SHORT COMMUNICATION

Erk-independent partial activation of AP-1 sites by the hepatitis B virus HBx protein

Frank Henkler, A. Ross Lopes, Mick Jones and Rajen Koshy

Imperial College of Science, Technology and Medicine, Hammersmith Campus, Department of Infectious Diseases, 7th floor Commonwealth Building, Ducane Road, London W12 0NN, UK

The hepatitis B virus X protein (HBx) is suggested to regulate transcription by stimulation of intracellular signalling pathways. We have analysed the effects of HBx on activation of the MAP kinase (Erk) and JNK/SAPK signalling pathways and confirm a stimulation of the Erk/MAP kinase in quiescent cells. However, a substantial Erk-independent activation of AP-1, and phosphorylation of c-Jun (serine-63), but not Erk-2, was induced by HBx in dividing, serum-maintained cells. These data suggest that HBx promiscuously activates Erk and JNK responsive pathways and that its overall effect on signalling may be influenced by external mitogenic stimuli.

The hepatitis B virus X protein (HBx) has been classified as a transcriptional activator since it is capable of up-regulating promoters of various cellular and viral genes (Twu et al., 1987; Spandau & Lee, 1988; Zahm et al., 1988). HBx is widely regarded as an aetiological factor in hepatitis B virus (HBV)-related carcinogenesis and its putative oncogenic risks are attributed to transcriptional activation of proto-oncogenes (for review see: Rossner, 1992; Henkler & Koshy, 1996) and a possible inactivation of the p53 tumour-suppressor protein (Feitelson et al., 1993; Wang et al., 1994; Truant et al., 1995). Since the effects of HBx are mediated via a wide range of transcription factor binding sites, promiscuous functional mechanisms have been postulated. Intracellular localization studies demonstrated a predominant cytoplasmic and a relatively minor nuclear population (Doria et al., 1995). Nuclear HBx is suggested to transactivate via binding to several transcription factors (Maguire et al., 1991; Williams & Andrisani, 1995; Antunovic et al., 1993; Qadri et al., 1995), whereas the cytoplasmic population was shown to trigger intracellular signalling pathways (Benn & Schneider, 1994; Doria et al., 1995; Kekule et al., 1992). The mechanisms of HBx responsive signalling are still not understood. Activation of various components of the Ras/MAP kinase signalling cascade including extracellular regulated kinase 2 (Erk-2) [also called MAP kinase 2] has been reported and is suggested to trigger the induction of AP-1 (Benn & Schneider, 1994; Doria et al., 1995) and cell cycle progression (Benn & Schneider, 1995). A related but distinct signalling pathway is controlled by the G-proteins, Rac, Rho and Ras, which leads to activation of at least two kinases [c-Jun N-terminal kinase (JNKs)] (Derijard et al., 1994; Coso et al., 1995 a, b). These kinases phosphorylate the activating domain of Jun at serine residues 63 and 73, stimulating DNA binding of AP-1 (Whitmarsh & Davis, 1996). The Erk and the JNK pathway are regulated separately, although cross-talk exists at G-protein and MAP kinase levels (Ramirez et al., 1997; Urch et al., 1997; Whitmarsh & Davis, 1996). The JNK pathway [also called SAPK (stress activated protein kinase)] is particularly associated with transduction of stress-related signals (Kyriakis et al., 1994; Sanchez et al., 1994; Whitmarsh & Davis, 1996), but can also activate Erk in tissue culture. Similarly, the Erk/MAP kinase pathway has been shown to trigger phosphorylation of Jun in some studies (Palverer et al., 1991); however, the N terminus of Jun is not regarded as a substrate of Erk/MAPK in vivo (Whitmarsh & Davis, 1996). HBx has been recently shown to trigger N-terminal phosphorylation of c-Jun in vitro (Benn et al., 1996) and is suggested to activate the JNK/SAPK pathway. The question arises as to whether induction of both Erk/MAPK and JNK signalling is necessary and required for transcriptional activation of AP-1 binding sites by HBx.

