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# Activation of Src Family Kinases by Hepatitis B Virus HBx Protein and Coupled Signaling to Ras

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**The HBx protein of hepatitis B virus (HBV) is a small transcriptional transactivator that is essential for infection by the mammalian hepadnaviruses and is thought to be a cofactor in HBV-mediated liver cancer. HBx stimulates signal transduction pathways by acting in the cytoplasm, which accounts for many but not all of its transcriptional activities. Studies have shown that HBx protein activates Ras and downstream Ras signaling pathways including Raf, mitogen-activated protein (MAP) kinase kinase kinase (MEK), and MAP kinases. In this study, we investigated the mechanism of activation of Ras by HBx because it has been found to be central to the ability of HBx protein to stimulate transcription and to release growth arrest in quiescent cells. In contrast to the transient but strong stimulation of Ras typical of autocrine factors, activation of Ras by HBx protein was found to be constitutive but moderate. HBx induced the association of Ras upstream activating proteins Shc, Grb2, and Sos and stimulated GTP loading onto Ras, but without directly participating in complex formation. Instead, HBx is shown to stimulate Ras-activating proteins by functioning as an intracellular cytoplasmic activator of the Src family of tyrosine kinases, which can signal to Ras. HBx protein stimulated c-Src and Fyn kinases for a prolonged time. Activation of Src is shown to be indispensable for a number of HBx activities, including activation of Ras and the Ras-Raf-MAP kinase pathway and stimulation of transcription mediated by transcription factor AP-1. Importantly, HBx protein expressed in cultured cells during HBV replication is shown to activate the Ras signaling pathway. Mechanisms by which HBx protein might activate Src kinases are discussed.**

Hepatitis B viruses (HBV) are small hepatotropic pararetroviruses that replicate by reverse transcription and establish persistent liver infection in humans and other animals (reviewed in reference 25). There is considerable interest in HBV because chronic infection is strongly associated with development of primary liver cancer (hepatocellular carcinoma [HCC]) in humans and mammals, which is one of the most prevalent forms of human cancer worldwide (4). A number of factors, including the inflammatory response, integration of viral genomes into cellular chromosomes, and expression of the viral HBx gene, have been linked to development of HCC during chronic infection (reviewed in references 14 and 70); the latter is the subject of this report. The HBx gene is found only in the mammalian hepadnaviruses and encodes a small (154-amino-acid, 17-kDa) polypeptide which is synthesized during HBV replication (70). The role of HBx in HBV infection has not been clearly defined, although genetic studies indicate that it is essential for infection by the woodchuck hepatitis B virus (WHV) (12, 75) and is therefore presumably required for infection by the other mammalian hepadnaviruses, including human HBV, all of which are highly related. A recent report provided a compelling correlation between expression of WHV HBx protein (WHx) and active replication of the virus in woodchuck hepatocytes (19). In addition to its essential but undefined function in infection, HBx is thought to play a role in the development of HCC during chronic infection. Some investigators have reported that transgenic mice showed a correlation between high levels of HBx expression and development of HCC (31, 33). Although other groups have not found any direct correlation between HBx and HCC (19, 36), they did observe that HBx expression increased sensitivity to tumorigenic effects of hepatocarcinogens two- to threefold (19, 60). It is therefore likely that early events of carcinoma are facilitated by HBx, but the process probably also involves a particular genetic background, a high level of HBx expression, and/or specific environmental factors.

Although the roles of HBx in viral infection and carcinoma have been elusive, a large number of studies concur that HBx acts as a weak to moderately strong transcriptional transactivator (reviewed in reference 70). HBx has been shown to transactivate a wide variety of cellular and viral transcriptional elements, including the HBV enhancers and the human immunodeficiency virus long terminal repeat (38, 61), class III promoters (2, 67), proto-oncogenes c-*myc* (3), N-*myc* (64), and c-*jun* (7, 49), and transcription factors AP-1 (5, 49, 59), NF-κB (44, 62, 66), and ATF/CREB (46, 69). HBx does not contain any structural motifs that convincingly suggest a function, nor does it directly bind DNA (70). In vitro binding studies detected an association between p53 and HBx which was reported to inactivate p53 function (65, 68). However, this association could not be demonstrated in vivo (1). HBx was also reported to bind to the human homolog of a UV-damaged DNA repair protein involved in nucleotide excision repair (35) and to the C7 subunit of the proteasome complex (28), which might comprise additional targets of HBx activity.

Some studies indicated that HBx might stimulate transcription at the level of the promoter by binding transcription factors or components of RNA polymerase II (26, 27), such as the RPB5 subunit of RNA polymerase (13), TATA-binding protein (53), or ATF/CREB (46, 69). Although no consensus yet exists concerning the mechanism of action of HBx, and most of these data have been demonstrated only in vitro or by using synthetic approaches, these data taken collectively suggest that

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HBx might regulate transcription by interacting with certain cellular factors in the nucleus. Alternatively, HBx was also shown to stimulate transcription indirectly by activating cellular signal transduction pathways (5, 18, 30, 44, 50). Consistent with this scenario, HBx has been localized largely if not entirely to the cytoplasm in transfected cells (22, 74) and in WHV-infected livers (19). Furthermore, HBx activates AP-1 and NF-κB only when located in the cytoplasm (22). An HBx protein engineered to relocate to the nucleus by incorporation of a nuclear localization signal (NLS) could activate the HBV enhancer but lost the ability to stimulate Ras signaling (22). Several groups have now demonstrated that HBx activates Ras and the Ras–Raf–mitogen-activated protein (MAP) kinase signal transduction cascade (5, 18, 50). Ras is the prototype member of the low-molecular-weight family of GTPases, cycling between an inactive GDP-bound state (RasGDP) and an active GTP-bound state (RasGTP) (reviewed in reference 9). Ras converts upstream activating signals into downstream phosphorylating events by recruiting the kinase Raf to the plasma membrane. Raf activates MAP kinase kinase kinase (MEK), which in turn activates the family of MAP kinases, including the extracellular-signal-regulated protein kinases (ERKs). Activation of the Ras-Raf-MAP kinase signaling pathway was found to be essential for HBx activation of AP-1 and NF-κB (5, 7, 18, 50, 62). Moreover, HBx was shown to stimulate quiescent cells to enter S phase by deregulation of cell cycle checkpoint controls in a Ras-dependent manner (6), indicating that activation of Ras by HBx probably plays a central role in defining many HBx activities.

The objective of this study was to determine how HBx protein activates Ras. Given that HBx protein obligately requires Ras to activate transcription in many cells (5–7, 18, 50), an understanding of the mechanism by which HBx stimulates Ras and establishes a signal transduction pathway might clarify the role of HBx during HBV infection and possibly in carcinoma. It is shown in this study that HBx activates Ras by increasing GTP uptake onto Ras without altering Ras-specific GTPase-activating protein (RasGAP) activity. HBx is shown to stimulate RasGTP formation by indirectly acting on the upstream activators of Ras, by stimulating the association of Shc, Grb2, and the Ras GTP exchange factor Sos. However, HBx protein could not be found to directly complex with any of these proteins. Instead, it is demonstrated that HBx activates Ras by functioning as an intracellular cytoplasmic activator of the Src family of nonreceptor tyrosine kinases. HBx activation of Src kinase, and subsequent Src activation of Ras, is thereby shown to be the essential link by which HBx activates Ras signaling, stimulating the Ras-Raf-MAP kinase pathway and transcriptional transactivation mediated by transcription factor AP-1.

