Hepatitis B Virus X Protein via the p38MAPK Pathway Induces E2F1 Release and ATR Kinase Activation Mediating p53 Apoptosis*[^5]

Received for publication, March 10, 2008, and in revised form, June 19, 2008. Published, JBC Papers in Press, July 7, 2008, DOI 10.1074/jbc.M801934200

Wen-Horng Wang, Ronald L. Hullinger, and Ourania M. Andrisani

From the Department of Basic Medical Sciences, Purdue University, West Lafayette, Indiana 47907

Hepatitis B virus (HBV) X protein (pX) is implicated in hepatocellular carcinoma (HCC) pathogenesis by an unknown mechanism. Deletions or mutations of genes involved in the p53 pathway are often associated with HBV-mediated HCC, indicating rescue from p53 apoptosis is a likely mechanism in HBV-HCC pathogenesis. Herein, we determined the mechanism by which pX sensitizes hepatocytes to p53-mediated apoptosis. Although it is well established that the Rb/E2F/ARF pathway stabilizes p53, and the DNA damage-activated ATM/ATR kinases activate p53, the mechanism that coordinates these two pathways has not been determined. We demonstrate that the p38MAPK pathway activated by pX serves this role in p53 apoptosis. Specifically, the activated p38MAPK pathway stabilizes p53 via E2F1-mediated ARF expression, and also activates the transcriptional function of p53 by activating ATR. Knockdown of p53, E2F1, ATR, or p38MAPKα abrogates pX-mediated apoptosis, demonstrating that E2F1, ATR, and p38MAPKα are all essential in p53 apoptosis in response to pX. Specifically, in response to pX expression, the p38MAPK pathway activates Cdk4 and Cdk2, leading to phosphorylation of Rb, release of E2F1, and transcription of ARF. The p38MAPK pathway also activates ATR, leading to phosphorylation of p53 on Ser-18 and Ser-23, transcription of pro-apoptotic genes Bax, Fas, and Noxa, and apoptosis. In conclusion, pX sensitizes hepatocytes to p53 apoptosis via activation of the p38MAPK pathway, which couples p53 stabilization and p53 activation, by E2F1 induction and ATR activation, respectively.

[^5]: This work was supported, in whole or in part, by National Institutes of Health Grant NIDDK 044533 (to O. M. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[^4]: The online version of this article (available at http://www.jbc.org) contains supplemental Figs. S4A, S4D, and S6C.

[^1]: To whom correspondence should be addressed: Purdue University, 625 Harrison St., West Lafayette, IN 47907-2026. Tel.: 765-494-8131; Fax: 765-494-0781; E-mail: andrisao@purdue.edu.

[^2]: The abbreviations used are: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinase; GST, glutathione S-transferase; ChIP, chromatin immunoprecipitation assay; JNK, c-Jun N-terminal kinase; WCE, whole cell extract; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kd, knockdown; Rb, retinoblastoma protein.
HBV X Protein Mediates p53 Apoptosis by ATR Activation

23); 4pX-1 cells conditionally express pX via the tet-off system. The significance of this cellular model is that pX expression is very low, resembling expression levels occurring in natural HBV infection (22). In our earlier studies we have shown that pX sensitizes 4pX-1 cells to apoptosis only when challenged by additional sub-apoptotic signals such as growth factor deprivation, by activating the p38MAPK pathway (21). Importantly, we have shown that pX expression induces sustained activation of the p38MAPK pathway within 6 hours following serum withdrawal (21). By contrast, in the absence of pX expression, serum withdrawal induces the p38MAPK pathway later, at 24 hours. Thus, the effect of pX on the activation of the p38MAPK pathway precedes the effect of serum withdrawal. Moreover, inhibition of the p38MAPK pathway by SB 202190 inhibits pX-mediated apoptosis. Accordingly, our earlier studies (21) have clearly established that the activation of the p38MAPK pathway by pX is necessary for initiation of pX-mediated apoptosis. The role of the p38MAPK pathway in pX-mediated apoptosis remains to be determined.

