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Hepatitis B Virus HBx Protein Activates Transcription Factor NF-κB by Acting on Multiple Cytoplasmic Inhibitors of *rel*-Related Proteins

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The HBx protein is a small polypeptide encoded by mammalian hepadnaviruses that is essential for viral infectivity and is thought to play a role in development of hepatocellular carcinoma during chronic hepatitis B virus infection. HBx is a transactivator that stimulates Ras signal transduction pathways in the cytoplasm and certain transcription elements in the nucleus. To better understand the activities of HBx protein and its mechanism of action, we have explored the manner by which HBx activates the transcription factor NF-κB during transient expression. We show that HBx induces prolonged formation, in a Ras-dependent manner, of transcriptionally active NF-κB DNA-binding complexes, which make up the family of Rel-related proteins, p50, p52, RelA, and c-Rel. HBx was found to activate NF-κB through two distinct cytoplasmic pathways by acting on both the 37-kDa IκBα inhibitor and the 105-kDa NF-κB1 precursor inhibitor protein, known as p105. HBx induces phosphorylation of IκBα, a three- to fourfold reduction in IκBα stability, and concomitant nuclear accumulation of NF-κB DNA-binding complexes, similar to that reported for human T-cell leukemia virus type 1 Tax protein. In addition, HBx mediates a striking reduction in cytoplasmic p105 NF-κB1 inhibitor and p50 protein levels and release of RelA protein that was sequestered by the p105 inhibitor, concomitant with nuclear accumulation of NF-κB complexes. HBx mediated only a slight reduction in the cytoplasmic levels of NF-κB2 p100 protein, an additional precursor inhibitor of NF-κB, which is thought to be less efficiently processed or less responsive to release of NF-κB. No evidence was found for HBx activation of NF-κB by targeting acidic sphingomyelinase-controlled pathways. Studies also suggest that stimulation of NF-κB by HBx does not involve activation of Ras via the neutral sphingomyelin-ceramide pathway. Thus, HBx protein is shown to activate the NF-κB family of Rel-related proteins by acting on two distinct NF-κB cytoplasmic inhibitors.

Mammalian hepatitis B viruses encode a small transcriptional transactivator known as the HBx protein, a product of the HBx gene (83, 85, 93). HBx is essential for viral infectivity (20, 95) and is a potential cofactor in viral carcinogenesis (11, 12, 38, 47–49). HBx does not bind DNA directly and possesses few distinguishing features in common with other transcription-regulatory proteins. HBx has been shown to activate transcription factors NF-κB (53, 56, 58, 60, 75, 76, 84, 94), AP-2 (74), AP-1 (39, 62, 74, 83), and possibly c/EBP (27, 58, 86). HBx also stimulates transcription by RNA polymerase III (4, 87).

Some studies postulate a transcriptional role for HBx at the promoter, on the basis its in vitro ability to bind several transcription factors and components of the transcriptional apparatus or to stimulate transcription when tethered to a DNA-binding factor (21, 36, 57, 68, 74, 86, 89). On the other hand, HBx also activates cytoplasmic signal transduction cascades (24, 45, 56, 62) including the Ras-Raf mitogen-activated protein kinase (MAPK) cascade (11, 12, 63), which is essential for activation of AP-1 by HBx (11, 24, 63). HBx was recently shown to be both a cytoplasmic and nuclear protein (26); cytoplasmic HBx activates the Ras-Raf-MAPK cascade, whereas nuclear HBx activates specific transcription elements.

Although HBx is known to activate NF-κB, the mechanism by which this occurs has not been well studied. Understanding how HBx activates NF-κB can aid in establishing the fundamental molecular actions of HBx and provide insights into its function during viral infection and carcinogenesis.

NF-κB is a heterodimeric transcription factor complex consisting of two proteins of 50 or 52 kDa (p50 and p52) and 65 kDa (p65, now called RelA) (6, 16, 33, 46, 73). All members of the Rel family of factors (p50, NF-κB1; p52, NF-κB2; p65, RelA; c-Rel; and RelB) possess a *rel* homology domain that confers DNA binding and protein dimerization (reviewed in reference 34). Inactive NF-κB is sequestered in the cytoplasm by binding to the labile cytoplasmic inhibitor IκBα (5–7), which masks the RelA nuclear localization signal (10, 32). IκBα is uncoupled from NF-κB in response to extracellular signals (reviewed in reference 34), and intracellular factors, including the human T-cell leukemia virus type 1 (HTLV-1) Tax protein (42, 79). Uncoupling of IκBα from NF-κB generally requires phosphorylation of IκBα (9, 17, 18, 37, 41, 79) followed by rapid degradation of IκBα in proteosomes (67, 82). After degradation, IκBα is quickly replenished by NF-κB-mediated transcription of the IκBα gene (22, 52, 78).

IκBα is one member of a family of NF-κB inhibitor proteins that bind RelA. Other members include IκBβ (92), Bcl-3 (66), and protein precursors of NF-κB1 (p105) and NF-κB2 (p100), which generate p50 and p52 Rel family members, respectively, after proteolytic processing (59, 69, 80). Of particular interest is the regulation of NF-κB by the abundant p105 and p100 NF-κB1 and NF-κB2 precursors. Studies have shown that these cytoplasmic proteins constitute an authentic additional mechanism for controlling NF-κB activation in cells. For example, despite complete degradation of IκBα by tumor necro-

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sis factor alpha (TNF-α), only part of the inactive cytoplasmic pool of NF-κB is released, with the remainder sequestered by p100 and p105 (80). Several studies also reported that HTLV-1 Tax protein liberates NF-κB from the p100 or p105 NF-κB1 and NF-κB2 precursors (42, 88), although others have found that Tax activation of NF-κB is prevented by Tax binding to p100 (14, 79). It is not known how the p100 and p105 precursors are regulated or whether they are differentially controlled. The mechanisms for release of NF-κB *rel*-related proteins from the cytoplasmic precursor inhibitors is also not understood, although there is a correlation between inducible phosphorylation of p105 and the release of *rel*-related proteins (31, 64).

We recently showed that HBx activates both transcription factors AP-1 and NF-κB by acting in the cytoplasm of cells (26). In this study we have explored the basis for cytoplasmic activation of NF-κB by HBx protein. We first show that HBx activates NF-κB heterodimers that consist of p50, p52, RelA (p65), and c-Rel proteins. HBx activation of NF-κB DNA-binding complexes was fully blocked by coexpression of a Ras dominant-interfering protein, similar to that reported previously for HBx activation of transcription factor AP-1. We then show that HBx strongly induces phosphorylation of IκBα but only a three- to fourfold decrease in the cytoplasmic stability of IκBα, similar to the reported effect of HTLV-1 Tax protein. Increased turnover of IκBα protein, however, was found to represent only part of the mechanism for HBx-mediated release of cytoplasmically sequestered Rel proteins. Expression of HBx also induced a significant loss of NF-κB1 p105 precursor protein from the cytoplasm of cells, accompanied by a commensurate release of cytoplasmically sequestered RelA protein and nuclear accumulation of NF-κB DNA-binding complexes. These findings therefore demonstrate that HBV HBx protein activates transcription factor NF-κB by acting on two distinct cytoplasmic NF-κB inhibitor pathways.

MATERIALS AND METHODS

Cell culture, transfection, and infections. Chang cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum and 50 μg of gentamicin sulfate per ml. Cells were starved for 24 h in DMEM–0.5% calf serum prior to infection with adenovirus (Ad) vectors. Cells at 60 to 70% confluency were infected in phosphate-buffered saline (PBS) plus 2% calf serum (1 ml/10-cm plate) with recombinant Ad vectors at 25 PFU per cell for 1 h at 37°C with occasional rotation. The cells were incubated in fresh DMEM–2% calf serum for various periods before analysis. Where indicated, cells were treated with human TNF-α at 20 ng/ml for the times indicated or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) at 20 μM for 30 min before harvest. Nieman-Pick disease skin fibroblasts (RF and RFCIII cells) were provided by R. Desnick, Mount Sinai School of Medicine, New York, N.Y. RF cells are cultured Nieman-Pick skin fibroblasts that lack detectable acidic sphingomeylinase (SMase) activity. RFCIII cells are RF cells transformed with the acidic SMase gene, and they express a high level of acidic SMase activity (77). Both lines were cultured in DMEM–20% fetal calf serum. Cells were treated with the sphingosine isomer (inhibitor) DL-*threo*-dihydrosphingosine (DHS; Sigma Chemical Co.) essentially as outlined previously (19). Briefly, 9 ml of DMEM was mixed with 1 ml of 1% bovine serum albumin (BSA; final concentration, 0.1%) and then with 10 to 50 μl of a 10 mM DHS stock prepared in ethanol (final concentration of DHS, 10 to 50 μM). The mixture was incubated at 37°C for 1 h and then used to replace the cell medium for 30 min. DHS medium was then removed and replaced with fresh medium, followed by infection of cells with Ad-HBx recombinants or treatment with TPA or TNF-α as described above. Combined transfection-Ad vector infection studies were carried out as described previously (11, 12) with 10 μg of plasmid DNA per 10-cm plate, followed by Ad infection. The assay of chloramphenicol acetyltransferase (CAT) activity was carried out as described previously (26, 56) with equal amounts of cell extracts. Quantitation of CAT assay products was performed by PhosphorImager analysis with samples that were within the linear range of activity (5 to 50% conversion).

