified by chromatography on Ni-NTA resin (Qiagen). The resin, after protein adsorption, was washed with one column vol. of urea (8 M), MES (pH 6.0, 50 mM), NaCl (0.5 M), DTT (2 mM) and washed further with 10 column vols of the same buffer minus urea. Elution with sodium acetate (pH 4.5, 0.05 M), NaCl (0.5 M), imidazole (200 mM) and subsequent dialysis against Tris–HCl (0.01 M, pH 8.3), 2-mercaptoethanol (MSH, 10 mM) gave pure HBx, as shown in Fig. 1 (b). We also expressed pTrcHis without insert and after using the same purification scheme as described for the hexaHis HBx, and tested the final eluate as a control in all the enzymic reactions, where it consistently tested negative, indicating that the findings we describe here relate to the HBx and not to an Escherichia coli contaminant. In addition, the fact that two entirely different purification schemes have...
Fig. 1. (a) Final Sephadex G75 purification of unfused HBx. Fractions 12, 13 and 14 were pooled as unfused HBx. (b) Purified pTrcHis fusion HBx. Both are Coomassie blue stained PAGE gels.

Fig. 2. AMP kinase activity of HBx. Lane 1: without HBx. Lane 2: with HBx, no additions. Lanes 3–5: 1, 10 or 100 μM-AMP added.

provided two HBx preparations which yielded identical results in the described experiments, supports our contention that the findings relate to the HBx.

Nucleotidyl hydrolase and phosphate transfer activities were studied in 10 ml of a reaction buffer containing Tris–HCl (pH 7.5, 40 mM), MgCl₂ (10 mM), KCl (2 mM), DTT (5 mM) (NTPase buffer) and containing [γ-³²P]NTP (10 μM, 0.1 mCi/μmol) and 100 ng of either unfused HBx or hexaHis HBx. Reactions were incubated for 60 min at 37 °C. Aliquots (2 μl) were applied to a polyethylene imine (PEI) thin layer plate, which was developed with 0.75 N-KH₂PO₄, pH 3.5. For the studies of phosphate transfer activities or of inhibition of enzymic activity by nucleotides, unlabelled nucleotides were added at the desired concentrations.

We found HBx to be a potent ATP-dependent AMP kinase. In Fig. 2 this activity is shown at varying AMP concentrations; at least 75% of the substrate ATP was converted to ADP, when the concentration of AMP was 100 μM. This activity is totally ATP-dependent; GTP does not substitute for ATP, and neither does HBx use GTP to phosphorylate GMP. The Kᵅ for AMP in this reaction is approx. 150 μM. The numerical values obtained may be physiologically significant, since the calculated concentration of ATP in the average cell is approx. 50–100 μM. The described AMP kinase activity is unique and is totally different from the mitochondrial enzymic activity which is totally dependent on GTP to convert metabolically generated energy into chemical energy via GTP-mediated phosphorylation of AMP. However, the structure of the mitochondrial enzyme, as exemplified by horse muscle adenyl kinase, does show sequence homology with the HBx, which is shown in Fig. 3. We found this homology after searching both the SwissProt and EMBL protein databases and using the FASTA (Pearson & Lipmann, 1988) and the BLAST (Altschul et al., 1990) methodologies for alignments. We aligned the HBx consensus sequence with the published sequence of beef heart mitochondrial matrix adenylate kinase (EC 2.7.4.10) using the PILEUP program (Wisconsin, GCG) and found that there was a direct 21-5% identity and a further 21% close functional similarity. Although some of the published binding sites for ATP in the mitochondrial enzyme (Diederichs & Schulz, 1991) also occur in the HBx sequence in the predicted places, some are missing and others differ to a varying extent.

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40
Kad
G ASARLRAAI MGAPGSQGT VSSRITKHFE
HBx consensus
MAAR--CQLD P-RDVLCLR P VGAES-GRPF- SGG--
HBx
MAARLCCQLD PARDVLLCR P VGAESCQRPF SGG--

90
LKHLSSGDLL RDNMLRGTEI GVLAKTFIDQ GKLIPDDVMT RLVLHELKNL
LG-L-SPS -SAVP-DHGA HLSLRLGP
LGTLSSSPS... ............ ............ PSAVPTDHGA HLSLRLGP..

140
TOYNWLDGQF PRTLPOAEAL DRAVQIDTVI NLNVPFEVIK QRLTARWHIP
VCAF SSAGPCALRF TSAR-METTV NAH--LPKVL HKRT
......VCAF SSAGPCALRF TSARMETTV NAHQILPKVL HKRT......

190
GSGRVYNIEF NPPKTMGIDD LTGEPLVQRE DDPETTVKR LKAYEAQTEP
LGL ---STTDLEA YFKDCLFKDW EELGEEI R LKVFR
......LGL SAMSTTDLEA YFKDCLFKDW EELGEEI... R LKVFR......

235
VLEYRRKKGV LETFSGTETN KIWPHVYAFI QTLPQRSQE TEVTP
VLGGCRHKLV C-PAPCNFF TSA
VLGGCRHKLV ............ .CAPAPCNFF ............ TSA...

Fig. 3. Sequence similarity of beef heart adenyl kinase (KAD) with HBx. The upper rows show the KAD sequence, the centre rows the HBx consensus sequence, while the lower rows show the HBx (strain AYW) sequence. Amino acid identities are shown in bold-type and underlined, while similarities are in bold-type. The substrate binding sites in KAD are shown in italics.

diphosphate ester kinase activities. These results are illustrated in Fig. 4, where the GTPase activity is shown in lane 2, while in lanes 3, 4 and 5 the addition of numerous concentrations of ADP demonstrates the diphosphate ester kinase activity. The \( K_m \) for GTP is 35 \( \mu \)M. The GTPase reaction is not inhibited by the addition of ATP, GDP or GMP. De-Medina et al. (1994) recently showed that HBx possesses both ATPase and ATP-GTP diphosphate ester kinase activities; however, they were unable to demonstrate any GTP related activities. The fact that in our experiments, which show HBx to be a GTPase, ATP did not appear to inhibit GTP hydrolysis supports the idea that GTP and ATP are hydrolysed at different sites on the HBx molecule. Both ATP and GTP hydrolysis play an important role in transcription activities; ATP hydrolysis is a source of energy driving helicase activity (Wang et al., 1992) and is used in the phosphorylation of RNA polymerase II (Chestnut et al., 1992), while GTP is involved in many receptor-linked signalling cascades. During normal in-
Fig. 4. GTPase activity and GTP-ADP diphosphate ester kinase activities of HBx. Lane 1: without HBx. Lane 2: with HBx, no additions. Lanes 3–5: 1, 10 or 100 μM-ADP added.

Infection, HBx is primarily, if not exclusively located in the cytoplasm of the affected cell (Benn & Schneider, 1994). As a logical outcome it appears unlikely that HBx is directly involved in nuclear processes, but may indirectly affect these. One way in which this could happen is through modulation of key cellular reactions by changes in delicately balanced nucleotide levels as a result of the unregulated and polyfunctional enzymic activities ascribable to the HBx. A number of nucleotide-mediated key reactions occur at the nuclear membrane and are involved in regulation of cellular activities, and it could be here that HBx affects the cell mechanism to direct the cell onto the pathway to abnormal proliferation. The finding of ATP-dependent binding of the TATA-binding protein by Qadri et al. (1995) may be relevant to the enzymic activities reported here, especially since the enzymic parameters of HBx are close to calculated cellular ATP concentrations.

References


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