## MATERIALS AND METHODS

**Cell culture.** Cell lines used in this study were obtained from the American Type Culture Collection. Chang cells, NIH 3T3 cells, and 293 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (CS) and 100 μg of gentamicin sulfate per ml. Serum starvation of Chang cells was carried out in 0.05% CS, and that for NIH 3T3 cells was carried out in 0.5% CS, for 16 to 24 h.

**Transfections and Ad infections.** The replication-defective recombinant adenoviruses (Ads) express, under the control of the cytomegalovirus (CMV) promoter, either wild-type (wt) HBx (AdCMV-X) or a mutant HBx mRNA in which all the AUGs have been deleted (AdCMV-Xo). The constructions of the recombinant AdCMV-X and AdCMV-Xo have been previously described (22). Ads were propagated and titers were determined in 293 cells before use. Cells were transfected by the calcium phosphate precipitation technique. Cells ($6 \times 10^5$ to $7.5 \times 10^5$ per 10-cm-diameter plate) were transfected for 7 to 10 h (20 μg of DNA in total), after which the medium was aspirated and replaced with fresh 10% CS–DMEM (22). Eighteen hours after transfection, cells were serum starved prior to analysis of kinase activities (0.05% CS for Chang cells; 0.5% CS for NIH 3T3 cells). Cells were infected with AdCMV-X or AdCMV-Xo at 25 PFU/cell for 1 h in 1 ml of phosphate-buffered saline–2% CS, after which 10 ml of DMEM containing 0.5% (NIH 3T3 cells) or 0.05% (Chang cells) CS was added and the cells were harvested at the indicated times.

**HBV and WHV replication.** Chang cells ($10^6$/10-cm-diameter plate) were transfected with 10 μg of an infectious head-to-tail genomic plasmid dimer of wt or HBx-deficient mutant human HBV (wtHBV or X-21HBV, respectively [8]) or a plasmid containing a genomic cDNA copy of wt or WHx-deficient genome (wtWHV or CWHV, respectively [75]). At 3 days posttransfection, cells were serum starved in 0.5% CS for 18 h, and then lysates were analyzed for ERK2 activity as described below.

**Antibodies and plasmids.** Ras-specific monoclonal antibody Y13-259 was provided by A. Pellicer (New York University [NYU]). Rabbit anti-Src, anti-Shc, and anti-Sos sera were provided by J. Schlessinger (NYU). Rabbit anti-Grb2, anti-ERK2, anti-Csk, and anti-Fyn antibodies were purchased from Santa Cruz, Inc. Plasmid phSos expressing the human *Sos* (h*Sos*) gene under the control of the CMV promoter was a gift from J. Schlessinger. Plasmid pCaCsk was a gift from D. Littman (NYU) and expresses the *CSK* gene under the control of the CMV promoter. pCMV-X expresses wt HBx under the control of the CMV promoter. pCMV-Xo contains a mutant HBx gene in which all of the AUGs in the open reading frame have been mutated, eliminating synthesis of HBx protein (22). HBx-NLS contains a functional N-terminal NLS and exclusively relocates HBx protein to the nucleus (22).

**Uptake of GTP onto Ras.** Subconfluent 10-cm-diameter dishes of NIH 3T3 cells were starved as described above prior to infection and cell permeabilization. At 8 h postinfection (p.i.), cells were washed once with 37°C phosphate-buffered saline and then harvested in 0.8 ml of freshly prepared permeabilization buffer {150 mM KCl, 37.5 mM NaCl, 6.25 mM $MgCl_2$, 0.8 mM $CaCl_2$, 1 mM EGTA, 1.25 mM ATP, 12.5 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 7.4)}. After 0.2 ml of streptolysin O (Sigma) was added to a final concentration of 0.4 U/ml, the mixture was incubated at 37°C for 5 min. Then 10 μCi of [α-$^{32}$P]GTP (800 Ci/mmol; New England Nuclear) was added, followed by stimulation with platelet-derived growth factor (PDGF; BB homodimer; 50 ng/ml; Intergen) when indicated, and the samples were incubated at 37°C for the indicated times. The cells were then lysed in buffer (1% Triton, 20 mM Tris HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM $Na_3VO_4$, 50 mM NaF, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml) containing 5 μg of Ras-specific antibody Y13-259 for 30 min on ice. Equal amounts of protein were incubated with bovine serum albumin-blocked 10% charcoal for 60 min, followed by incubation with protein G-agarose. The immunoprecipitates were extensively washed with 20 mM Tris HCl (pH 7.5)–10 mM $MgCl_2$, and the nucleotides bound to Ras were eluted in 20 mM Tris HCl (pH 7.5)–20 mM EDTA–2% sodium dodecyl sulfate (SDS) by incubation at 65°C for 5 min. Nucleotides eluted from Ras were spotted onto polyethyleneimine cellulose (J. T. Baker), separated by ascending thin-layer chromatography (TLC) for 6 h in 0.75 M $KH_2PO_4$, autoradiographed, and quantitated by analysis with a Molecular Dynamics PhosphorImager and ImageQuant software.

**RasGAP assay.** NIH 3T3 cells were serum starved as described above. Following infection, S100 fractions were prepared as described previously (40). Equal amounts of protein extracts (prewarmed to 37°C) were incubated with 1 μg of bacterially produced c-H-Ras bound to [α-$^{32}$P]GTP (described below) that had been immobilized to an activated silica matrix (provided by A. Wolfman, Cleveland Clinic [48]). At the indicated times, the samples were removed, and 1 ml of cold p21 buffer (48) was added to stop the reaction. After microcentrifugation for 30 s, the nucleotides bound to Ras were eluted and analyzed by TLC and PhosphorImager quantitation as described above. c-H-Ras was labeled in vitro with [α-$^{32}$P]GTP by incubating the immobilized Ras in p21 buffer containing 20 mM EDTA and 10 μCi of [α-$^{32}$P]GTP on ice for 5 min. $MgCl_2$ was then added to 20 mM, followed by addition of 1 ml of cold p21 buffer. Following centrifugation and a p21 wash, immobilized c-H-Ras was stored in p21 buffer until use.

**Immunoprecipitation and Western immunoblotting.** Cells were lysed in 1% Triton–20 mM HEPES (pH 7.4)–150 mM NaCl–10% glycerol–1 mM $Na_3VO_4$–50 mM NaF–1 mM PMSF–10 μg of aprotinin per ml–10 μg of leupeptin per ml. Equal amounts of protein were immunoprecipitated on ice for 2 h, and the immune complexes were collected by incubation with protein A-agarose (Santa Cruz) for 60 min at 4°C. Immunoprecipitates were washed three times with HNTG (20 mM HEPES [pH 7.4], 150 mM NaCl, 0.1% Triton, 10% glycerol), resuspended in Laemmli sample buffer, heated to 95°C for 10 min, resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and immunoblotted to nitrocellulose by using standard techniques. Immunoblots were visualized by using an enhanced chemiluminescence (ECL) system (Amersham).