The Ras/Erk kinase signalling pathway utilizes a protein kinase phosphorylation cascade leading to an activation of MAP kinase (Erk-1 and Erk-2) (de Vries-Smits et al., 1992; Whitmarsh & Davis, 1996). Activated Erk-1/2 is phosphorylated and translocated into the nucleus, where it triggers activation of various transcription factors. In our experiments, HBx-induced phosphorylation of Erk-1 was assayed by an adapted Western blot analysis using low bis-acrylamide PAGE to allow efficient separation of phosphorylated and non-phosphorylated endogenous Erk-1 (Fig. 1A).

CCL-13 cells (2.5 × 10⁶) were split into 9 cm dishes

Author for correspondence: Frank Henkler. Present address: National Institute for Medical Research, Division of Membrane Biology, The Ridgeway, Mill Hill, London NW7 1AA, UK. Fax + 44 181 906 4477. e-mail fhenkle@nimr.mrc.ac.uk

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Effects of HBx on Erk-1 and Erk-2. (A) Activation of Erk-1 by HBx. Western blot analysis. The proteins were separated by modified SDS–PAGE overnight; migration of the 26 kDa protein marker was at least 17.5 cm on a 10% polyacrylamide gel. Phosphorylated Erk has a lower electrophoretic mobility and is detected as a band of apparently higher molecular mass. Erk-1 was detected with a monoclonal antibody (Transduction Laboratories, Catalogue #M12325), and visualized by chemiluminescence (ECL, Amersham). (Exposure was imaged using Adobe Photoshop 4.0.) Lanes 1–3, cells were transfected with pcDNA3; lane 2, control cells which were kept in 10% FCS throughout the experiment to assess basal Erk-1 activation in CCL-13 cells; lane 3, positive control, 10 min TPA (100 ng/ml); lanes 4–6, HBx-expressing cells, transfected with pcCMVX: 1 µg (lane 4), 5 µg (lane 5) or 10 µg (lane 6), respectively. The total amount of DNA used was 25 µg in each transfection. (B) Activation of Erk-2 by HBx. Western blot analysis. Cells were transfected with 5 µg pcCMVErk(tag) (lanes 1–6) and co-transfected with: lanes 1 and 2, pcDNA3; lane 3, 10 µg pcCMVX(nuc); lane 4: 1 µg pcCMVX; lane 5, 5 µg pcCMVX; lane 6, 10 µg pcCMVX. The total amount of DNA was adjusted to 25 µg in each transfection with pcDNA3. Cells in lane 2 were stimulated with TPA (100 ng/ml), 10 min before harvest. Phosphorylation of Erk-2 was analysed by Western blotting using the Myc-tag-specific antibody 9E10. Immunocomplexes were visualized with the ECL-detection system (imaged with Adobe Photoshop 4.0). The phosphorylated and non-phosphorylated Erk populations are indicated on the right-hand side. (C) Effects of HBx on Erk-2 phosphorylation in serum-maintained and serum-deprived cells. Western blot analysis. CCL-13 cells were transfected with 5 µg pcCMVErk(tag) (lanes 1–6) and co-transfected with 5 µg pcCMVX (lanes 3 and 5) or 5 µg pcCMVX(nuc) (lanes 4 and 6). Cells in lanes 5 and 6 were serum starved for 16 h, 26 h post-transfection, and stimulated with 2.5% serum for 3 h. All other cells were maintained in 10% serum throughout the entire experiment. Control cells in lane 2 were treated with 100 ng/ml TPA for 10 min before harvest. Phosphorylation of tagged Erk-2 was analysed by mobility shift/Western blotting. (D) CCL-13 cells were serum starved for 36 h (lanes 1–3) and stimulated with TPA (lane 2) or 10% serum (lane 3) before harvest. Cells in lane 4 were maintained in 10% serum throughout the entire experiment. Phosphorylation of endogenous Erk-1 was analysed by mobility shift/Western blotting. (E) Detection of HBx by immunochemical staining. Expression of HBx was verified by immunochemical staining, as described in the text. Left panel: CCL-13 cells transfected with pcDNA3; Right panel: CCL-13 cells transfected with 5 µg pcCMVX. Expression of HBx is indicated as brown staining; cells were counterstained with haematoxylin.
Effects of HBx on Erk/MAPK and JNK