Preparation of cytoplasmic and nuclear extracts. Cytoplasmic and nuclear extracts were prepared as described previously (1). Briefly, cells infected with Ad vectors or treated with TNF-α or TPA were washed with PBS and collected by centrifugation. Cell pellets were resuspended in 400 μl of cold buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml), swollen on ice for 10 min, and vortexed for 10 s, and samples were centrifuged for 10 s at 12,000 × *g*. Supernatants were reserved as cytoplasmic fractions. Nuclear pellets were resuspended in 100 μl of cold buffer B (20 mM HEPES [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml) and incubated on ice for 20 min, and nuclear extracts were obtained by centrifugation at 12,000 × *g* for 2 min at 4°C.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts (5 μg) were incubated for 30 min at 23°C in 15 μl of buffer C [10 mM HEPES (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, 3 μg of poly(dI-dC)] with 10 fmol of ^{32}P-5′-end-labeled double-stranded DNA (dsDNA) oligonucleotide (10^6 cpm per reaction). The dsDNA oligonucleotide for probes or competitors containing an NF-κB-binding site corresponded to the sequence 5′-GATCCAGAGGGGCCACTTTCCGAGAGGA-3′ (70). DNA–NF-κB complexes were resolved from free labeled DNA by electrophoresis in 4.5% polyacrylamide gels containing 50 mM Tris-HCl (pH 8.5), 200 mM glycine, and 1 mM EDTA. The gels were dried, autoradiographed, and quantitated by PhosphorImager analysis with ImageQuant software (Molecular Dynamics). For the antibody competitor assay, analysis was carried out with specific antibodies against the p50, p52, and p65 subunits of NF-κB (Santa Cruz, Inc.), by adding them to the binding-reaction mixture before adding labeled oligonucleotide for 15 min at 4°C. Cold competitor assays were carried out by adding a 100-fold molar excess of unlabeled dsDNA NF-κB oligonucleotide simultaneously with the labeled probe.

Analysis of IκBα. Immunoblot analysis of cytosolic extracts prepared as described above was carried out with 100 μg of protein lysate resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide) (SDS-PAGE). The proteins were electrophoretically transferred to nitrocellulose and immunoblotted with antibody to the C-terminal portion of IκBα (provided by W. Greene). Immune complexes were visualized by the ECL chemiluminescence system (Amersham) and quantitated by densitometry. Phosphorylation of IκBα was detected by its lower electrophoretic mobility. Decay rates for IκBα were determined by addition of 50 μg of cycloheximide per ml to cells after infection with Ad vectors or treatment with TNFα followed by immunoblot analysis. Decay rates were calculated from the quantitation of bands obtained from three independent experiments, assuming a biological first-order decay constant.

Western immunoblotting. For Western immunoblot studies, nuclear and cytoplasmic lysates were prepared by disruption of cells in 0.5% Nonidet P-40–20 mM Tris-HCl (pH 8.0)–15 mM KCl at 4°C. A 50-μg portion of nuclear protein was resolved by SDS-PAGE (15% polyacrylamide) and transferred to nitrocellulose filters. The filters were preincubated for 2 h at 23°C in Tris-buffered saline (TBS) containing 3% BSA at followed by a 3-h incubation with antibody directed to IκBα, p105, or p100 protein (Santa Cruz, Inc.). The filters were washed three times for 10 min each in TBS–0.5% Nonidet P-40 and incubated for 10 min in TBS containing 2% BSA. The proteins were then detected by the ECL chemiluminescence system and autoradiographed at −70°C.

RESULTS

Characterization of NF-κB DNA-binding complexes activated by HBx protein. To study the kinetics of NF-κB induction by HBx, the HBx gene was expressed from a replication-defective Ad vector under the control of the cytomegalovirus (CMV) promoter. We previously showed that these vectors are genetically silent except for expression of the transgene during the short course of these experiments and that the HBx protein does not act by first inducing the expression of Ad promoters (11–13, 26). The kinetics for HBx activation of NF-κB were determined by infecting Chang cells with Ad vectors expressing the wild-type HBx gene or an HBx mutant encoding an mRNA deleted of all AUG codons that fails to synthesize HBx protein (HBxo) (11–13, 26). Nuclear extracts were prepared at different times after infection, and induction of NF-κB DNA-binding activity was examined by EMSA with a ^{32}P-labeled oligonucleotide probe containing a single NF-κB-binding site or an oligonucleotide with a mutant site that does not bind NF-κB (70). HBx induced a strong increase in NF-κB DNA-binding activity, which initiated 3 h after expression, plateaued between 5 and 10 h and remained evident at 24 h (Fig. 1A). Cells expressing the control HBxo gene did not demonstrate any activation of NF-κB. As found previously for HBx activation of AP-1, induction of active NF-κB was still sustained past 24 h after expression of HBx (data not shown). HBx activation of

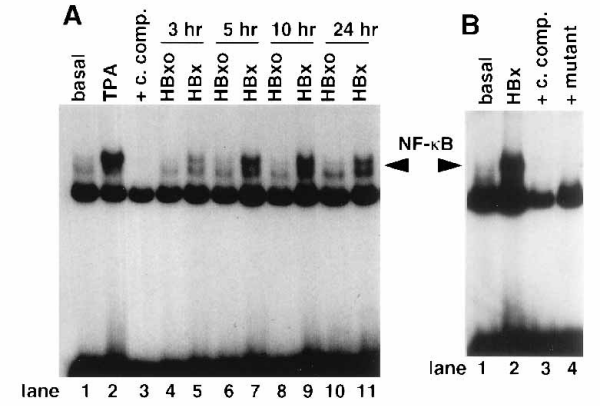

FIG. 1. Kinetics of HBx induction of NF-κB DNA-binding activity. Serum-starved Chang cells were infected with Ad CMV-X (wt HBx) or Ad CMV-Xo (HBxo) at 25 PFU per cell, and nuclear extracts were prepared at the times indicated. (A) Equal protein amounts of extract were used to measure NF-κB DNA-binding activity by EMSA with a ^{32}P-labeled oligonucleotide probe containing one binding site. Binding reactions were performed with 5 μg of nuclear extract, 3 μg of poly(dI-dC), and 10 fmol of labeled oligonucleotide for 30 min at 23°C. For competition studies, a 100-fold molar excess of cold competitor (+c. comp.) probe was added. (B) Studies were performed as described for panel A but with an oligonucleotide containing a mutated NF-κB DNA-binding site. Basal refers to unstimulated, uninfected quiescent Chang cells. TPA treatment of cells was performed at 20 μM for 30 min before harvest. Protein-DNA complexes were resolved by electrophoresis in 4% polyacrylamide gels, visualized by autoradiography, and quantitated by PhosphorImager analysis.

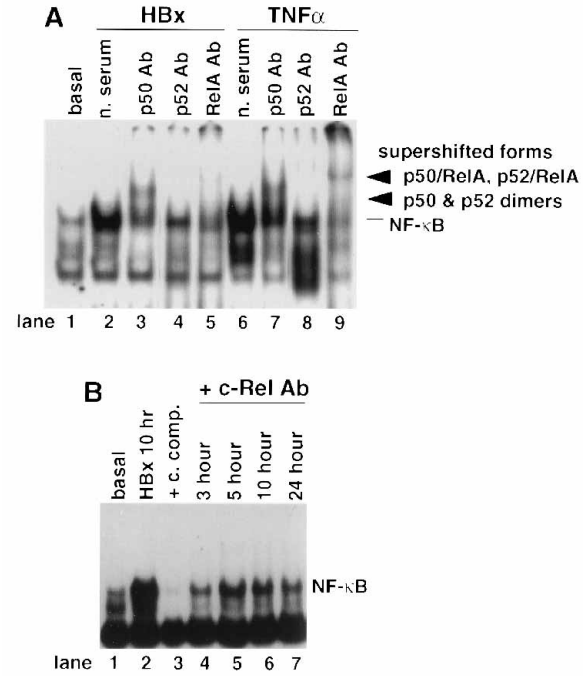

FIG. 2. Composition of NF-κB DNA-binding complexes induced by HBx protein. Serum-starved Chang cells were infected with Ad CMV-X at 25 PFU per cell for 10 h or treated with human TNF-α at 20 ng/ml for 20 min. Nuclear extracts were prepared, and NF-κB DNA-binding activity was detected by EMSA as described in the legend to Fig. 1. Specific polyclonal antibodies (Ab) were added to binding-reaction mixtures corresponding to p50 (NF-κB1), p52 (NF-κB2), and RelA (A) or to c-Rel (B) prior to addition of labeled oligonucleotide probe. The presence of the respective *rel*-related polypeptide is detected by partial or full ablation of the shift or the presence of a supershifted complex. Controls consist of quiescent Chang cell extracts (basal) and normal serum addition (n. serum). The gel was electrophoresed longer than in the other figures to resolve the complexes. The strong nonspecific band is therefore not shown.