Herein we demonstrate that the p38MAPK pathway, activated by pX following growth factor withdrawal, induces p53 stabilization by E2F1-mediated ARF expression. The p38MAPK pathway also induces the transcriptional activity of p53 by activating ATR, leading to phosphorylation of Ser-18 and Ser-23 of murine p53. Knockdown of p53, E2F1, ATR, or p38MAPKα abrogates pX-mediated apoptosis, identifying the essential role of the p38MAPK pathway, E2F1, and ATR in p53 apoptosis. We conclude that activation of the p38MAPK pathway by the weakly oncogenic pX couples the stabilization and activation of p53, via ARF induction and ATR activation, respectively, leading to p53-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture—The 4pX-1 cell line, derived from AML12 cells, was propagated as described (22) in medium containing 5 μg/ml tetracycline to turn-off pX expression. Apoptotic conditions are as described (21). Briefly, confluent 4pX-1 cells were treated with 5 μg/ml tetracycline for 24 hours in medium containing 10% fetal calf serum; apoptosis was initiated by switching cultures to 2% fetal calf serum within 5 μg/ml tetracycline. Concentrations of inhibitors were: SB202190 (CalBiochem), 5 μM; PFT-α (BioMoL), 10 μM; caffeine, 8 mM; and cycloheximide, 10 μM.

Transient Transfections—Transient transfections of luciferase reporter plasmids were performed using FuGENE 6 (21). DNA was added to apotopic 4pX-1 cultures 8 hours prior to serum withdrawal; cells were harvested after 24 hours. Assays were performed in triplicate and quantified per μg of protein extract. NFkB-Luc, NFAT-Luc, p53-Luc were purchased from Stratagene. Fas (−1.7 kb)-Luc was kindly provided by Dr. Owens-Schaub. Construction of Clonal 4pX-1-p53α/M, 4pX-1-E2F1α/MM, 4pX-1-ATRα/MM, and p38MAPKα/MM Cell Lines—4pX-1 cells were transfected with pSilencer CMV-Puro (Ambion) containing the p53 sequence forming a short hairpin (26), or the E2F1 and ATR sequences listed below. The p38MAPKα-shRNA vector was purchased from Open Biosystems. Clonal stable cell lines were isolated by puromycin (1.0 μg/ml) selection and screened by Western blot assays employing antibodies for p53, E2F1, ATR and p38MAPKα. Clonal stable cell lines exhibiting the highest level of knockdown were selected for further analyses. Clonal stable cell lines isolated from the same cultures exhibiting absence of knockdown referred to as pseudo-knockdown cell lines (pseudokd), serve as the negative control: E2F1shRNA: 5'-GATCCACGGAGGCTGGATCGTAGGATTCTAACGACTCCAGTATCCAGGCTCCCGTTTTTTGGAAT-3'; ATRshRNA: 5'-GATTCCTGATCATCTTGTGAATTCAGAGACCTTACAAAGATGATGATT'TT-3'.

Real-time Quantitative PCR—Real-time quantitative PCR was performed as described (23), employing 18 S RNA as internal control. Primers used were: ARF: Fwd, 5’-ATAGC-TTCAGCTCAAGACGAG-3’ and Rev, 5’-AAGCCACATGCTCAGACA-3’; ASPP2: Fwd, 5’-GGAGATCGAGACATGAAATAGC-3’ and Rev, 5’-ATCCTGGCCTTCTTCAGC-3’; CHK2: Fwd, 5’-AACCTGAAGAACCTGTTC-3’ and Rev, 5’-TCGAGAATTATTCACGAC-3’; pX: Fwd, 5’-TCCAGCAATGTCAACGACC-3’ and Rev, 5’-CCAATTTAGCCTACAGCCTCC-3’. DNA fragmentation assays were performed as described (21). In vitro Cdk4 kinase assays were performed as described (23).