**Kinase assays.** To assay for MAP kinase activity, cells were lysed in freshly prepared lysis buffer (10 mM Tris HCl [pH 7.5], 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM $Na_3VO_4$, 50 mM NaF, 1 mM PMSF, 40 mM $PP_i$, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml), and MAP kinase was immunoprecipitated by using anti-ERK2 antibodies (1 μg/ml) from equal amounts of protein. After four washes with lysis buffer and two with kinase buffer (20 mM HEPES [pH 7.4], 10 mM $MgCl_2$), the immunoprecipitates were incubated with kinase buffer containing 0.5 mg of myelin basic protein (MBP; Sigma) per ml, 10 μM ATP, and 5 μCi of [γ-$^{32}$P]ATP for 30 min at 30°C. The reactions were stopped by the addition of sample loading buffer, resolved by SDS-PAGE (15% gel), visualized by PhosphorImager analysis, and quantitated

by using ImageQuant software. Src kinase assays were carried out as follows. NIH 3T3 or Chang cells were lysed in buffer containing 1% Nonidet P-40, 150 mM NaCl, 20 mM Tris HCl [pH 8], 2.5 mM EDTA, 1 mM $Na_3VO_4$, 50 mM NaF, 1 mM PMSF, 10 μg of aprotinin per ml, and 10 μg of leupeptin per ml. Src and Fyn kinase activities were measured by immunoprecipitating either c-Src or Fyn from equal amounts of cell lysate. The immunoprecipitates were washed four times with lysis buffer, once with 20 mM HEPES (pH 7.4), and once with kinase buffer (20 mM HEPES [pH 7.4], 10 mM $MnCl_2$). Immunoprecipitates were then resuspended in kinase buffer containing 0.2 μg of acid-denatured enolase (rabbit muscle; Sigma), 20 μCi of [γ-$^{32}$P]ATP, and 10 μM ATP and incubated at 30°C for 30 min. Kinase reactions containing enolase were stopped with 2× sample loading buffer, heated for 10 min at 95°C, and resolved by 10% SDS-PAGE (10% gel). Luciferase assays were performed as instructed by the manufacturer (Promega).

**Preparation of nuclear extracts.** Nuclear extracts were prepared according to a modified Dignam protocol (63). Cell pellets were resuspended in 250 μl of cold buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM PMSF, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml), swollen on ice for 10 min, vortexed for 10 s, and centrifuged for 10 s at 12,000 × *g*. Nuclear pellets were resuspended in 30 μl of cold buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 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.

## RESULTS

It has been previously shown that HBx activates p21$^{ras}$ (5, 50), which is required both for HBx stimulation of transcription factors AP-1 (5, 50) and NF-κB (63) and for deregulation of cell cycle checkpoints in serum-starved cells (5). To understand the role that Ras plays in defining HBx activities, the mechanism by which HBx activates Ras was investigated. The sequence of HBx protein does not provide any mechanistic clues to understanding how HBx stimulates Ras. Fundamental studies were therefore conducted to characterize both the mechanism of Ras activation by HBx and its duration.

There are several regulatory steps at which HBx could stimulate Ras activity. Ras is positively regulated by guanine nucleotide exchange factor, called hSos in humans, which converts inactive RasGDP into active RasGTP complex, and negatively regulated by RasGAP, which stimulates GTP hydrolysis. hSos is generally stimulated in response to activation of tyrosine kinase receptors, while RasGAP can be downregulated by specific signals (23). HBx might therefore stimulate Ras either by activating hSos or by inhibiting RasGAP. Activation of tyrosine kinase receptors by autocrine factors recruits Grb2 (growth factor receptor-bound protein 2) (43) and Sos to Ras (41), inducing its activation (1). HBx might therefore participate in or facilitate Grb2-Sos-Ras complex formation. The protein Shc is activated by Src kinase tyrosine phosphorylation, which in turn stimulates Ras by associating with Grb2-Sos (52, 55). HBx might therefore activate Ras by activating Src. In fact, the polyomavirus middle T (MT) antigen activates Ras by binding to c-Src (16), inducing tyrosine phosphorylation of Shc and subsequent binding of Shc to Grb2 (21). Studies presented below determined the point of involvement of HBx protein in Ras regulation.

**HBx stimulates GTP loading onto Ras rather than blocking RasGAP activity.** HBx protein expressed from a heterologous promoter transactivates transcription in many cell types, including NIH 3T3 cells, which have been used extensively to investigate Ras signaling. Wild-type HBx and a mutant lacking all AUG codons (HBxo) were expressed in serum-starved NIH 3T3 cells by introduction on a replication-defective recombinant Ad vector that lacks E1A and E1B genes (AdCMV-X and AdCMV-Xo, respectively). Ad vectors do not activate Ras in the absence of HBx expression (5, 7) and do not overexpress HBx protein if used at low multiplicities of infection (64). The activity of MAP kinase was assayed as a sensitive measure of the Ras-Raf-MAP kinase cascade. Cells were infected with

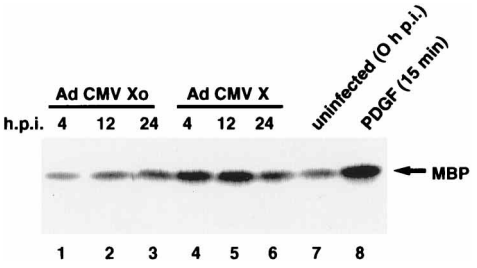


FIG. 1. Kinetic analysis of HBx activation of MAP kinase in NIH 3T3 cells. NIH 3T3 cells were cultivated in DMEM with 0.5% CS for 18 h (serum starved) and then infected with 25 PFU of AdCMV-X or -Xo vector per ml for the indicated times. Cell extracts were then prepared, ERK2 was immunoprecipitated from equal amounts of protein extracts, and pellets were assayed for the ability to phosphorylate MBP with [γ-$^{32}$P]ATP. Labeled MBP was resolved by SDS-PAGE (15% gel) followed by PhosphorImager analysis. Stimulation of cells with PDGF as a positive control was carried out with 50 ng/ml for 15 min.

AdCMV-X or -Xo and then assayed for HBx activation of MAP kinase by immunoprecipitating MAP kinase and incubating the pellets with MBP and [γ-$^{32}$P]ATP. HBx but not HBxo activated Ras-Raf-MAP kinase signaling in NIH 3T3 cells by 4 h, an effect which persisted strongly up to 12 h (Fig. 1). The kinetics for HBx activation of MAP kinase in NIH 3T3 cells was similar to the kinetics in other cell lines tested such as Chang and HepG2 (5, 7), indicating that HBx stimulation of Ras can be studied interchangeably in NIH 3T3 and Chang cells.