NF-κB DNA-binding activity was specific, since it was ablated by an excess of unlabeled cold competitor, and did not bind to a mutated NF-κB site (Fig. 1B). The invariant fast-migrating band was a nonspecific complex observed in NF-κB EMSA studies. As shown previously (26, 56) (see Fig. 3B), the NF-κB complexes induced by HBx are active and strongly stimulate the transcription of an NF-κB-dependent CAT reporter construct.

Next, the composition of NF-κB complexes induced by HBx at 6 h was probed. Either normal serum or polyclonal antibodies directed against p50, p52, and RelA proteins were added prior to addition of labeled oligonucleotide. The specific antibodies generally block the formation of NF-κB DNA-binding complexes, although some supershifting to lower-mobility complexes is also observed (Fig. 2A). Antibodies to p50 or p52 proteins each partially blocked the formation of native NF-κB complexes. HBx therefore activated the formation of NF-κB complexes containing p50 and p52 proteins. Antibodies to RelA protein also partially prevented the formation of NF-κB complexes (Fig. 2A). Similarly, NF-κB complexes induced by 15-min TNF-α treatment of cells contained p50, p52, and RelA proteins. Antibodies to c-Rel protein also partially blocked the formation of NF-κB DNA-binding complexes, particularly those formed early (3 h) after expression of HBx (Fig. 2B). These results indicate that HBx activates authentic NF-κB DNA-binding complexes containing the family of Rel-related proteins and does so for a prolonged period. It was not technically possible to add all four antibodies simultaneously to ablate the NF-κB shift because of volume and concentration constraints.

Effect of Ras inhibition on HBx activation of NF-κB DNA-binding activity. Previous studies demonstrated that HBx stimulation of transcription factor AP-1 requires its establishing a Ras-Raf-MAPK signalling cascade (11, 26, 63). Studies were therefore carried out to determine the requirement for the Ras signalling cascade in activation of NF-κB by HBx when expressed either by transient transfection of plasmids or from recombinant Ad vectors (Fig. 3A). Chang cells were cotransfected with a plasmid expressing a dominant-interfering mutant of Ras (Ras Asn-17), which prevents the formation of Ras-GTP complexes (28), and with plasmids expressing HBx or HBxo genes for 24 h prior to preparation of nuclear extracts and assay for NF-κB DNA-binding activity. In other studies, cells were first transfected with the Ras dominant-negative mutant plasmid and then infected with Ad CMV-X or Ad CMV-Xo vectors. Previous studies have shown that transfection followed by infection drives the dominant-interfering expression plasmid into the same cells infected by Ad recombinant vectors (11, 12, 91). The strong HBx induction of NF-κB DNA-binding activity was fully blocked by coexpression of the Ras dominant-interfering protein in both transiently transfected and Ad vector-transduced cells (Fig. 3A). The slight difference in appearance of NF-κB complexes is a result of different electrophoresis conditions. As expected, the Ras dominant-negative mutant also blocked HBx stimulation of a CAT reporter controlled by four NF-κB-binding sites and a basal promoter (Fig. 3B) (26, 56). Transfected HBx induced strong activation of NF-κB-dependent CAT activity, which was fully blocked by cotransfection with the Ras dominant-negative mutant (Fig. 3B). The Ras mutant is not merely down-regulating the synthesis of HBx, a point which was previously excluded (11). Both genes are controlled by the same (CMV) promoter, which is not significantly affected by the Ras mutant, as assayed by using a β-galactosidase reporter construct under

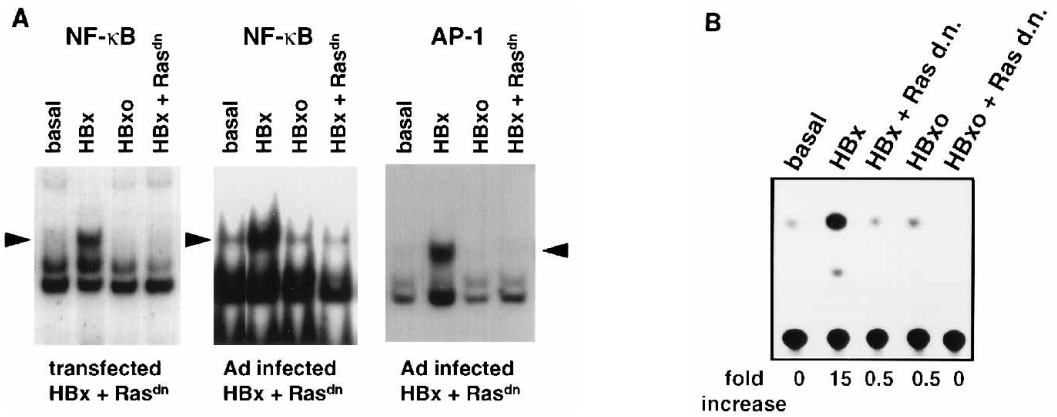

FIG. 3. (A) HBx requires Ras activity to induce NF-κB DNA-binding complexes. Chang cells were transfected with Ad Ras Asn-17 plasmid and plasmids expressing HBx or HBxo or superinfected later with Ad CMV-X or Ad CMV-Xo, and nuclear extracts were prepared for EMSA. Treatment with 20 μM TPA was continued for 30 min. (B) HBx induces transcriptionally active NF-κB through a Ras pathway. Chang cells were cotransfected with plasmids expressing HBx or HBxo, the Ras dominant-negative (d.n.) inhibitor and the CAT gene under the control of the CMV promoter, extracts prepared and CAT activity determined as described (26, 56).

CMV promoter control. These results therefore demonstrate that during transient expression of HBx in Chang cells, induction of NF-κB, like that of AP-1, requires the activation of Ras signalling cascades.

HBx induces constitutive phosphorylation and moderate degradation of the cytoplasmic pool of IκBα. The biochemical basis for HBx activation of NF-κB was examined by determining whether HBx expression leads to phosphorylation and degradation of IκBα, one of several cytoplasmic inhibitors of NF-κB. Release of NF-κB by phosphorylation and degradation of IκBα in turn induces the synthesis of new IκBα, thereby establishing an autostimulatory loop (2, 22, 79, 80). Long-term activation of NF-κB therefore actually results in an increased cytoplasmic abundance of IκBα, which is phosphorylated and more rapidly degraded. Thus, an increased level of IκBα protein which is phosphorylated and turns over more rapidly is indicative of protracted NF-κB activation. Phosphorylation of IκBα causes a slight, slower electrophoretic perturbation in the mobility of the 37-kDa protein, which can be detected by immunoblot analysis of IκBα resolved by SDS-PAGE (see e.g., references 79 and 80). Compared with control cells that do not express HBx, a steady-state increase in phosphorylated IκBα was evident in HBx-expressing cells at 6 h and was still sustained at 30 h, accounting for roughly 30 to 50% of the IκBα protein by this time (Fig. 4). The total amount of IκBα protein

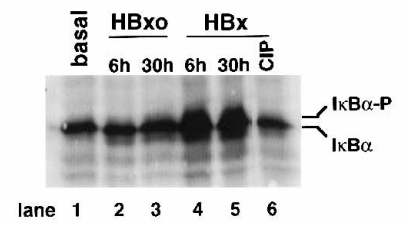

FIG. 4. Inducible phosphorylation of IκBα by HBx protein. Quiescent Chang cells (basal) were infected with Ad-X vectors at 25 PFU per cell for 6 or 30 h. Whole-cell extracts were prepared in the presence of phosphatase inhibitors (10 mM sodium vanadate and 50 mM sodium floride) and denatured, and equal protein amounts were resolved by SDS-PAGE (12% polyacrylamide) for an extended time to resolve the lower-electrophoretic-mobility form of phosphorylated (IκBα-P) protein from the nonphosphorylated (IκBα) protein. After electrophoresis, proteins were transferred to nitrocellulose, immunoblotted with an antibody directed to the C terminus of IκBα, and detected by enhanced chemiluminescence and autoradiography.

also increased by about 50% by 30 h after HBx expression, an effect indicative of induction of new IκBα gene expression mediated by activated NF-κB (2, 22, 79, 80). No such increase in IκBα phosphorylation or steady-state protein level was observed in HBxo-expressing cells (Fig. 4). Treatment of proteins from 10-h HBx-expressing extracts with alkaline phosphatase prior to electrophoresis largely eliminated the lower-mobility form of IκBα, demonstrating, as expected, that the slower HBx-inducible species resulted from phosphorylation of the faster-migrating 37-kDa IκBα protein. The kinetics for phosphorylation of IκBα in HBx-expressing cells correlates with the kinetics for nuclear accumulation of NF-κB DNA-binding activity shown in Fig. 1.