Western Blot Analyses—Western blot analyses were performed employing whole cell extracts (WCE) isolated from apoptotic cultures (10^7 cells), grown in 200 μl of 1× SDS loading buffer. Western blot analyses were performed using Amersham Biosciences ECL reagent. Antibodies used were purchased from: p53 (Vector); phospho-Ser18-p53, phospho-Ser23-p53, and phospho-Ser389-p53, Rb, phospho-Ser800/804-Rb, active caspase3, phospho-Ser18-p53, phospho-Ser23-p53, and phospho-

RESULTS

pX expression Induces p53-mediated Apoptosis—The HBV X protein sensitizes the 4pX-1 hepatocyte cell line to apoptosis after growth factor withdrawal by inducing sustained activation of the p38MAPK pathway (21). Specifically, 4pX-1 cultures expressing pX via the Tet-off expression system (22), exhibit sustained activation of the p38MAPK pathway within 6 hours following growth factor withdrawal. By contrast, in the absence of pX expression, serum withdrawal induces the p38MAPK pathway later at 24 hours. Thus, the effect of pX on the activation of the p38MAPK pathway precedes the effect of serum withdrawal. Moreover, inhibition of the p38MAPK pathway by SB 202190
inhibits pX-mediated apoptosis. Thus, our earlier studies (21) established that the activation of the p38MAPK pathway by pX is necessary for initiation of pX-mediated apoptosis. Moreover, the pX-mediated activation of the p38MAPK pathway induces expression of TNFR1/TNF-α, Fas/Fasl, and p53-regulated genes Fas, Bax, and Noxa (21). Expression of pro-apoptotic p53-regulated genes in response to pX suggested the involvement of p53 in pX-mediated apoptosis, in agreement with earlier observations (30). The mechanism by which pX stabilizes and activates pro-apoptotic p53 transcription is unknown.

To obtain initial indications whether pX activates p53 transcription, the p53-responsive luciferase constructs p53-Luc, pBax-Luc (23), and pfas-Luc (31) were transiently transfected in apoptotic 4pX-1 cells. All p53-responsive promoters display induction in response to pX expression (Fig. 1A). Treatment with 10 μM PFT-α, known to specifically inhibit p53 transcriptional activity (29), inhibits induction by pX from the p53-dependent reporter, without significantly inhibiting NFKB- or NFAT-driven luciferase expression, suggesting pX activates p53 (Fig. 1A). To confirm the effect of pX on p53 transcriptional activation and pX-mediated apoptosis, we examined expression of the endogenous p53-regulated genes Bax and p21, in a time course, following incubation of 4pX-1 cells in apoptotic conditions by growth factor withdrawal (Fig. 1B). pX induces expression of both p21 and Bax, as early as 3 h following incubation in apoptotic conditions. Importantly, by 12 h pX mediates a 3-fold increase in the expression of pro-apoptotic Bax in comparison to a 1.6-fold increase in expression of the growth arrest gene p21, suggesting the involvement of p53 in apoptosis, in response to pX expression.

To determine whether p53 activation is involved in pX-dependent apoptosis, radioactive DNA fragmentation assays were performed in apoptotic 4pX-1 cultures, as a function of PFT-α addition (Fig. 1C). PFT-α profoundly inhibits onset of apoptosis in response to pX (Fig. 1C). Likewise, PFT-α inhibits pX-dependent caspase3 activation and apoptosis, assayed by the cell-permeable fluorogenic Z-DEVD-FMK caspase3 substrate (Fig. 1D).

To directly demonstrate involvement of p53 in pX-mediated apoptosis, a 4pX-1 p53 knockout (kd) cell line was generated, 4pX-1-p53kd, displaying more than 90% silencing of p53. p53-responsive luciferase plasmids transfected in the 4pX-1-p53kd cell line do not exhibit pX-dependent induction. Importantly, this cell line is resistant to apoptosis in response to pX expression (Fig. 1D), assayed by immunofluorescence microscopy, using the cell-permeable fluorogenic Z-DEVD-FMK caspase3 substrate (21). Together, these results (Fig. 1) support that pX activates p53 and induces p53-mediated apoptosis.