Lane 3 – compare with lanes 2 and 5). This latter observation was surprising, since HBx trans-activates AP-1 binding sites up to 12-fold under these conditions (unpublished observation). It is notable that Erk-phosphorylation is a transient response to mitogenic signalling, since treatment of serum-starved CCL-13 cells with 10% serum triggered a rapid and strong phosphorylation of Erk-1 (Fig. 1D, lane 3), whereas we typically detect no or very little phosphorylation in serum-maintained cells (lane 4). We conclude that HBx cannot attenuate nor sustain transient effects of serum on Erk-phosphorylation and operates through mechanisms distinct from TPA, as was previously proposed by Murakami et al. (1994).

A requirement of Erk induction for HBx-mediated trans-activation of AP-1 binding sites was analysed in CCL-13 cells, which were treated with PD98059 (New England Biolabs), a selective inhibitor of MEK which specifically inhibits the Erk/MAP kinase signalling pathway (Dudley et al., 1995). CCL-13 cells were transfected with pCMVErk-2(tag) (Fig. 2D) and treated with 80 μM PD98059 for 8 h (lane 3) and 1 h (lane 4). Cells were further treated with 50 ng/ml TPA for 5 min (lanes 2–4) prior to harvest, 36 h post-transfection, and phosphorylation of Erk-2 was analysed by Western blot analysis. Treatment with 80 μM PD98059 was sufficient for a complete inhibition of Erk-2 phosphorylation after 1 h (lane 4), and the inhibitor was efficient for at least 8 h (lane 3). Further, CCL-13 cells were transfected with a reporter plasmid containing three AP-1 binding sites (3 × AP-1 CAT) (Fig. 2A, B, lanes 1–5) and co-transfected with pCMVX (lanes 3–5). Positive control cells in lane 2 were transfected with the c-Jun expression vector pRSVjun. The precipitates were removed after 12 h and fresh medium (lanes 1, 2 and 4) or medium containing 100 μM PD98059 (lane 3 and 5) was added to the plates. Control cells (lane 3) were harvested after a further 1 h to determine the basal AP-1 activation, prior to the treatment with MEK inhibitor. Medium containing PD98059 was applied every 8 h for 24 h, 12 h post-transfection. Medium containing PD98059 was replaced every 8 h. Cells were harvested 36 h post-transfection (except in lane 3). Cells in lane 3 were harvested 13 h post-transfection to determine the basal level of CAT expression, activated by AP-1, before inhibition of the Erk/MAP kinase pathway was carried out. (A) Analysis of a typical CAT assay thin-layer chromatograph by autoradiography. An overnight exposure is shown (imaged with Adobe Photoshop 4.0). (B) Spots of acetylated chloramphenicol were quantified using a Packard liquid scintillation analyser (1900CA). Relative transactivations (lane 1 = 1) were averaged from four independent experiments. (C) To verify the inhibitory effect of PD98059, each plate was treated with 50 ng/ml TPA for 5 min, immediately before harvest, in two experiments. These lysates were used to determine CAT activities and to analyse phosphorylation of endogenous Erk-1 by mobility shift/Western blotting. The number of the lanes corresponds with (A) and (B). (D) CCL-13 cells were transfected with 5 μg pCMVErk(tag) (lanes 1–4) and treated with 80 μM PD98059, 28 h (lane 3) and 35 h (lane 4) post-transfection, and harvested 36 h post-transfection and analysed by Western blotting for phosphorylation of Erk-2. Cells in lanes 2–4 were stimulated with 50 ng/ml TPA for 5 min, before harvest.
HBx triggers serine-63 phosphorylation of c-Jun. (A) Western blot analysis. CCL-13 cells (9 cm plates) were transfected with 4 µg pRSVjun (lanes 1–5) and co-transfected with HBx expression vectors (lanes 3–5) as described below. c-Jun phosphorylation was analysed by Western blotting using a serine-63 phospho-Jun specific antiserum (New England Biolabs) and visualized with the ECL-detection system. Lane 1, negative control cells transfected with pcDNA3; lane 2, positive control cells treated with TPA (10 ng/ml) and anisomycin (5 µg/ml); lane 3, cells co-transfected with 1 µg pCMVX(nuc); lane 4, cells transfected with 8 µg pCMVX (wild-type) and then treated with PD98059 3 h prior to harvest; lane 5, cells co-transfected with 8 µg pCMVX. (B) Western blot analysis. The overall expression levels of Jun in samples, described in (A), were analysed by Western blot analysis, using an anti-Jun rabbit polyclonal antiserum (Santa Cruz Biotechnology) and visualized using the ECL-detection system.