To explore whether phosphorylation of IκBα mediated by HBx protein also resulted in a decrease in IκBα protein stability, the half-life of the protein in cells treated with cycloheximide to block de novo protein synthesis was determined. Serum-starved Chang cells expressing HBx or HBxo genes for 7 h were treated with cycloheximide for increasing periods, and the levels of IκBα protein were compared. Control cells were treated with TNF-α for 15 min followed by a 15-min incubation, shown previously to induce rapid degradation of IκBα (2, 30, 79). Extracts were produced at the indicated times after cycloheximide treatment, proteins were resolved by SDS-PAGE, and the level of IκBα protein was determined by immunoblot analysis (Fig. 5). In untreated control cells, and cells expressing the HBxo gene, the pool of IκBα protein displayed a long half-life ($t_{1/2} \sim 240$ min). In contrast, in HBx-expressing cells, IκBα decayed about three- to fourfold more rapidly ($t_{1/2} \sim 60$ to 80 min). A similar three- to fourfold decrease in the stability of the pool of IκBα was reported in Jurkat cells expressing HTLV-1 Tax protein (51). In cells treated with TNF-α, IκBα degradation was very rapid ($t_{1/2} < 20$ min), an effect which is consistent with other studies from different cell lines (2, 30, 79). However, in Jurkat cells, IκBα protein typically turned over with a shorter half-life, which might reflect cell type differences or growth differences, since quiescent Chang cells were used in our study. Nevertheless, these results demonstrate that expression of HBx protein is associated with an increase in phosphorylation of IκBα and a three- to fourfold-accelerated turnover of the IκBα inhibitor protein.

HBx protein induces release of RelA from NF-κB1 precursor protein p105, an additional cytoplasmic inhibitor of NF-κB activation. The decay rate of IκBα induced by HBx protein was

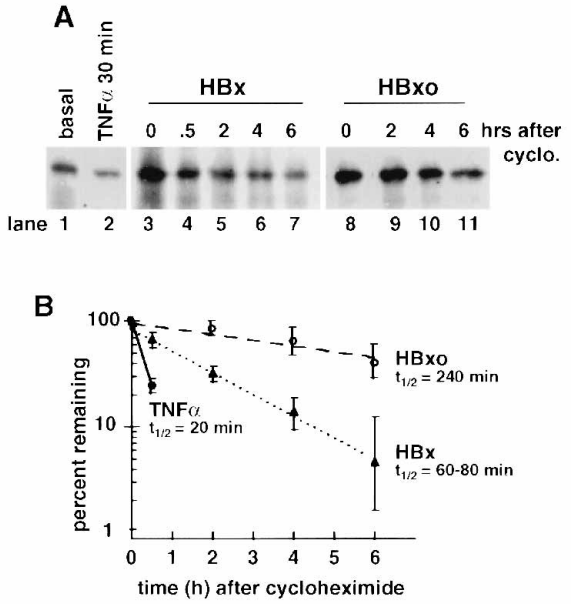

FIG. 5. Rates of IκBα protein turnover in Chang cells expressing wild-type HBx or HBxo proteins. Quiescent Chang cells were infected with 25 PFU of Ad CMV-X or Ad CMV-Xo per ml for 7 h or treated with 20 ng of TNF-α per ml for 30 min, and then 50 μg of cycloheximide (cyclo.) per ml was added for 0 to 6 h to prevent de novo protein synthesis. (A) Whole-cell extracts were prepared, equal protein amounts were resolved by SDS-PAGE (12% polyacrylamide) and transferred to nitrocellulose, and IκBα protein was detected by immunoblotting with an antibody directed to the C terminus of IκBα and enhanced chemiluminescence. (B) Protein bands were quantitated by PhosphorImager analysis. Data were plotted relative to the amount of IκBα protein present in the time zero lane for each sample and represent the average of three independent studies. Error bars represent the extent of experimental error from three trials.

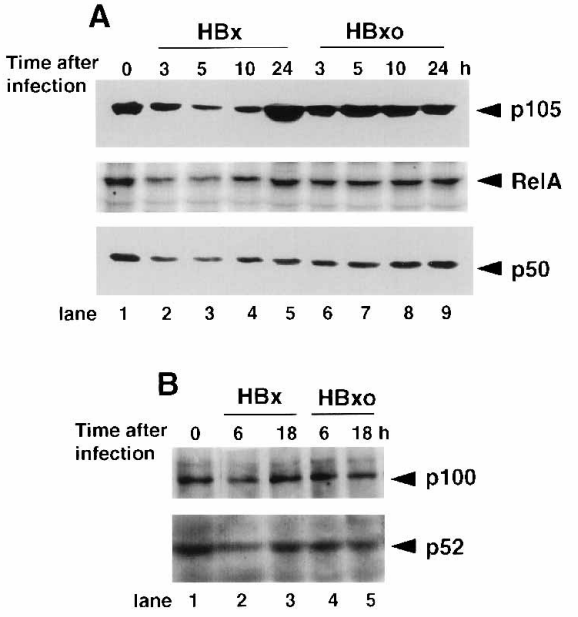

FIG. 6. HBx induces a coordinate decrease in the cytoplasmic level of NF-κB1 precursor p105 inhibitor and release of cytoplasmically sequestered RelA and p50 proteins. Quiescent Chang cells infected with Ad CMV-X or Ad CMV-Xo vectors for 1 to 24 h were processed into cytoplasmic and nuclear fractions. Nuclear fractions were used for detection of NF-κB DNA-binding activity corresponding to that in Fig. 1. Equal protein amounts of cytoplasmic fractions were resolved by SDS-PAGE (12% polyacrylamide), transferred to nitrocellulose, and immunoblotted with antibodies specific for NF-κB1 precursor p105, RelA, or p50 proteins (A) or p100 or p52 proteins (B). Immune complexes were detected by enhanced chemiluminescence and quantitated by densitometry.

considerably lower than that of IκBα induced by TNF-α treatment of cells and was similar to that reported for HTLV-1 Tax protein (51). We therefore questioned whether the marked increase in nuclear NF-κB DNA-binding activity induced by HBx could be fully attributed to release from IκBα or might also be associated with the release of Rel proteins from other cytoplasmic inhibitors of NF-κB. The activity of NF-κB is regulated by cytoplasmic sequestration with two types of inhibitors: (i) 37-kDa IκBα and (ii) p105, the precursor of p50 (NF-κB1), and p100, the precursor of p52 (NF-κB2). Both p105 and p100, as well as IκBα, bind RelA and *rel*-related proteins, mediating their cytoplasmic retention through a common C-terminal ankyrin motif (3, 14, 42, 55, 59, 61, 65, 67, 69, 71, 80). The regulation of p100 and p105 inhibitors is poorly understood, although some evidence suggests that the regulation of p100, p105, and IκBα may be distinct and may constitute alternate mechanisms for controlling the activation of NF-κB (59, 61, 69, 80).

The possibility that HBx targets alternate NF-κB inhibitors was probed by examining the steady-state cytoplasmic levels of p105, p50, and RelA as a function of time during NF-κB activation. Chang cells infected with Ad vectors expressing wild-type HBx or HBxo mutant genes were harvested at different times after introduction of the HBx genes, nuclear and cytoplasmic fractions were isolated, and equal amounts of cytoplasmic proteins were resolved by SDS-PAGE and subjected to immunoblot analysis with antibodies specific for p105, p50, and RelA polypeptides. Nuclear accumulation of NF-κB DNA-binding activity corresponded to the profile of NF-κB activation shown in Fig. 1. HBx induced a striking loss of p105 in the cytoplasm of cells (~10-fold) by 5 h after expression, which was sustained at 10 h and recovered above control cell levels (time zero) by 24 h (Fig. 6A). The loss of cytoplasmic p105 correlated with the nuclear accumulation of NF-κB DNA-binding activity (Fig. 1) and the increase in IκBα phosphorylation (Fig. 4). The decrease in cytoplasmic RelA protein levels also tracked with the decreased cytoplasmic level of p105 (Fig. 6A). Moreover, the increase in cytoplasmic p105 levels at 24 h paralleled a similar increase in RelA levels at this same time in HBx-expressing cells. As expected, there was no change in cytoplasmic p105 and RelA levels in cells expressing the HBxo gene. During the same period, cytoplasmic p50 levels also decreased in parallel with those of RelA and p105 but to a slightly lesser extent. Although the reason for the weaker change in the p50 protein levels is not known, it may reflect a greater cytoplasmic pool of p50 protein than RelA/p105 or perhaps stimulation of p105 processing by HBx, consistent with RelA release. Regardless, these data demonstrate that expression of HBx induced a coordinate decrease in the cytoplasmic level of p105 NF-κB1 precursor protein, release of cytoplasmically sequestered RelA protein, and nuclear accumulation of NF-κB DNA-binding activity, all with parallel kinetics.