To determine whether HBx stimulates RasGTP complex formation by increasing GTP exchange onto Ras, studies measured the rate of [α-$^{32}$P]GTP uptake onto Ras in permeabilized cells expressing HBx. This assay detects only in vitro addition of newly added [$^{32}$P]GTP onto Ras by activated Sos (10). Serum-starved NIH 3T3 cells were infected with AdCMV-X or AdCMV-Xo, and cells were permeabilized with streptolysin O at 8 h p.i. and labeled in vitro with [α-$^{32}$P]GTP for the indicated times, Ras was immunoprecipitated, and the level of nucleotides bound to Ras was analyzed by ascending TLC. HBx moderately enhanced both the rate of addition and the level of [$^{32}$P]GTP and [$^{32}$P]GDP bound to Ras compared to HBxo-expressing cells (Fig. 2A), indicating that HBx stimulated the uptake of GTP onto Ras. GTP-to-GDP hydrolysis on Ras occurs after initial nucleotide loading due to the intrinsic GTPase activity of Ras, accounting for the presence of radiolabeled GDP. Three independent [α-$^{32}$P]GTP loading experiments were carried out and quantitated by PhosphorImager analysis to provide data on the relative rate and level of RasGTP loading by HBx (Fig. 2B). HBx expression increased GTP exchange onto Ras by an average of 1.7-fold, compared to a transient 2.0-fold increase by PDGF stimulation of uninfected cells. The level of GTP exchange onto Ras by PDGF, an established strong activator of Ras, is consistent with the two- to fourfold stimulation of previous reports (10, 73). An immunoblot of cell extracts for p21$^{ras}$ (Fig. 2C) indicated that its level was not enhanced by HBx, excluding the possibility that HBx increased Ras protein levels. These data therefore demonstrate that HBx stimulates addition of GTP onto Ras and that HBx stimulates Ras for a prolonged time (more than 8 h) but to a lower level than the transient stimulation by growth factors. Similar results were also obtained in Chang cells (data not shown).

The effect of HBx on RasGAP activity was investigated by preparing fractions containing GTPase activity from AdCMV-X- or -Xo-infected cells at 8 and 10 h p.i. as described previously (40). Lysates were incubated with purified [α-$^{32}$P]GTP-

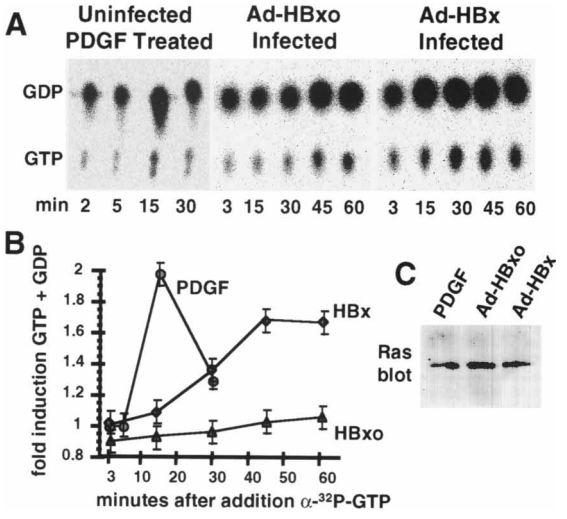


FIG. 2. HBx stimulates GTP loading onto Ras. (A) Effect of HBx on RasGTP loading. Serum-starved NIH 3T3 cells were infected with AdCMV-X or -Xo, cells were permeabilized at 8 h p.i. with streptolysin O and labeled in vitro with [α-$^{32}$P]GTP, Ras was immunoprecipitated from equal amounts of protein extract, and the nucleotides bound to Ras were eluted and analyzed by TLC and PhosphorImager analysis. (B) Average effect of HBx-induced GTP loading onto Ras. Data from three independent experiments as shown in panel A were quantitated by PhosphorImager analysis using ImageQuant software. Calculated standard errors are shown (approximately ± 10% of mean values). Locations of GDP and GTP were determined by inclusion of nonradioactive standards (not shown). PDGF stimulation of control cells was carried out with 50 ng/ml for the indicated times. (C) Western immunoblot analysis of p21$^{ras}$. Equal protein amounts of cell lysates were resolved by SDS-PAGE, transferred to a membrane, and blotted with anti-Ras antiserum Y13-259, using the ECL system for visualization.

labeled c-Ras, and then Ras-bound nucleotides were analyzed by TLC (Fig. 3A). This experiment measures cellular RasGAP activity since it quantitates the level of nucleotides which remain bound to Ras after incubation with cellular GTPase (40). None of the samples displayed a significant change in the rate of RasGTP turnover compared to untreated cells (Fig. 3). It can be concluded that HBx does not influence RasGAP, with the qualification that we were not able to find any conditions for fibroblasts which altered RasGAP activity.

**HBx indirectly induces prolonged stimulation of Ras and upstream Ras activators.** Activation of Ras signaling by epidermal growth factor, insulin, v-*src*, and $Ca^{2+}$ influx into PC12 cells has been shown to be mediated predominantly through formation of a complex between the adapter molecules Shc and Grb2-Sos (52, 56). However, HBx could not be detected in a direct complex with Grb2, Shc, or Sos when immunoprecipitated from cells or in in vitro binding studies using glutathione *S*-transferase-linked proteins at artificially high concentrations (data not shown). Instead, experiments determined that HBx stimulates Ras by indirectly inducing prolonged association of the upstream activators of Ras. To investigate Shc/Grb2 activation, serum-starved NIH 3T3 cells were infected with AdCMV-X or -Xo, Grb2 was immunoprecipitated, and associated proteins were resolved by gel electrophoresis. Membranes were immunoblotted with anti-Grb2, Shc, or hSos antibodies (Fig. 4A). Stimulation of cells with PDGF induced a strong but transient association between Shc and Grb2, which was maximal at 15 min (Fig. 4) and undetectable by 2 h (data not shown). HBx but not HBxo induced a moderate increase in coupling of Shc to Grb2, which was still strongly evident 8 h after HBx expression. Similarly, PDGF stimulated strong coupling of hSos to the Grb2-Shc complex, whereas HBx induced a more moderate increase and HBxo induced no increase at all. Equal amounts of Grb2 protein were immunoprecipitated in all samples (Fig. 4A). HBx did not increase the cellular levels of Shc or Sos proteins, since immunoblots obtained from cell extracts failed to detect any change in their abundance (Fig. 4B). These data therefore indicate that expression of HBx induces increased GTP exchange on Ras, at least in part through an increase in Shc-Grb2-Sos complex formation. HBx stimulation of Ras was unlike growth factor stimulation because it was sustained rather than transient and occurred at a measurably lower level.