p53 Stabilization by pX Requires Activation of the p38MAPK Pathway—Our earlier studies (21) have linked apoptosis by pX to activation of the p38MAPK pathway. Because our results now show that p53 mediates apoptosis in response to pX expression (Fig. 1), we investigated the link between the p38MAPK pathway and p53. A p38MAPKα knockdown cell line (4pX-1-p38MAPKαkd) was constructed, exhibiting ~80% reduction in the endogenous p38MAPKα (Fig. 2A). Employing the 4pX-1 and 4pX-1-p38MAPKαkd cell lines, we investigated whether pX stabilizes p53, and if p53 stability is dependent on activation of the p38MAPK pathway. Confluent, 4pX-1, and 4pX-1-p38MAPKαkd cultures were grown as a function of pX expression, and apoptosis was initiated by serum withdrawal (21). Cycloheximide was added 2 h after onset of apoptosis. p53 protein levels were determined by Western blot analyses and quantified in a time course, following cycloheximide addition (Fig. 2, B and C). In 4pX-1 cells grown in the absence of pX, 50% of p53 remains 30 min after cycloheximide addition, reaching basal levels by 120 min. By contrast, in the presence of pX, 80% of p53 remains at the 30-min interval, decreasing to 60% by 60 min, and reaching the basal level 180 min after cycloheximide addition. The half-life of p53 is estimated to be ~30 min in apoptotic 4pX-1 cultures without pX, whereas in the presence of pX, the p53 half-life is increased to 75 min, demonstrating that pX stabilizes p53. Interestingly, in 4pX-1-p38MAPKαkd cells expressing pX, knockdown of the p38MAPKα decreased the half-life of p53 to 45 min, indicating the p38MAPK pathway plays a role in pX-mediated stabilization of p53. Similar conclusions were derived from pulse-chase studies of p53, employing in vivo metabolic labeling with [35S]methionine, in conjunction with treatment using the p38MAPK pathway inhibitor SB202190 (data not shown).

The p38MAPK Pathway Activated by pX Promotes E2F1 Function via Cdk4/Cdk2 Activation—The E2F family of transcription factors is sequestered from function by binding to unphosphorylated Rb. Activated Cdk4 and Cdk2 phosphorylate Rb, releasing E2Fs which mediate transcription of either proliferative S-phase genes, or pro-apoptotic genes such as Arf (7, 32). To obtain initial indications whether pX induces E2F1 activity, the E2F1-responsive APAF-Luc (33) and CyclA-Luc (34) reporters were transiently transfected in apoptotic 4pX-1 cultures. pX expression promoted a 3–4-fold luciferase induction (Fig. 3A), suggesting that pX mediates E2F1 release. Based on these observations, we determined the phosphorylation status of Rb in pX-expressing cells grown in apoptotic conditions. Enhanced Rb phosphorylation was observed in apoptotic 4pX-1 cultures expressing pX, detected by Western blot analyses with the phospho-Rb Ser-800/804 antibody (Fig. 3B). Specifically, in pX-expressing 4pX-1 cells, phosphorylation of Rb increased by 8-fold starting 0 to 12 h after growth factor withdrawal. By contrast, in 4pX-1-p38MAPKαkd cells expressing pX, only a 1.5-fold increase in Rb phosphorylation is observed (Fig. 3B), supporting that the pX-activated p38MAPK pathway is required for Rb phosphorylation.

Because Rb phosphorylation is mediated by G1 phase cyclin-dependent kinases, we examined by in vitro immunocomplex kinase assays, the level of Cdk4 activation as a function of pX expression (Fig. 3C). Employing extracts isolated from apoptotic 4pX-1 cells, we demonstrate that Cdk4 activation is maintained for 12 h following onset of apoptosis in the presence of pX. Inhibition of the p38MAPK pathway by SB202190 suppresses this pX-dependent Cdk4 activation, whereas inhibition of the JNK pathway by treatment with SP600125 has only a small effect (Fig. 3C). To further confirm these results, we also examined the activation state of Cdk2, and its link to the p38MAPK pathway. A critical step in Cdk2 activation, similar to Cdc2 activation (35), is dephosphorylation of Tyr-15 (36). Employing extracts from the 4pX-1 and 4pX-1-p38MAPKαkd
HBV X Protein Mediates p53 Apoptosis by ATR Activation