The effect of HBx expression on the cytoplasmic levels of NF-κB2 p100 protein was also examined. The cytoplasmic level of p100 protein was analyzed by Western immunoblot analysis of equal amounts of cytoplasmic extracts with p52/p100 N-terminal specific antibody. HBx expression induced only a very slight reduction in the cytoplasmic level of p100 and p52 proteins (by 6 h) after HBx expression, and the level then recovered (Fig. 6B). HBxo expression induced no such alteration. Although both p105 and p100 precursor proteins undergo inducible phosphorylation, it has been shown previously that

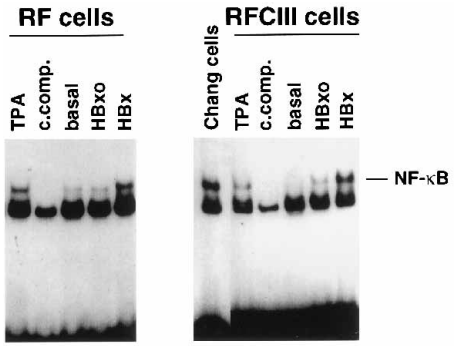

FIG. 7. HBx induces NF-κB DNA-binding complexes in cells lacking acidic SMase activity. Nieman-Pick disease skin fibroblasts deficient in acidic SMase activity (RF cells) or expressing a high level of acidic SMase by retrovirus-mediated transformation with the acidic SMase gene (RFIIIC cells) were infected with Ad CMV-X or Ad CMV-Xo at 25 PFU per cell for 12 h. Nuclear extracts were prepared, and NF-κB DNA-binding activity was detected by EMSA. The cold-competitor (c. comp.) assay and treatment of cells with TPA were carried out as described in the legend to Fig. 1. Basal refers to untreated and uninfected RF and RFIIIC cells, which grow very slowly. The Chang cell sample corresponds to a nuclear extract of TPA-treated cells. One-fourth as much Chang cell sample was loaded onto the gels as the amount of RF and RFIIIC cell extracts.

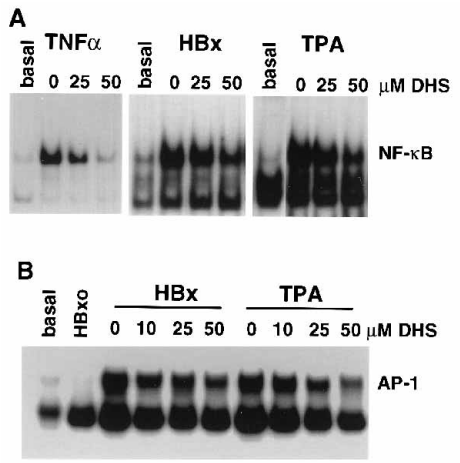

FIG. 8. Inhibition of sphingosine kinase does not block HBx induction of NF-κB. Quiescent Chang cells were treated with 10 to 50 μM DHS coupled to 0.1% BSA in DMEM for 30 min. The cells then received no further treatment (basal) or were treated with 20 μM TPA for 30 min or 20 ng of TNF-α per ml for 20 min or infected with 25 PFU of Ad CMV-X or Ad CMV-Xo per cell for 7 h. Equal protein amounts of nuclear extracts were examined for DNA-binding activity by EMSA corresponding to NF-κB (A) or AP-1 (B) transcription factors. DNA-protein complexes were resolved by PAGE (4% polyacrylamide) and then visualized and quantitated by PhosphorImager analysis. Basal refers to cells treated with DHS that received no further treatment.

p100 protein is either less efficiently processed or less responsive to release of Rel proteins (64).

Role of sphingomyelin-ceramide pathways in HBx activation of NF-κB. Studies have shown that TNF-α and interleukin-1β can activate the inactive, cytoplasmic pool NF-κB by initiating the plasma membrane conversion of sphingomyelin to ceramide by stimulating SMase (reviewed in references 35 and 50). Degradation of sphingomyelin to ceramide is carried out by a plasma membrane-associated neutral SMase (35, 50) and possibly also by a lysosomal acidic SMase (72). Ceramide in turn might activate NF-κB via activation of Raf and/or MAPKs (29, 54) or by production of phosphatidic acid, which is thought to stimulate Ras (25, 50). Since the mechanism for the release of RelA from NF-κB precursor proteins is not known, we investigated whether HBx stimulates the release of NF-κB by acting through a sphingomyelin-ceramide conversion pathway.

To investigate whether HBx might activate NF-κB via a pathway controlled by acidic SMase, we made use of a Neimann-Pick disease cell line deficient in acidic SMase activity (RF cells) and a derivative line in which a high level of acidic SMase activity was restored by retrovirus-mediated transformation of RF cells with the acidic SMase gene (RFCIII cells) (77). The cells were infected with Ad vectors expressing either wtHBx or HBxo genes, and nuclear extracts were prepared at 12 h for NF-κB EMSA (Fig. 7). A kinetic analysis of NF-κB activation in these cell lines had established this to be an optimal time point for induction (data not shown). HBx, but not HBxo, induced a specific NF-κB DNA-binding activity in both acidic SMase-negative (RF) and -positive (RFIIIC) cell lines. The DNA-binding activity was shown to correspond to authentic NF-κB because it comigrated with that induced by HBx in Chang cells or by TPA in RF and RFIIIC cells and could be specifically inhibited by an excess of unlabeled NF-κB oligonucleotide. These data therefore exclude HBx activation of NF-κB via a pathway controlled by acidic (lysosomal) SMase.

It was then determined whether HBx might activate NF-κB by stimulating neutral SMase activity. In particular, we asked whether HBx acts through sphingosine kinase and production of sphingosine-1-phosphate to stimulate Ras (25, 50). DHS is an antagonist of at least several sphingosine-1-phosphate activities, including production of phosphatidic acid but probably not calcium mobilization (19, 25, 90). Cells were treated with DHS and then infected with Ad-HBx vectors, or treated with TNF-α or TPA, and the effect of different concentrations of DHS on the activation of NF-κB or AP-1 was determined. Induction of AP-1 by TPA or HBx was used to determine the general influence of the drug on cell toxicity, since AP-1 has previously been shown to be induced by HBx through a Ras-dependent pathway (11, 13, 63). In Chang cells, DHS at 25 μM caused a ~5-fold impairment of TNF-α induction of NF-κB and DHS at 50 μM (a concentration thought to be cytotoxic to most cells) caused a ~25-fold impairment (Fig. 8A). The induction of NF-κB by TPA or HBx in Chang cells was only slightly impaired by DHS pretreatment. Nuclear accumulation of NF-κB DNA-binding activity was not reduced in HBx-expressing or TPA-treated cells exposed to 25 μM DHS, and at 50 μM DHS it was reduced ~3-fold compared with a 25-fold decrease for TNF-α. To determine whether the slight reduction in NF-κB induction observed in TPA-treated or HBx-expressing cells was due to general toxicity of DHS, the induction of AP-1 DNA-binding activity by HBx or TPA was examined (Fig. 8B). Pretreatment of Chang cells with DHS impaired induction of AP-1 by both HBx and TPA to a similar extent at a 50 μM dose, i.e., about threefold, the same reduction observed for induction of NF-κB. These data suggest that a neutral sphingomyelin-ceramide pathway is not likely to be involved in Ras-dependent HBx activation of NF-κB.

DISCUSSION

Several distinct mechanisms by which activation of cytoplasmically sequestered NF-κB can occur have been described. These include release from the prototypic 37-kDa IκBα cytoplasmic inhibitor (5–7; reviewed in reference 34), and release from the NF-κB1 and NF-κB2 precursor proteins, p105 and p100, respectively, which share a C-terminal ankyrin motif that causes retention of *rel*-related proteins in the cytoplasm (10, 32). Release of *rel*-related proteins from IκBα is mediated by

its phosphorylation and subsequent degradation, which can be induced (directly or indirectly) through a variety of distinct pathways involving protein kinase C, Ras, Raf, dsRNA activated protein kinase (PKR), c-Src, and ceramide-SMase-sphingosine kinase activities, among others (reviewed in reference 81). The mechanism for *rel*-related protein release from p105 and p100 precursor proteins is still not well known. For p105, activation of NF-κB correlates with phosphorylation of the precursor and possibly enhanced proteolytic processing (8, 31, 64).