**HBx is a cytoplasmic intracellular activator of the Src family of tyrosine kinases.** Given that HBx did not associate with Ras or Ras upstream activator Sos, Grb2, or Shc, the results described above are most consistent with a model in which HBx stimulates Ras indirectly by acting on distant activators such as the nonreceptor tyrosine kinases (9). It is well established that Ras can be activated by both receptor and nonreceptor tyrosine kinases. HBx was previously shown to stimulate Ras by acting in the cytoplasm (22), raising the possibility that HBx acts through receptor or non-receptor-linked tyrosine kinases. Treatment of cells with genistein, a specific inhibitor of tyrosine kinases, was found to block HBx stimulation of RasGTP loading (unpublished results), implicating activation of tyrosine kinases as an essential component in HBx activity. Previous results excluded the possibility that HBx activates receptor tyrosine kinases by inducing the secretion of autocrine factors (5). However, non-receptor-linked tyrosine kinases, such as the Src family, have been shown to activate Ras signaling pathways. Numerous examples of activation of Ras signaling through stimulation of Src exist. For instance, v-*src* mediates strong tyrosine phosphorylation of Shc (51), causing

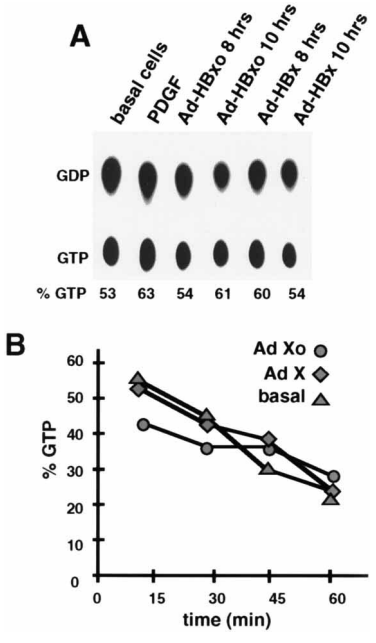


FIG. 3. HBx does not alter cellular RasGAP activity. Lysates were prepared from serum-starved NIH 3T3 cells infected with AdCMV-X or -Xo as described in Materials and Methods. (A) Lysates from cells 8 or 10 h p.i. were incubated with [α-$^{32}$P]GTP-labeled c-Ras for 10 min at 37°C; the remaining nucleotides bound to Ras were eluted and analyzed by TLC and autoradiography. (B) Lysates from cells 8 h p.i. were incubated with [α-$^{32}$P]GTP-labeled c-Ras for 15, 30, 45, and 60 min at 37°C and analyzed as in panel A. The nucleotides released were quantitated by using ImageQuant software. Data shown are the averages of three independent experiments.

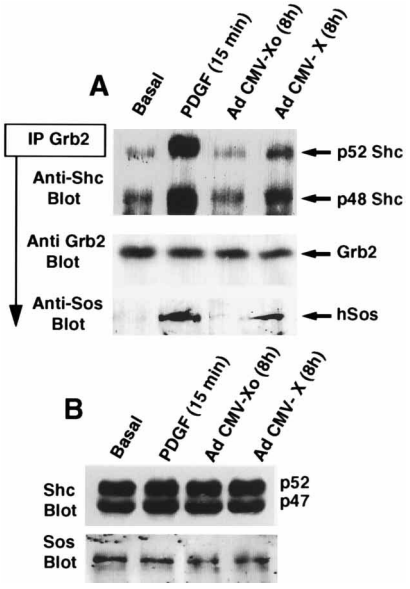


FIG. 4. Effect of HBx on formation of Grb2-Shc-Sos Ras activation complex. Serum-starved NIH 3T3 cells were infected with AdCMV-X or -Xo. (A) Grb2 was immunoprecipitated (IP) from equal amounts of protein extracts at 8 h p.i., immune pellets were resolved by SDS-PAGE, and proteins were transferred to nitrocellulose and immunoblotted with antibodies to Shc, Grb2, or hSos. (B) Western immunoblot analysis of Shc and Sos protein levels. Equal protein amounts of cell lysates obtained for panel A were resolved by SDS-PAGE and immunoblotted with antibodies to Shc or Sos. Immunoblotted proteins were detected by using the ECL system.

association with Grb2 and activation of Ras signaling (55). Similarly, the transforming polyomavirus MT antigen binds to c-Src (17), inducing tyrosine phosphorylation of Shc, subsequent binding of Shc to Grb2, and activation of Ras (21). Src family kinases are also activated by the G-protein-coupled thrombin receptor and are implicated in G-protein activation of the Ras/MAP kinase cascade by relaying signals through Shc and Grb2 (45). Influx of $Ca^{2+}$ into PC12 cells activates Src, which in turn induces binding of Shc to Grb2 (56) and activation of Ras (54). Additionally, Src may be involved in stimulation of Ras signaling during cell adhesion molecule signaling events (29). In summary, signals that stimulate the Src family of kinases can lead to activation of Ras signaling. Studies were therefore carried out to determine whether HBx activates the Src family of nonreceptor tyrosine kinases.

c-Src is the prototypic member of the Src family of nonreceptor tyrosine kinases, which include the widely expressed Src, Fyn, Yes, and Lyn kinases and the four hematopoietic restricted kinases, Hck, Fgr, Lck, and Blk (42). Src family kinases share common structures, which include a short amino-terminal membrane anchor, a unique domain characteristic of each individual kinase, an SH3 domain which binds proline-rich sequences, an SH2 domain that typically binds proteins containing a consensus YEEI motif (61), a catalytic domain which has kinase activity, and a short carboxy-terminal tail containing the major regulatory tyrosine residue. Src family kinases are all regulated similarly; the repressed state is associated with phosphorylation of a carboxy-terminal tyrosine (Y-527 in c-Src) by the C-terminal Src kinase (Csk) (reviewed in reference 24). Dephosphorylation of the carboxy-terminal tail tyrosine by CD45 in T lymphocytes or the receptor protein tyrosine phosphatase $\alpha$ in fibroblasts stimulates Src kinase activity.

HBx was found to strongly stimulate the Src family of kinases in serum-starved NIH 3T3 cells. NIH 3T3 cells were infected with AdCMV-X or -Xo for 8 h, endogenous c-Src was immunoprecipitated from equal amounts of extracted cell protein, immune complex pellets were washed extensively, and an in vitro kinase assay was carried out with immunoprecipitated c-Src (autophosphorylation assay) or enolase (transphosphorylation assay) as the substrate (Fig. 5). Expression of HBx, but not HBxo, induced a specific and strong increase in the ability of c-Src to carry out both autophosphorylation and transphosphorylation of enolase (Fig. 5A). Stimulation of cells with PDGF for 5 min, a prototypic Src induction protocol, induced a similarly strong activation. The observed PDGF-induced activation of c-Src is comparable to that reported by other groups (16, 45, 62) and indicates that HBx expression induced significant activation of c-Src. Identical results were observed in Chang and HepG2 cells (data not shown). Immunoblot analysis of cell lysates demonstrated that HBx did not detectably increase Src protein levels, indicating that Src activity rather than abundance was induced by HBx (Fig. 5A). To determine whether HBx also activates other Src family members, endogenous Fyn was immunoprecipitated from equal amounts of cell lysates, and the immunoprecipitated pellets were subjected to in vitro auto- and transphosphorylation assays (Fig. 5B). Expression of HBx induced both auto- and trans-Fyn tyrosine kinase activation comparable to activation of Fyn following PDGF treatment, which was not observed in the HBxo-expressing cells. No increase in Fyn protein levels was observed in HBx-expressing cells (data not shown). A kinetic analysis demonstrated that HBx mediated Src and Fyn activation in NIH 3T3 cells starting at 3 to 4 h after expression, an effect