A. transient transfections of p53-Luc (100 ng), pBax-Luc (100 ng), pFas-Luc (100 ng), pNFκB-Luc (100 ng), and NFAT-Luc (100 ng) plasmids in apoptotic 4pX-1 cells grown as a function of pX expression, with (+) or without (−) PFT-α (10 μM), as indicated. pX expression is via the Tet-off system, by tetracycline removal (5 μg/ml). Results are expressed as pX-dependent induction, −Tet/+Tet ratio, quantified from three independent assays performed in triplicate. B. immunoblot of Bax and p21 employing WCE from apoptotic 4pX-1 cultures grown with (+) or without (−) pX. Actin is the internal control. Quantification is by the Scion software, relative to actin. C. pX-dependent apoptosis, −Tet/+Tet ratio, by Phospholmager quantification of three independent radioactive DNA fragmentation assays of apoptotic 4pX-1 cultures (21) grown with (+) or without (−) PFT-α, as indicated. D. upper left panel, Western blot of p53 using WCE from 4pX-1 and 4pX-1-p53kd cell lines grown with (+) or without (−) pX or PFT-α (10 μM), as indicated. Lower left panel, immunofluorescence microscopy using the fluorigenic Z-DEVD-FMK caspase3 substrate (21) and 4pX-1 and 4pX-1-p53kd cells grown in apoptotic conditions for 24 h with (+) or without (−) pX or PFT-α (10 μM), as indicated. Lower right panel shows flow cytometric quantification of caspase3-positive cells in 4pX-1 and 4pX-1-p53kd cell lines, using the fluorigenic Z-DEVD-FMK caspase3 substrate (21). Error bars in A, C, and D represent the S.D.
HBV X Protein Mediates p53 Apoptosis by ATR Activation

Our previous studies have shown that pX induces Ser-18 phosphorylation of p53 within 1 h following onset of apoptosis (21). Ser-18 phosphorylation participates in p53 stabilization by disrupting interaction with cell lines, we monitored by Western blot analyses the phosphorylation state of Tyr-15 of Cdk2 as a function of pX expression (Fig. 3D). In 4pX-1 cells grown in apoptotic conditions for 12 h, pX expression results in Tyr-15 dephosphorylation of Cdk2, indicating pX mediates activation of Cdk2. By contrast, the inhibitory Tyr-15 phosphorylation is maintained in 4pX-1 cells not expressing pX, as well as in pX-expressing cells in which p38MAPKα has been knocked-down (4pX-1-p38MAPKαkd cells). We interpret these results to mean that pX cannot activate Cdk2 in the absence of the p38MAPK pathway. We conclude activation of the p38MAPK by pX expression mediates activation of Cdk4 and Cdk2, in turn phosphorylating Rb.

Phosphorylation of Rb results in release of E2F leading to transcription of E2F-responsive genes. Functional E2F1 binding sites have been mapped in the promoters of Arf (7), ASPP2 (37), and Chk2 genes (38). To determine whether pX expression induces the release of E2F1, we examined by ChIP assays the association of endogenous E2F1 with the promoters of Arf (7), ASPP2 (37), and Chk2 genes (38). ChIP assays with the E2F1 antibody were performed employing the 4pX-1 cell line and an E2F-1 4pX-1 knockdown cell line (4pX-1-E2F1kd) displaying nearly 70% depletion of endogenous E2F1 (Fig. 4A), grown in apoptotic conditions as a function of pX expression. E2F1 ChIP assays were analyzed both by quantitative real-time PCR and agarose gel electrophoresis of the PCR products (Fig. 4B). We observe increased association of endogenous E2F1 with the Arf, ASPP2, and Chk2 promoters in the presence of pX in the 4pX-1 cell line, but not in the 4pX-1E2F1kd cell line (Fig. 4B). In addition, pX-dependent transcriptional induction of the endogenous Arf, ASPP2, and Chk2 genes, 12 h following onset of apoptosis, was observed only in 4pX-1 cells, and not in the 4pX-1-E2F1kd cell line (supplemental Fig. S4B for Fig. 4B). Accordingly, both assays support that pX expression induces expression of endogenous E2F1-regulated genes including the Arf gene.