We explored the manner by which HBV HBx protein activates NF-κB and characterized the DNA-binding complexes that were formed. These data should help to establish the fundamental activities of HBx protein, its mechanism of action, and the molecular partners with which it interacts. By introducing the HBx gene via a replication-defective Ad vector, we found that HBx rapidly induces NF-κB DNA-binding complexes, which persist for a prolonged period (Fig. 1; >24 to 30 h). HBx induced NF-κB complexes containing all of the *rel*-related proteins typically found in fibroblasts: p50 p52, RelA, and c-Rel (Fig. 2). We could find no evidence for HBx activation of the acidic sphingomyelinase pathway, which has been implicated in TNF-α activation of NF-κB (reviewed in references 35 and 50). HBx stimulated the formation of NF-κB in Niemann-Pick disease skin fibroblasts that are deficient in acidic SMase, and it did so to a level similar to that of cells that were genetically identical except for restoration of acidic SMase activity (Fig. 7). The possible role of the ceramide pathway controlled by neutral SMase and sphingosine kinase in Ras-dependent HBx activity was examined by use of the sphingosine-1-phosphate inhibitor, DHS, which prevents the formation of phosphatidic acid and potential activation of Ras. DHS did not specifically block HBx activation of NF-κB (Fig. 8). We found that inhibition of HBx induction of Ras activation strongly blocked the ability of HBx to activate NF-κB (Fig. 3). HBx was shown previously to rapidly activate the Ras-Raf-MAPK signalling cascade (within 3 to 5 h), which is essential for its activation of AP-1 (Fig. 3) (11–13, 63). Thus, the data presented in Fig. 3 and those published previously collectively indicate that HBx activation of NF-κB, like that of AP-1, requires HBx stimulation of the Ras signalling pathway. The results found here are seemingly in contrast to those of Chirillo et al. (23). Although the reason for the difference is not apparent, several possible explanations arise. First, it is possible that HBx activates NF-κB by inherently different mechanisms in Chang cells (used in our study) and HeLa cells. Second, it is not known whether the same level of Ras activation is required for induction of both AP-1 and NF-κB. The extent of Ras suppression by transfection of a dominant-interfering expression plasmid was not directly measured here or by Chirillo et al. (23). It is perhaps possible that a lower or residual level of Ras activation is sufficient to activate NF-κB. Third, it is possible that the HBx plasmid used by Chirillo et al. (23) generated dsRNA, which is a common occurrence during transfection (43, 44), and which can activate NF-κB through PKR, independently of Ras signalling cascades (reviewed in reference 81).

Since activation of NF-κB is often mediated by phosphorylation of IκBα, which promotes rapid degradation of the inhibitor through a proteosome-mediated pathway (67, 82), we investigated whether this constitutes the major mechanism for HBx activation of NF-κB. HBx strongly and rapidly induced the phosphorylation of 30 to 50% of the pool of IκBα protein, which was maintained at steady state (Fig. 4). Surprisingly, this resulted in only a three- to fourfold decrease in total IκBα protein stability, as determined by pulse-chase analysis (Fig. 5). In this regard, HBx protein and HTLV-1 Tax protein seem to display similar effects on the pool of IκBα protein with regard to phosphorylation and stability (51). These data suggest that enhanced degradation of IκBα could account for only a portion of the NF-κB protein activated by HBx. An alternate mechanism of control, in which the cytoplasmic sequestration of RelA by NF-κB1 p105 precursor protein was a target of HBx activity, was also found (Fig. 4). Therefore, in addition to IκBα degradation, HBx coordinately induced a striking decrease in cytoplasmic levels of p105, RelA, and p52 proteins, commensurate with increased nuclear accumulation of NF-κB DNA-binding activity. Parenthetically, in the study by Chirillo et al. (23), HBx also induced only a moderate decrease in IκBα levels, and overexpression of IκBα abrogated the induction of NF-κB by only about 50%, consistent with the activation of NF-κB by alternate mechanisms.

It is not known how HBx causes the release of RelA from p105 or how the reduction in cytoplasmic p105 levels takes place. Little is known of the mechanisms for regulation of p105 and p100 NF-κB inhibitor proteins. Since p105 is expressed in cells at a level similar to that of other NF-κB proteins (15), the cytoplasmic sequestration of *rel*-related proteins probably constitutes a significant means of regulating nuclear importation of active NF-κB (reviewed in reference 81). In our studies, HBx activation of Ras signalling cascades appears to be essential for activation of NF-κB, suggesting that the p105 precursor protein is also a target of cytoplasmic signalling pathways. Moreover, evidence indicates that the release of *rel*-related proteins from p105 correlates with its phosphorylation (31, 64), although this mechanism has not been directly proven. As with IκBα, phosphorylation of p105 could then lead to degradation of p105 or to proteolytic processing to remove the ankyrin-rich domain and release of *rel*-related proteins. Like HBx, the mechanism by which HTLV-1 Tax protein induces the displacement of *rel*-related proteins from NF-κB precursor inhibitor proteins is not known (40, 42, 61, 88). Like HBx, Tax protein also induces phosphorylation and rapid turnover of IκBα (40). Although Tax can associate with IκB and NF-κB proteins (42, 88), it is not clear how this is related to activation of NF-κB. In particular, evidence has been presented implicating indirect activation of NF-κB by Tax through an effect on cell signalling cascades (41). Thus, there may be similarities between the mechanisms used by HBx and Tax to induce the activation of NF-κB.

Our previous results demonstrated that HBx must act in the cytoplasm to activate NF-κB (26). Coupled to the results presented here, it is clear that HBx acts on the normally inactive, cytoplasmically sequestered pool of NF-κB proteins. Moreover, we can also largely exclude the possibility that HBx acts directly on NF-κB proteins, for example by causing their physical dissociation from IκB inhibitors. This is supported by three lines of evidence: (i) inhibition of Ras activity blocks HBx activation of NF-κB; (ii), HBx induces coupled phosphorylation and degradation of IκBα; and (iii) HBx could not be found directly associated with any NF-κB proteins (data not shown). Studies are now currently characterizing the mechanism for HBx-Ras-mediated release of NF-κB sequestered by both IκBα and p105 precursor proteins.