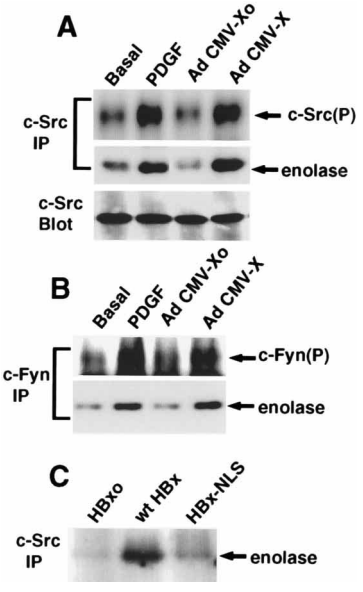


FIG. 5. HBx induces activation of c-Src and Fyn. Serum-starved NIH 3T3 cells were infected with AdCMV-X or -Xo; c-Src or Fyn was immunoprecipitated; the pellet was washed and assayed by incubation with the substrate enolase and [$\gamma$-$^{32}$P]ATP. Products were resolved by SDS-PAGE (10% gel) and then visualized and quantitated by PhosphorImager analysis. c-Src (A) or Fyn (B) was immunoprecipitated (IP) 8 h p.i. from NIH 3T3 cell lysates expressing HBx or HBxo. Where indicated, cells were incubated with PDGF (100 ng/ml) for 5 min. Also shown in panel A is a Western immunoblot analysis of c-Src protein levels in the immunoprecipitated c-Src pellet prior to kinase assay. (C) Transfected HBx activates c-Src in the cytoplasm. Chang cells were transfected with 10 $\mu$g of pCMV-Xo (HBxo), pCMV-X (wt HBx), or pCMV-XNLS (nuclear HBx) expression plasmid and serum starved for 24 h; c-Src was immunoprecipitated, subjected to an in vitro transphosphorylation assay, and then analyzed as described above. Similar results were obtained with NIH 3T3 cells.

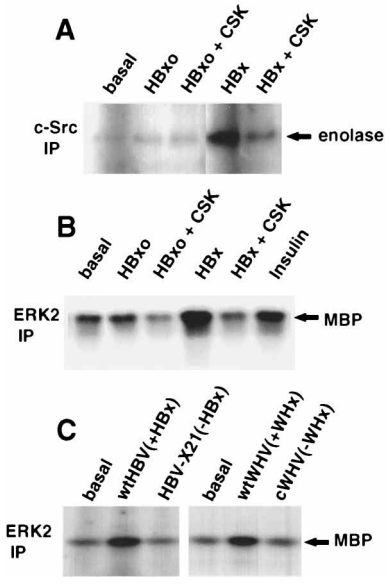


FIG. 6. HBx stimulation of Ras involves activation of Src kinases. Chang cells were transfected for 18 h with 8 μg of plasmid pCMV-Xo (HBxo) or pCMV-X (wtHBx), with or without 8 μg of plasmid pCsk or carrier DNA, and then serum starved for 18 h. (A) c-Src was immunoprecipitated (IP) from equal amounts of cell lysates and subjected to an in vitro transphosphorylation assay with [γ-$^{32}$P]ATP. (B) ERK2 was immunoprecipitated from equal amounts of cell lysates analyzed by in vitro phosphorylation of MBP by using [γ-$^{32}$P]ATP. Labeled substrate proteins were resolved by SDS-PAGE (15% gel) and then visualized and quantitated by PhosphorImager analysis. Cells were stimulated with 100 μg of insulin (Intergen) per ml for 10 min. (C) Chang cells were transfected for 3 days with 10 μg of plasmid DNA as indicated and serum starved for 18 h, and then ERK2 activation was analyzed as described above.

which persisted for more than 12 h (data not shown). These results demonstrate that HBx induces a sustained activation of different members of the Src family of nonreceptor tyrosine kinases.

**HBx activates c-Src in the cytoplasm.** To ensure that the activation of Src was not an unforeseen consequence of infection with the recombinant Ads, and to determine whether HBx activates c-Src in the cytoplasm or nucleus, Chang cells were transfected with plasmids expressing either wt HBx, mutant HBxo, or the nucleus-targeted HBx variant known as HBx-NLS. HBx-NLS contains a functional NLS which causes the protein to become exclusively relocated to the nucleus (22). Following transfection, cells were serum starved for 24 h, c-Src was immunoprecipitated from equal amounts of cell lysate, and an in vitro kinase assay was carried out with enolase as a transphosphorylation substrate (Fig. 5C). Src kinase activity was strongly activated in serum-starved cells expressing wild-type HBx for 24 to 36 h but not in those expressing HBxo or HBx-NLS. These results are consistent with the ability of HBx to activate Ras signaling cascades only in the cytoplasm. They also confirm that it is HBx which activates c-Src and that a cytoplasmic location is essential for HBx activity. The plasmids all synthesize HBx mRNAs containing the Ad late 5′ noncoding region (tripartite leader), which permits efficient translation under serum-free conditions as used here (58). Thus, these data demonstrate that HBx is capable of activating c-Src without a need for serum for a prolonged period of time (24 to 36 h) and only when located in the cytoplasm. It should be noted that conditioned media from cells expressing HBx for 8 h did not activate c-Src in control cells, excluding the possibility that HBx initiates Src activation by inducing secretion of autocrine factors (data not shown).

**HBx activation of Ras signaling requires Src family kinases.** Csk is a tyrosine kinase that specifically phosphorylates the carboxy-terminal tyrosine of Src kinases and returns them to an inactive state. Overexpression of Csk can be used to block Src family kinases and potentially inhibit the ability of HBx to activate the Ras cascade. Several independent lines of investigation were carried out to determine whether HBx activation of the Src family of kinases is required for stimulation of Ras signaling by HBx. Chang cells were transfected at low density with HBx or HBxo expression plasmid in the presence or absence of a plasmid expressing Csk controlled by the tripartite leader 5′ noncoding region, which enables efficient protein expression during serum starvation (71). Extracts were prepared 24 h later, and immunoprecipitated c-Src was tested for transphosphorylation of the substrate enolase (Fig. 6A). Expression of Csk fully inhibited the ability of HBx to induce c-Src activity, as evidenced by suppression of enolase labeling in an in vitro transphosphorylation assay. Equal amounts of Csk were synthesized in transfected cells, as determined by Western immunoblotting (data not shown). It was next found that activation of Src kinases is essential for HBx stimulation of the Ras-Raf-MAP kinase signal cascade. Chang cells were transfected at low density with plasmids expressing HBxo, HBx, or HBx with or without Csk and serum starved for 20 h. Activation of immunoprecipitated ERK2 was assayed by incubation with MBP and [γ-$^{32}$P]ATP (Fig. 6B). Expression of HBx induced a strong activation in the ability of MAP kinase to phosphorylate MBP that was abolished upon cotransfection with Csk. Similar results were obtained for other cell lines tested, including HepG2 and NIH 3T3 (data not shown). Thus, these data clearly demonstrate that HBx requires activation of the Src family of kinases for induction of the Ras signaling cascade.