E2F1-induced ARF expression (7) promotes the dissociation of p53 from Mdm2 and, in turn, the stabilization of p53. Because E2F1 release is mediated by Rb phosphorylation, and the p38MAPK pathway via Cdk2/4 activation (Fig. 3, C and D) phosphorylates Rb (Fig. 3B), we examined the induction of ARF protein in the 4pX-1, 4pX-1-E2F1kd, and 4pX-1-p38MAPKαkd cell lines in response to pX expression; cell lysates were prepared in a time course following the onset of apoptosis by serum withdrawal. In 4pX-1 cells expressing pX, ARF expression is observed at time 0 h, and maintained for 24 h following the onset of apoptosis by serum withdrawal (Fig. 4C), in agreement with the pX-dependent activation of Cdk4/Cdk2 and phosphorylation of Rb (Fig. 3, B–D). In the 4pX-1-E2F1kd cell line used as our control, pX expression does not induce ARF (Fig. 4C). Importantly, in the 4pX-1-p38MAPKαkd cell line, ARF induction is also not observed in response to pX expression, clearly demonstrating that activation of the p38MAPK pathway by pX is necessary for E2F1 release and expression of ARF. pX expression increased the p53 protein level only in the 4pX-1 cell line, and not in the 4pX-1-E2F1kd and 4pX-1-p38MAPKαkd cell lines, which lack pX-mediated ARF expression (Fig. 4D). Moreover, ARF is important for the observed p53 stabilization, because only in 4pX-1 cells, and not in the 4pX-1-E2F1kd and 4pX-1-p38MAPKαkd cells, p53 co-immunoprecipitates with ARF (supplemental Fig. S4D for Fig. 4D). Together, these results link the stabilization of p53 with the activation of the p38MAPK pathway by pX.
Mdm2 and in promoting p53 nuclear retention (39) and association with CBP (40). Ser-18 phosphorylation of p53 is mediated by ATM/ATR kinases (10, 41).

Accordingly, we investigated whether pX induces activation of ATR and ATM in apoptotic 4pX-1 cultures. Immunoblot analyses employing the phosphospecific ATR and ATM antibodies show pX-dependent activation of ATR in the 0–3-h interval following the onset of apoptosis by serum withdrawal, without detectable ATM activation (Fig. 5A). In agreement with the role of ATR-mediated Ser-18 phosphorylation in p53 stability, a 2–3-fold increase in the p53 level is observed within 1–2 h following onset of pX-dependent apoptosis. Importantly, this increase in p53 stability in the presence of pX, is suppressed to a basal level following addition of the ATM/ATR-specific inhibitor caffeine (Fig. 5A). To confirm these observations we constructed an ATR knockout 4pX-1 cell line (4pX-1-ATR\textsuperscript{kd}) displaying nearly 70% reduction in the level of the ATR protein (Fig. 5B), and determined the level of p53 as a function of pX expression. In contrast to 4pX-1 cells, which exhibit increased p53 with pX expression, ATR knockdown in the 4pX-1-ATR\textsuperscript{kd} cell line abrogates this increase (Fig. 5C).