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REFERENCES

1. **Andrews, N. C., and D. V. Faller.** 1991. A rapid micropreparation technique for extraction of DNA binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res. **19:**2499.
2. **Arenzana-Seisdedos, F., J. Thompson, M. S. Rodriguez, F. Bachelerie, D. Thomas, and T. T. Hay.** 1995. Inducible nuclear expression of newly synthesized IκBα negatively regulates DNA-binding and transcriptional activities of NF-κB. Mol. Cell. Biol. **15:**2689–2696.
3. **Arima, N., J. A. Molitor, M. R. Smith, J. H. Kim, Y. Daitoku, and W. C. Greene.** 1991. Human T-cell leukemia type I Tax induces expression of the Rel-related family of κB enhancer-binding proteins: evidence for a pretranslational component of regulation. J. Virol. **65:**6892–6899.
4. **Aufiero, B., and R. J. Schneider.** 1990. The hepatitis B virus X-gene product transactivates both RNA polymerase II and III promoters. EMBO J. **9:**497–504.
5. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity of an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53:**211–217.
6. **Baeuerle, P. A., and D. Baltimore.** 1989. The 65-kDa subunit of active NF-κB is required for inhibition of NF-κB by IκB. Genes Dev. **3:**1689–1698.
7. **Baeuerle, P. A., M. Lenardo, J. W. Pierce, and D. Baltimore.** 1988. Phorbol ester induced activation of the NF-κB transcription factor involves dissociation of an apparently cytoplasmic NF-κB/inhibitor complex. Cold Spring Harbor Symp. Quant. Biol. **53:**789–798.
8. **Beg, A. A., and A. S. Baldwin.** 1993. The IκB proteins: mutifunctional regulators of Rel/NF-κB transcription factors. Genes Dev. **7:**2064–2070.
9. **Beg, A. A., T. S. Finco, P. V. Nantermet, and A. S. Baldwin.** 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. **13:**3301–3310.
10. **Beg, A. A., S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. S. Baldwin.** 1992. IκB intertacts with the nuclear localization sequence of the subunits of NF-κB: a mechanism for cytoplasmic retention. Genes Dev. **6:**1899–1913.
11. **Benn, J., and R. J. Schneider.** 1994. Hepatitis B virus HBx protein activates Ras-GTP complex formation and establishes a Ras, Raf, MAP kinase signalling cascade. Proc. Natl. Acad. Sci. USA **91:**10350–10354.
12. **Benn, J., and R. J. Schneider.** 1995. Hepatitis B virus HBx protein deregulates cell cycle checkpoint controls. Proc. Natl. Acad. Sci. USA **92:**11215–11219.
13. **Benn, J., F. Su, M. Doria, and R. J. Schneider.** Hepatitis B virus HBx protein induces transcription factor AP-1 by activation of extracellular signal-regulated and c-Jun N-terminal mitogen-activated protein kinases. J. Virol., in press.
14. **Beraud, C., S.-C. Sun, P. Ganchi, D. W. Ballard, and W. C. Greene.** 1994. Human T-cell leukemia virus type I Tax associates with and is negatively regulated by the NF-κB2 p100 gene product: implications for viral latency. Mol. Cell. Biol. **14:**1374–1382.
15. **Blank, V., P. Kourilsky, and A. Israel.** 1991. Cytoplasmic retention, DNA binding and processing of the NF-κB p50 precursor are controlled by a small region in its C-terminus. EMBO J. **10:**4159–4167.
16. **Bours, V., J. Villalobos, P. R. Burd, K. Kelly, and U. Siebenlist.** 1990. Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the rel oncogene and to cell-cycle motifs. Nature (London) **348:**76–80.
17. **Brockman, J. A., D. C. Scherer, T. A. McKinsey, S. M. Hall, X. Qi, W. Y. Lee, and D. W. Ballard.** 1995. Coupling of a signal response domain in IκBα to multiple pathways for NF-κB activation. Mol. Cell. Biol. **15:**2809–2818.
18. **Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist.** 1993. Mutual regulation of the transcriptional activator NF-κB and its inhibitor IκBα. Proc. Natl. Acad. Sci. USA **90:**2532–2536.
19. **Buehrer, B. N., and R. M. Bell.** 1992. Inhibition of sphingosine kinase in vitro and in platelets. J. Biol. Chem. **267:**3154–3159.
20. **Chen, H., S. Kaneko, R. Girones, R. W. Anderson, W. E. Hornbuckle, B. C. Tennant, P. J. Cote, J. L. Gerin, R. H. Purcell, and R. H. Miller.** 1993. The woodchuck hepatitis virus X gene is important for establishment of virus infection in woodchucks. J. Virol. **67:**1218–1226.
21. **Cheong, J.-H., M.-K. Yi, Y. Lin, and S. Murakami.** 1995. Human RPB5, a subunit shared by eukaryotic nuclear polymerases, binds human hepatitis B virus X protein and may play a role in X transactivation. EMBO J. **14:**143–150.
22. **Chiao, P. J., S. Miyamoto, and I. Verma.** 1994. Autoregulation of IκBa activity. Proc. Natl. Acad. Sci. USA **91:**28–32.
23. **Chirillo, P., M. Falco, P. L. Puri, M. Artini, C. Balsano, M. Levrero, and G. Natoli.** 1996. Hepatitis B virus pX activates NF-κB-dependent transcription through a Raf-independent pathway. J. Virol. **70:**641–646.
24. **Cross, J. C., P. Wen, and W. J. Rutter.** 1993. Transactivation by hepatitis B virus X protein is promiscuous and dependent on mitogen activated cellular serine/threonine kinases. Proc. Natl. Acad. Sci. USA **90:**8078–8082.
25. **Desai, N. N., H. Zhang, A. Olivera, M. E. Mattie, and S. Spiegel.** 1992. Sphingosine-1-phosphate, a metabolite of sphingosine, increases phosphatidic acid levels by phospholipase D activation. J. Biol. Chem. **267:**23122–23128.
26. **Doria, M., N. Klein, R. Lucito, and R. J. Schneider.** 1995. Hepatitis B virus HBx protein is a dual specificity cytoplasmic activator of Ras and nuclear activator of transcription factors. EMBO J. **14:**4747–4757.
27. **Faktor, O., and Y. Shaul.** 1990. The identification of hepatitis B virus X gene responsive elements reveals functional similarity of X and HTLV-I tax. Oncogene **5:**867–872.
28. **Feig, L. A., and G. M. Cooper.** 1988. Inhibition of NIH 3T3 cell proliferation by a mutant Ras protein with preferential affinity for GDP. Mol. Cell. Biol. **8:**3235–3243.
29. **Finco, T. S., and A. S. Baldwin.** 1993. Kappa B site dependent induction of gene expression by diverse inducers of nuclear factor kappa B requires Raf-1. J. Biol. Chem. **268:**17676–17679.
30. **Finco, T. S., A. A. Beg, and A. S. Baldwin.** 1994. Inducible phosphorylation of IκBa is not sufficient for its dissociation from NF-κB and is inhibited by protease inhibitors. Proc. Natl. Acad. Sci. USA **91:**11884–11888.
31. **Fujimoto, K., H. Yasuda, Y. Sato, and K.-I. Yamamoto.** 1995. A role for phosphorylation in the proteolytic processing of the human NF-κB1 precursor. Gene **165:**183–189.
32. **Ganchi, P. A., S.-C. Sun, W. C. Greene, and D. W. Ballard.** 1992. IκB/MAD-3 masks the nuclear localization signal of NF-κB p65 and acts with the C-terminal activation domain to inhibit NF-κB p65 DNA binding. Mol. Cell. Biol. **3:**1339–1352.
33. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to rel and dorsal. Cell **62:**1019–1029.
34. **Grilli, M., J. Chiu, and M. Lenardo.** 1993. NF-kB and Rel: participants in a multiform transcriptional regulatory system. Int. Rev. Cytol. **143:**1–62.
35. **Hannun, Y. A.** 1994. The sphingomyelin cycle and the second messenger function of ceramide. J. Biol. Chem. **269:**3125–3128.
36. **Haviv, I., D. Vaizel, and Y. Shaul.** 1995. The X protein of hepatitis B virus coactivates potent activation domains. Mol. Cell. Biol. **15:**1079–1085.
37. **Henkel, T., T. Machleidt, L. Alkalay, M. Kronke, Y. Ben-Neriah, and P. A. Baeuerle.** 1993. Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. Nature (London) **365:**182–185.
38. **Hohne, M., S. Schaefer, M. Seifer, M. A. Feitelson, D. Paul, and W. H. Gerlich.** 1990. Malignant transformation of immortalized transgenic hepatocytes after transfection with hepatitis B virus DNA. EMBO J. **9:**1137–1145.
39. **Hu, K.-Q., J. M. Vierling, and A. Siddiqui.** 1990. Trans-activation of HLA-DR gene by hepatitis B virus X gene product. Proc. Natl. Acad. Sci. USA **87:**7140–7144.
40. **Kanno, T., K. Brown, G. Franzoso, and U. Siebenlist.** 1994. Kinetic analysis of human T-cell leukemia virus type I Tax-mediated activation of NF-κB. Mol. Cell. Biol. **14:**6443–6451.
41. **Kanno, T., K. Brown, and U. Siebenlist.** 1995. Evidence in support of a role for human T-cell leukemia virus type I Tax in activating NF-κB via stimulation of signalling pathways. J. Biol. Chem. **270:**11745–11748.
42. **Kanno, T., G. Franzoso, and U. Siebenlist.** 1994. Human T-cell leukemia virus type I Tax-protein-mediated activation of NF-κB from p100 (NF-κB2)-inhibited cytoplasmic reservoirs. Proc. Natl. Acad. Sci. USA **91:**12634–12638.
43. **Kaufman, R. J., M. V. Davies, V. K. Pathak, and J. W. B. Hershey.** 1989. The phosphorylation state of eukaryotic initiation factor 2 alters translational efficiency of specific mRNAs. Mol. Cell. Biol. **9:**946–958.
44. **Kaufman, R. J., and P. Muhrta.** 1987. Translational control mediated by eucaryotic initiation factor 2 is restricted to specific mRNAs in transfected cells. Mol. Cell. Biol. **7:**1568–1571.
45. **Kekule, A. S., U. Lauer, L. Weiss, B. Luber, and P. H. Hofschneider.** 1993. Hepatitis B virus transactivator HBx uses a tumor promoter signalling pathway. Nature (London) **361:**742–745.
46. **Kieran, M., V. Blank, F. Logeat, J. Vanderkerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israel.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell **62:**1007–1018.
47. **Kim, C.-M., K. Koike, I. Saito, T. Miyamura, and G. Jay.** 1991. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature (London) **353:**317–320.
48. **Koike, K., K. Moriya, S. Iino, H. Yotsuyanagi, Y. Endo, T. Miyamura, and K. Kurokawa.** 1994. High-level expression of hepatitis B virus HBx gene and heptocellular carcinogenesis in transgenic mice. Hepatology **19:**810–819.
49. **Koike, K., K. Moriya, H. Yotsuyanagi, S. Iino, and K. Kurokawa.** 1994. Induction of cell cycle progression by hepatitis B virus HBx gene expression in quiescent mouse fibroblasts. J. Clin. Invest. **94:**44–49.
50. **Kolesnick, R., and D. W. Golde.** 1994. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signalling. Cell **77:**325–328.
51. **Lacoste, J., L. Petropoulos, N. Pepin, and J. Hiscott.** 1995. Constitutive phosphorylation and turnover of IκBα in human T-cell leukemia virus type I-infected and Tax-expressing T cells. J. Virol. **69:**564–569.
52. **LeBail, O., U. R. Schmidt, and A. Israel.** 1993. Promoter analysis of the gene encoding the IκBα/MAD-3 inhibitor of NF-κB: positive regulation by members of the rel/NF-κB family. EMBO J. **12:**5043–5049.
53. **Levrero, M., C. Balsano, G. Natoli, M. L. Avantaggiati, and E. Elfassi.** 1990. Hepatitis B virus X protein transactivates the long terminal repeats of human immunodeficiency virus types 1 and 2. J. Virol. **64:**3082–3086.