Control studies indicated that Csk selectively blocks HBx activation of Src-Ras signaling, rather than causing a generalized negative transcriptional effect in cells. It should be noted that Csk and HBx are both transcribed from the CMV promoter, making it unlikely that Csk merely downregulates HBx expression. This was directly examined by transfecting Chang cells with two reporter constructs expressing β-galactosidase (β-Gal), one controlled by the CMV promoter and another controlled by the Ad major late promoter (MLP), with and without coexpression of Csk (Table 1). The MLP is largely unresponsive to HBx (3, 65) and is therefore indicative of general transcriptional activity. Coexpression with Csk only moderately reduced expression of β-Gal (24% from the CMV promoter; 16% from the MLP). These results therefore indi-

TABLE 1. Effect of Csk expression on basal transcriptional activity

| Construct[a] | β-Gal activity (%)[b] |
|---|---|
| pCMV-βgal | 100 |
| pCMV-βgal + pCsk | 76 |
| pMLP-βgal | 100 |
| pMLP-βgal + pCsk | 84 |

[a] Plasmid pCMV-βgal expresses the β-Gal gene under the control of the CMV promoter. Plasmid pCsk expresses the *csk* gene under CMV promoter control. Plasmid pMLP-βgal expresses the β-Gal gene under the control of the Ad minimal MLP element.

[b] Determined from whole-cell lysates. Samples without coexpression of Csk were set at 100% activity. Results are averages of three independent experiments in which Chang cells were transfected with 10 μg of each plasmid or control pGem DNA.

cate that Csk specifically suppresses HBx activation of Src and Ras, rather than causing a general downregulation of transcription.

Studies were also carried out to determine whether HBx stimulates Src-Ras-MAP kinase signaling when expressed during HBV replication in cultured cells (i.e., in vitro replication). Although it is not possible to infect cultured cells with HBV, two different replicative DNA forms of the virus can be transfected into cultured cells and support in vitro viral replication. A head-to-tail genomic dimer of the HBV genome has been shown to productively replicate and produce HBV particles (8). A head-to-tail dimer is required due to the overlapping nature of the circular HBV genome. Alternatively, cells can be transfected with plasmids containing a full-length cDNA copy of the HBV or WHV genome fused to the CMV promoter, which also establishes productive viral replication (75). Chang cells were therefore transfected with a head-to-tail genomic dimer of wt HBV or a mutant that does not express functional HBx (wtHBV or HBV-X21, respectively [8]) or a wt pregenomic cDNA of WHV or one containing a mutant HBx gene (wtWHV or CWHV, respectively [75]). We have recently demonstrated the replication of HBV and WHV from either genomic DNA form (32). Three days following transfection, cells were serum starved for 18 h, and lysates were prepared and examined for activation of ERK2 by immunoprecipitation and in vitro phosphorylation of MBP (Fig. 6C). ERK2 was stimulated only in cells containing replicating WHV or HBV if WHx or HBx was also expressed.

**HBx transactivation of transcription factor AP-1 involves essential activation of Src kinases.** It was previously shown that HBx activation of the transcription factor AP-1, including both the c-Fos and c-Jun components, occurs through a Ras-dependent pathway in a variety of cell lines that were investigated (5, 7, 22, 49, 50). Here it is shown that HBx activation of AP-1 DNA binding activity and AP-1-directed transcription both involve an essential requirement for HBx activation of Src tyrosine kinases. Chang cells were transfected with plasmids expressing HBx or HBxo, with or without cotransfection of Csk, and serum starved, and nuclear extracts were prepared for electrophoretic mobility shift assay using equal amounts of nuclear extracts and a $^{32}$P-labeled double-stranded DNA probe containing one AP-1 binding site, as described previously (7). Cotransfection of increasing amounts of the Csk-expressing plasmid suppressed HBx induction of AP-1 DNA binding complexes in a titratable manner (Fig. 7A). Finally, cells were cotransfected with plasmids expressing HBx or HBxo and an AP-1-dependent luciferase reporter under the control of a minimal promoter, with and without a Csk expression plasmid (Fig. 7B). Coexpression with Csk blocked HBx-induced activation of AP-1-dependent transcription, again demonstrating that activation of Src kinases is essential for transcriptional transactivation by HBx.

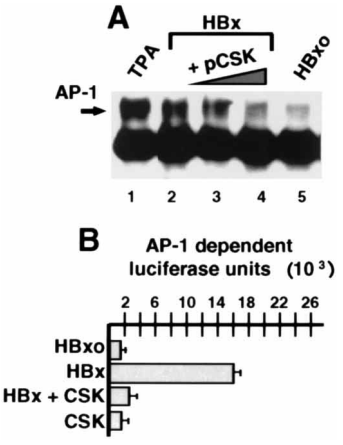


FIG. 7. Transcriptional activation of AP-1 by HBx involves activation of Src kinases. Chang cells were transfected with 8 μg of plasmid pCMV-X or pCMV-Xo, with or without cotransfection of pCsk for 18 h, and then serum starved for 18 h. (A) Cell extracts were prepared for AP-1 electrophoretic mobility shift assay as described previously, using a $^{32}$P-labeled double-stranded DNA oligonucleotide probe containing one AP-1 binding site (7). Reactions were carried out with 3 μg of nuclear extract, labeled oligonucleotide, and 1 μg of poly(dI-dC) for 30 min at 23°C. Protein-DNA complexes were resolved by electrophoresis on 4% polyacrylamide gels and visualized by PhosphorImager analysis. As a positive control for AP-1 stimulation, cells were treated with 20 μM tetradecanoyl phorbol acetate (TPA) for 30 min. (B) Cells were transfected as described above, but the mixture contained in addition 3 μg of plasmid pAP-1Luc, which encodes the luciferase reporter under the control of four AP-1 binding sites and a minimal TATA box promoter. Serum-starved cells were harvested for 18 h after transfection, and the level of expression of the luciferase reporter was assayed. Results are the averages of three independent experiments.

## DISCUSSION

This study has investigated the mechanism by which HBx activates Ras. There are three main conclusions from these results. First, HBx activates Ras for a prolonged time by increasing GTP uptake onto Ras (Fig. 2), probably without altering RasGAP activity (Fig. 3). Second, HBx stimulates association of the upstream Ras-activating proteins Shc, Grb2, and Sos but does not directly enter into a complex with them (Fig. 4). Third, HBx activates the Src family of tyrosine kinases (shown for c-Src and Fyn [Fig. 5]), through which it stimulates Ras (Fig. 6) and induces transactivation of transcription mediated by transcription factor AP-1 (Fig. 7). Thus, HBx was found to be a constitutive intracellular activator of Src and Fyn tyrosine kinases, both when expressed alone and during in vitro viral replication, through which it stimulates signal transduction pathways and AP-1-mediated transcription.