In apoptotic 4pX-1 cultures, the early effect of pX is activation of the p38MAPK pathway, regulating expression of the p53 pro-apoptotic genes Bax, Fas, and Noxa, and initiating apoptosis (21). Because our results (Fig. 5A) show that p53 activation by pX requires activation of ATR, we investigated whether the
FIGURE 4. pX induces E2F1 release and ARF expression. A, Western blot of E2F1 using WCE from 4pX-1 and 4pX-1-E2F1kd cell lines grown with (+) pX in apoptotic conditions for 2 h. Quantification is by the Scion software, relative to actin. B, ChIP employing E2F1-specific antibody and apoptotic 4pX-1 and 4pX-1-E2F1kd cells grown with (+) or without (−) pX, with PCR primers for the E2F1 binding sites of ARF, Chk2, and ASPP2 promoters. Left panel, quantification of ChIP assays by real-time PCR from three independent assays. Relative to IgG, the pX-dependent induction is significant (p < 0.005). Right panel, agarose gel electrophoresis of PCR products from ChIP assays immunoprecipitated with E2F1 antibody or IgG using apoptotic 4pX-1 cells grown with (+) or without (−) pX. C, Western blot of ARF using WCE from apoptotic 4pX-1, 4pX-1-E2F1kd, and 4pX-1-p38MAPKαkd cell lines grown with (+) or without (−) pX, as indicated. Actin is the internal control. Quantification relative to actin is by the Scion software. D, Western blot of p53 using WCE isolated from apoptotic 4pX-1, 4pX-1-E2F1kd, and p38MAPKαkd cell lines grown with (+) or without (−) pX, as indicated. Quantification relative to actin is by the Scion software.
activation of ATR is dependent on the p38MAPK pathway. Employing the 4pX-1-p38MAPKkd cell line, we examined by Western blot analyses the activation of ATR in response to pX expression (Fig. 6A). In 4pX-1 cells, pX expression mediates a 2-fold induction in ATR activation; by contrast, knockdown of p38MAPK/H9251 significantly reduces this pX-mediated activation of ATR (Fig. 6A).

To confirm these results, we employed 4pX-1, 4pX-1-ATRkd, and 4pX-1-p38MAPKkd cell lines and mapped the p53 phosphorylations required for p53 transcriptional activation. These include phosphorylations on Ser-18 and Ser-23 required for nuclear retention (39) and association with CBP (40), as well as ensuing modifications on Ser-389 of murine p53 (42). p53 was immunoprecipitated using lysates isolated from 4pX-1, 4pX-1-ATRkd, and 4pX-1-p38MAPKkd cells at 2 h following onset of pX-dependent apoptosis. Moreover, to have an internal control for the immunoprecipitation reactions, the actin antibody was added together with the p53 antibody. The p53/actin immunoprecipitates were analyzed by Western blots using p53 and phospho-p53-specific antibodies. In the 4pX-1 cell line, pX induces p53 stabilization and phosphorylations on Ser-18, Ser-23, and Ser-389. By contrast, p53 protein and Ser-18, Ser-23, and Ser-389 modifications are not detected, either in the ATR or the p38MAPKkd knockdown cell lines (Fig. 6B). These results couple activation of the p38MAPK pathway to the activation of ATR by pX, in mediating the stabilization (Fig. 2) and the transcriptional modifications of p53 (Fig. 6B).

To investigate whether activation of the p38MAPK pathway and activation of ATR by pX induce p53 transcription, we...
examined by real-time PCR expression of endogenous p53-regulated genes Bax, Fas, and Noxa, employing the p53, ATR, and p38MAPKα knockdown cell lines (Fig. 6C). The 4pX-1-ATRkd and 4pX-1-p38MAPKαkd cell lines as well as the control 4pX-1 and 4pX-1-p53kd cell lines were grown as a function of pX for 12 h following the onset of apoptosis. In the 4pX-1 cell line, pX induces expression of Bax and Noxa mRNAs by nearly 4-fold and Fas mRNA expression by 7-fold (21). By contrast in the p53, ATR, or p38MAPKα knockdown cell lines, pX mediates only a minimal induction of these mRNAs (Fig. 6C). Inhibition of the p38MAPK pathway by addition of SB 202190 or inhibition of ATR by caffeine abrogated the induction of Fas, Bax, and Noxa in response to pX (supplemental Fig. S6C for Fig. 6C). These results provide conclusive evidence of the essential role of the p38MAPK pathway and the activation of ATR in p53 pro-apoptotic gene transcription induced by pX.