JNK/SAPK signalling was analysed by Western blotting, using an antibody (New England Biolabs catalogue #916L) which specifically recognizes phosphorylated Jun (serine-63), but not the non-phosphorylated protein (Fig. 3A). Since the endogenous levels of Jun were not sufficient to detect any phosphorylation, cells were transfected with pRSVjun (lanes 1–5) and cotransfected with pCMVX(nuc) (lane 3) or pCMVX (lanes 4 and 5), respectively. HBx caused moderate N-terminal phosphorylation of Jun (lane 5), as compared with the combined effect of anisomycin and TPA, used as a positive control (lane 2). Again, only wild-type HBx, but not the nuclear-targeted variant, was capable of inducing phosphorylation of c-Jun (lane 3 – compare with lane 5), showing that activation of JNK responsive signalling is only triggered by the cytoplasmic HBx population. These observations corroborate another recent report suggesting the activation of the JNK kinase pathway by HBx (Benn et al., 1996). HBx-stimulated phosphorylation of Jun was not diminished in cells treated with 80 µM PD98059 (lane 4), which may indicate that its activating effect on JNK signalling is maintained, despite inhibition of the Erk/MAP kinase pathway. Total expression of Jun was analysed by Western blotting, using a rabbit polyclonal antiserum (Santa Cruz Biotechnology) (Fig. 3B), and was found to be similar in each analysed sample. Western blot analysis indicated different populations of phosphorylated Jun. A strong band shift was repeatedly found in control cells treated with TPA and anisomycin using both the phospho-63 serine monoclonal antibody and the rabbit polyclonal antiserum (Fig. 3A, B, lane 2). Although serine-63 phosphorylated Jun is present in both bands, a more complex phosphorylation pattern was observed, reflecting possible phosphorylation of C-terminal serine residues, or alternatively an additional protein modification of Jun.

The effects of HBx on a variety of signalling pathways such as Erk/MAPK (Benn & Schneider, 1994), JNK (Benn et al., 1996) and Src-kinases (Klein & Schneider, 1997) have been well-documented. However, the pathological implications of these complex interactions are not understood. The requirement of the classical Erk/MAP kinase pathway for AP-1 activation by HBx was suggested when cotransfection with ras and dominant negative mutant recombinant plasmids (Natoli et al., 1994) or prolonged serum depletion (Cross et al., 1993) eradicated the capacity of HBx to stimulate transcription. As Erk/MAP is essential for c-Fos expression (Gille et al., 1992), perpetual blockage of Erk/MAPK could lead to depression of AP-1 levels. However, as we demonstrate here, a temporary inhibition of Erk/MAPK does not abolish activation of AP-1. This suggests that HBx can activate AP-1 by alternative mechanisms. By means of in vitro phosphorylation assays, Benn et al. (1996) have previously shown that HBx activates JNK/SAPK. Our results demonstrating the phosphorylation of c-Jun within transfected cells corroborate and substantiate those observations. The data suggest further that the signalling effects of HBx may be modulated by cell cycle or growth control mechanisms, because the capacity of HBx to stimulate Erk/MAP kinase was only observed in quiescent cells (2.5% serum). In contrast, HBx failed to trigger phosphorylation of Erk-2 in mitotically active cells (10% serum), but induced serine 63-phosphorylation of c-Jun and Erk-independent activation of AP-1. The JNK/SAPK pathway may constitute the predominant target of HBx, especially in hepatocytes exposed to external mitogenic or inflammatory stimuli, such as in chronic hepatitis B infection. We speculate that this may have physiological significance in the progression of HBV-induced liver disease.

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