A variety of different HBx activities have been reported over the past few years. The work presented here has extended previous investigations which showed that HBx activates cellular signal transduction pathways, particularly the Ras-Raf-MAP kinase cascade (5, 18, 50), which leads to transcriptional transactivation (5, 7, 50, 62) and the release of cells from quiescence (5, 6, 34, 50). Our work has not addressed other reported HBx activities, such as interactions with nuclear proteins involved in transcriptional control or DNA repair. A cytoplasmic location was previously found to be essential for HBx activation of Ras and of transcription factors AP-1 and NF-κB (22). In the cell systems used in our studies, and in natural infection of hepatocytes by WHV, HBx has been described as a predominantly cytoplasmic protein (19, 22, 72), although others have found greater concentration of HBx in the nucleus (39, 57). The data presented here clearly establish that HBx which was relocated exclusively to the nucleus (HBx-NLS) could no longer activate Src signaling (Fig. 5C). These experiments therefore establish that in our systems, HBx activates Src-Ras signaling in the same intracellular compartment in which it largely resides.

Investigation of the molecular mechanism by which HBx activates Ras revealed several interesting features. By measuring the uptake of GTP onto Ras, it was found that expression of HBx induced an increase in GTP exchange onto Ras that was less pronounced than that observed for growth factor (PDGF) stimulation (Fig. 2). However, unlike growth factor, HBx did not activate Ras signaling in a sharp, transient peak of activity (Fig. 2B) but instead did so over a more prolonged period of time and to a lower extent. The prolonged but lower

level of Ras activation by HBx compared to PDGF stimulation of cells is also consistent with the more modest induction by HBx of complex formation between Shc and Grb2-Sos (Fig. 4). Despite differences in magnitude of activation, the data presented here establish that HBx modifies Ras-activating proteins in a manner consistent with the molecular mechanism described for Ras activation following both receptor and nonreceptor tyrosine kinase stimulation (21, 56).

Since HBx did not activate Ras by inducing secretion of autocrine factors (5) but did activate Shc (Fig. 4), we investigated whether HBx activates the Src family of tyrosine kinases. c-Src activation by HBx was demonstrated both by infection with recombinant Ad vectors (Fig. 5), which we have previously shown to rapidly introduce and express HBx protein (6, 7, 22), and by transfection of HBx expression plasmids into cell lines including Chang cells (Fig. 5C and 6). Elevated Src kinase activity but not abundance was detected whether HBx was expressed from an Ad vector or from a plasmid, clearly demonstrating that expression of the HBx protein stimulates the kinase activity of the Src family regardless of the vector from which HBx is expressed. The activation of Src and Fyn by HBx appears to be prolonged, compared to the transient activation of Src that occurs in response to stimulation by growth factors, UV light, activated G-protein-coupled receptors, $Ca^{2+}$ influx, and adhesion of fibroblasts on fibronectin (29, 56). As observed previously for activation of Ras, HBx also induced sustained activation of Src without the need for serum and did not involve production of autocrine factors, since conditioned media obtained from 24-h HBx-expressing cells did not stimulate Src (data not shown). HBx may therefore be similar to the transforming polyomavirus MT antigen, which also induces continuous intracellular activation of c-Src (15).

HBx stimulation of Src kinases was found to be essential for its ability to activate Ras, because overexpression of Csk, a potent inhibitor of Src kinases, blocked HBx stimulation of Ras signaling and activation of the MAP kinase ERK-2 (Fig. 6). Coexpression of HBx with kinase-inactive, dominant-negative forms of Src or Fyn proteins expressed independently impaired HBx activation of ERK-2 by about half, consistent with activation of both kinases in Ras-Raf-MAP kinase signaling by HBx (data not shown). These results further confirmed that HBx requires Src kinase activity for induction of downstream Ras signaling. Collectively, the results presented in this report establish that HBx activation of the Src family of kinases is an essential link to HBx activation of Ras signaling. The observation that HBx induces prolonged activation of Src-Ras signaling raises the fundamental question whether this activity represents the essential requirement for HBx during HBV replication. Recent studies from our lab confirm the activation of c-Src by HBx expressed from replicating WHV genomes in cultured cells and suggest that it is an important activity for viral replication (32).

The Src kinases are involved in inducing a variety of cellular processes, including mitogenesis, differentiation, and cell movement. In particular, activation of Src kinases can promote cell cycling or differentiation in different contexts (24). HBx stimulation of Src might therefore be critical for replication of HBV either by preserving the differentiated state of the infected cell or by inducing $G_0$ cells to enter $G_1$ or early S phase, as described for polyomavirus and some other DNA tumor viruses. Alternatively, HBx activation of Src might induce undefined signaling pathways that impact on HBV infection. The activation of Src signaling could also influence the oncogenic and pathogenic potential of HBV. In this regard, we have recently shown that HBx, whether expressed alone or from a replicating WHV genome in cultured cells, potently sensitizes cells to apoptotic killing by tumor necrosis factor alpha (64). Thus, HBx activation of Src could have a profound effect on the killing of infected cells by the inflammatory response and the development of HCC, in addition to its essential role in HBV infection.

An essential question which we are now addressing concerns the molecular mechanism by which HBx activates the Src family of tyrosine kinases and how HBx activated Src signals to Ras. Recent unpublished work in our lab has found that HBx does not stimulate the activity of the positively acting Src phosphatase (receptor protein tyrosine phosphatase $\alpha$), nor does it mediate complex formation between Src and receptor tyrosine kinases. However, HBx was found to stimulate tyrosine phosphorylation of Shc, which presumably provides the link between Src activation and Ras activation. It is also possible that HBx activates Src kinases directly. For instance, we note that human HBV HBx proteins generally contain the well-conserved motif EELGEE(I/V), which is essential for activity (13, 70). This sequence vaguely resembles an SH2 binding motif except for a critical tyrosine residue which typically promotes binding when phosphorylated (16, 61). Although we have not yet detected interaction in vivo between HBx and c-Src, direct binding of HBx and Src remains a possibility. For example, only a small and difficult to detect fraction of polyomavirus MT antigen interacts with c-Src, and the presence of the tyrosine in MT is not critical (47). However, because the WHV WHx protein does not conserve the same motif found in HBV HBx, other mechanisms of Src activation must also be considered. It is possible that HBx acts by increasing intracellular $Ca^{2+}$, by stimulating G-protein-coupled receptor interactions, or by acting on the calcium-regulated Src activity kinase known as Pyk2 (37), which cooperates with Src to link G-protein-coupled receptors to Ras signaling pathways (20). Given the importance of Src activation in defining HBx activities, it seems likely that HBx induction of Src kinases will be found to play an essential role during the life cycle of mammalian hepadnaviruses and possibly during development of HCC.

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