54. **Li, S., and J. M. Sedivy.** 1993. Raf-1 protein kinase activates the NF-κB transcription factor by dissociating the cytoplasmic NF-κB-IκB complex. Proc. Natl. Acad. Sci. USA **90:**9247–9251.
55. **Liptay, S., R. M. Schmid, E. G. Nabel, and G. J. Nabel.** 1994. Transcriptional regulation of NF-κB2: evidence for κB-mediated positive and negative autoregulation. Mol. Cell. Biol. **14:**7695–7703.
56. **Lucito, R., and R. J. Schneider.** 1992. Hepatitis B virus X protein activates transcription factor NF-κB without a requirement for protein kinase C. J. Virol. **66:**983–991.
57. **Maguire, H. F., J. P. Hoeffler, and A. Siddiqui.** 1991. HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein-protein interactions. Science **252:**842–844.
58. **Mahe, Y., N. Mukaida, K. Kuno, M. Akiyama, N. Ikeda, K. Matshushima, and S. Murakami.** 1991. Hepatitis B virus X protein transactivates human interleukin-8 gene through acting on nuclear factor κB and CCAAT/enhancer -binding protein-like cis elements. J. Biol. Chem. **266:**13759–13763.
59. **Mercurio, F., J. A. DiDonato, C. Rosette, and M. Karin.** 1993. p105 and p98 precursor proteins play an active role in NF-κB mediated signal transduction. Genes Dev. **7:**705–718.
60. **Meyer, M., W. H. Caselmann, V. Schluter, R. Schreck, P. H. Hofschneider, and P. A. Baeuerle.** 1992. Hepatitis B virus transactivator MHBst: activation of NF-κB, selective inhibition by antioxidants and integral membrane localization. EMBO J. **11:**2991–3001.
61. **Munoz, E., G. Courtois, P. Veschambre, P. Jalinot, and A. Israel.** 1994. Tax induces nuclear translocation of NF-κB through dissociation of cytoplasmic complexes containing p105 or p100 but does not induce degradation of IκBα/MAD3. J. Virol. **68:**8035–8044.
62. **Natoli, G., M. L. Avantaggiati, P. Chirillo, A. Costanzo, M. Artini, C. Balsano, and M. Levrero.** 1994. Induction of the DNA-binding activity of c-Jun/c-Fos heterodimers by the hepatitis B virus transactivator pX. Mol. Cell. Biol. **14:**989–998.
63. **Natoli, G., M. L. Avantaggiati, P. Chirillo, P. L. Puri, A. Ianni, C. Balsano, and M. Levrero.** 1994. Ras- and raf-dependent activation of c-jun transcriptional activity by the hepatitis B virus transactivator pX. Oncogene **9:**2837–2843.
64. **Naumann, M., and C. Scheidereit.** 1994. Activation of NF-κB in vivo is regulated by multiple phosphorylations. EMBO J. **13:**4597–4607.
65. **Naumann, M., F. G. Wulczyn, and C. Scheidereit.** 1993. The NF-κB precursor p105 and the proto-oncogene product Bcl-3 are IκB molecules and control nuclear translocation of NF-κB. EMBO J. **12:**213–222.
66. **Ohno, H., G. Takimoto, and W. McKeithan.** 1990. The candidate proto-oncogene bcl-3 is related to genes implicated in cell lineage determination and cell cycle control. Cell **60:**991–997.
67. **Palombella, V. J., O. J. Rando, A. L. Goldberg, and T. Maniatis.** 1994. The ubiquitin-proteosome pathway is required for processing the NF-κB1 precursor protein and activation of NF-κB. Cell **78:**773–785.
68. **Qadri, I., H. F. Maguire, and A. Siddiqui.** 1995. Hepatitis B virus transactivator protein X interacts with the TATA-binding protein. Proc. Natl. Acad. Sci. USA **92:**1003–1007.
69. **Rice, N. R., M. L. MacKichan, and A. Israel.** 1992. The precursor of NF-κB p50 has IκB-like functions. Cell **71:**243–253.
70. **Ron, D., A. R. Brasier, K. A. Wright, J. E. Tate, and J. F. Habener.** 1990. An inducible 50-kDa NF-κB-like protein and a constitutive protein both bind the acute-phase response element of the angiotensinogen gene. Mol. Cell. Biol. **10:**1023–1032.
71. **Scheinman, R. I., A. A. Beg, and A. S. Baldwin.** 1993. NF-κB p100 (Lyt-10) is a component of H2TF1 and can function as an IκB-like molecule. Mol. Cell. Biol. **13:**6089–6101.
72. **Schutze, S., K. Potthoff, T. Machleidt, D. Berkovic, K. Wiegmann, and M. Kronke.** 1992. TNF activates NF-κB by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingmyelin breakdown. Cell **71:**765–776.
73. **Sen, R., and D. Baltimore.** 1986. Inducibility of K immunoglobulin enhancer binding protein NF-κB by a posttranslational mechanism. Cell **47:**921–928.
74. **Seto, E., P. J. Mitchell, and T. S. B. Yen.** 1990. Transactivation by the hepatitis B virus X protein depends on AP-2 and other transcription factors. Nature (London) **344:**72–74.
75. **Seto, E., T. S. B. Yen, B. M. Peterlin, and J.-H. Ou.** 1988. Trans-activation of the human immunodeficiency virus long terminal repeat by the hepatitis B virus X protein. Proc. Natl. Acad. Sci. USA **85:**8290–8286.
76. **Siddiqui, A., R. Gaynor, A. Srinivasan, J. Mapoles, and R. W. Farr.** 1989. Trans-activation of viral enhancers including long terminal repeat of the human immunodeficiency virus by the hepatitis B virus X protein. Virology **169:**479–484.
77. **Suchi, M., T. Dinur, R. J. Desnick, S. Gatt, L. Pereira, E. Gilboa, and E. H. Schuchman.** 1992. Retroviral-mediated transfer of the human acid sphingomyelinase cDNA: correction of the metabolic defect in cultured Niemann-Pick disease cells. Proc. Natl. Acad. Sci. USA **89:**3227–3231.
78. **Sun, S., P. A. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. Science **259:**1912–1915.
79. **Sun, S.-C., J. Elwood, C. Beraud, and W. C. Greene.** 1994. Human T-cell leukemia virus type I Tax activation of NF-κB/Rel involves phosphorylation and degradation of IκBα and RelA (p65)-mediated induction of the c-*rel* gene. Mol. Cell. Biol. **14:**7377–7384.
80. **Sun, S.-C., P. A. Ganchi, C. Beraud, D. W. Ballard, and W. C. Greene.** 1994. Autoregulation of the NF-κB transactivator RelA (p65) by multiple cytoplasmic inhibitors containing ankyrin motifs. Proc. Natl. Acad. Sci. USA **91:**1346–1350.
81. **Thanos, D., and T. Maniatis.** 1995. NF-κB: a lesson in family values. Cell **80:**529–532.
82. **Traenckner, E. B.-M., S. Wilk, and P. A. Baeuerle.** 1994. A proteosome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylated form of IκBα that is still bound to NF-κB. EMBO J. **13:**5433–5441.
83. **Twu, J.-S., M.-Y. Lai, D.-S. Chen, and W. S. Robinson.** 1993. Activation of protooncogene c-jun by the X protein of hepatitis B virus. Virology **192:**346–350.
84. **Twu, J.-S., and W. S. Robinson.** 1989. Hepatitis B virus X gene can transactivate heterologous viral sequences. Proc. Natl. Acad. Sci. USA **86:**2046–2050.
85. **Twu, J. S., and R. H. Schloemer.** 1987. Transcriptional *trans*-activating function of hepatitis B virus. J. Virol. **61:**3448–3453.
86. **Unger, T., and Y. Shaul.** 1990. The X protein of the hepatitis B virus acts as a transcription factor when targeted to its responsive element. EMBO J. **9:**1889–1895.
87. **Wang, H.-D., C.-H. Yuh, C. V. Dang, and D. L. Johnson.** 1995. The hepatitis B virus X protein increases the cellular level of TATA-binding protein which mediates transactivation of RNA polymerase III genes. Mol. Cell. Biol. **15:**6720–6728.
88. **Watanabe, M., M.-A. Muramatsu, H. Hirai, T. Suzuki, J. Fujisawa, M. Yoshida, K.-I. Arai, and N. Arai.** 1993. HTLV-I encoded Tax in association with NF-κB precursor p105 enhances nuclear localization of NF-κB p50 and p65 in transfected cells. Oncogene **8:**2949–2958.
89. **Williams, J. S., and O. M. Andrisani.** 1995. The hepatitis B virus X protein targets the basic region-leucine zipper domain of CREB. Proc. Natl. Acad. Sci. USA **92:**3819–3823.
90. **Wolff, R. A., R. T. Dobrowsky, A. Bielawska, L. M. Obeid, and Y. A. Hannun.** 1994. Role of ceramide-activated protein phosphatase in ceramide-mediated signal transduction. J. Biol. Chem. **269:**19605–19609.
91. **Yoshimura, K., M. A. Rosenfeld, P. Seth, and R. G. Crystal.** 1993. Adenovirus-mediated augmentation of cell transfection with unmodified plasmid vectors. J. Biol. Chem. **268:**2300–2303.
92. **Zabel, U., and P. A. Baeuerle.** 1990. Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. Cell **61:**255–265.
93. **Zahm, P., P. H. Hofschneider, and R. Koshy.** 1988. The HBV X-ORF encodes a transactivator: a potential factor in viral hepatocarcinogenesis. Oncogene **3:**169–177.
94. **Zhou, D.-X., A. Taraboulous, J.-H. Ou, and T. S. B. Yen.** 1990. Activation of class I major histocompatibility complex gene expression by hepatitis B virus. J. Virol. **64:**4025–4028.
95. **Zoulim, F., J. Saputelli, and C. Seeger.** 1994. Woodchuck hepatitis virus X protein is required for viral infection in vivo. J. Virol. **68:**2026–2030.