p38MAPKα, E2F1, and ATR Are Essential in p53 Apoptosis Induced by pX—To investigate whether both E2F1 release and ATR activation mediated by p38MAPK activation are essential in p53 apoptosis in response to pX, we employed the 4pX-1 cell line as well as the 4pX-1-p53kd, 4pX-1-E2F1kd, 4pX-1-ATRkd, and 4pX-1-p38MAPKαkd cell lines in apoptosis assays. Apoptosis in response to pX expression was assayed by Western blots monitoring the level of activated, cleaved caspase3, in a time course following onset of apoptosis by growth factor withdrawal (Fig. 7A). In addition, apoptotic assays were performed in pseudo-knockdown 4pX-1 cell lines for p53, E2F1, ATR, and p38MAPKα (Fig. 7, B and C). These pseudo-knockdown cell lines were isolated in parallel with each of the respective knockdown cell lines. This control was performed to exclude effects of puromycin selection and propagation on the observed phenotype of each of the knockdown cell lines. Furthermore in Fig. 7D, we demonstrate that all knockdown cell lines exhibit tetracycline-regulated expression of pX.

In 4pX-1 cells a 3- and 5-fold activation of caspase3 is observed at 12 and 24 h following the onset of apoptosis, respectively. By contrast, pX-dependent caspase3 activation is nearly undetectable in the 4pX-1-p53kd cell line, supporting that p53 mediates apoptosis in response to pX expression. Likewise, the absence of pX-dependent caspase3 activation in the 4pX-1-E2F1kd and 4pX-1-ATRkd cell lines demonstrates the essential role of E2F1 and ATR in stabilizing and activating p53, respectively. Finally, minimal activation of caspase3 is detected in the 4pX-1-p38MAPKαkd cell line. By contrast, all the pseu-
HBV X Protein Mediates p53 Apoptosis by ATR Activation

A. 4pX-1 cell line

| active Caspase3 relative intensity | 0.03 | 0.05 | 0.24 | 0.16 | 0.04 | 0.05 | 0.37 | 1.28 |
|-----------------------------------|------|------|------|------|------|------|------|------|
| actin                             |      |      |      |      |      |      |      |      |

| 4pX-1-p53kd cell line |
|------------------------|
| active Caspase3 relative intensity | 0.03 | 0.04 | 0.04 | 0.03 | 0.03 | 0.12 | 0.16 |
| actin                   |      |      |      |      |      |      |      |

| 4pX-1-E2F1kd cell line |
|------------------------|
| active Caspase3 relative intensity | 0.02 | 0.02 | 0.09 | 0.20 | 0.02 | 0.02 | 0.17 | 0.16 |
| actin                   |      |      |      |      |      |      |      |      |

| 4pX-1- ATRkd cell line |
|------------------------|
| active Caspase3 relative intensity | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.07 | 0.06 |
| actin                   |      |      |      |      |      |      |      |

| 4pX-1-p38MAPKαkd cell line |
|----------------------------|
| active Caspase3 relative intensity | 0.03 | 0.03 | 0.09 | 0.11 | 0.05 | 0.04 | 0.22 | 0.38 |
| actin                        |      |      |      |      |      |      |      |      |

B. 4pX-1 cell line

| active Caspase3 relative intensity | 0.02 | 0.1 | 0.02 | 0.7 |
|-----------------------------------|------|-----|------|-----|
| actin                             |      |      |      |      |

| 4pX-1-p53 pseudokd cell line |
|------------------------------|
| active Caspase3 relative intensity | 0.02 | 0.1 | 0.02 | 0.6 |
| actin                         |      |      |      |      |

| 4pX-1-E2F1 pseudokd cell line |
|-------------------------------|
| active Caspase3 relative intensity | 0.01 | 0.1 | 0.01 | 0.6 |
| actin                          |      |      |      |      |

| 4pX-1- ATR pseudokd cell line |
|-------------------------------|
| active Caspase3 relative intensity | 0.01 | 0.1 | 0.02 | 0.6 |
| actin                          |      |      |      |      |

| 4pX-1-p38MAPKα pseudokd cell line |
|-----------------------------------|
| active Caspase3 relative intensity | 0.02 | 0.15 | 0.02 | 0.7 |
| actin                             |      |      |      |      |

C. 4pX-1 4pX-1-p53 pseudokd

| p53                | 1     | 1     |
|--------------------|-------|-------|
| actin              |       |       |

| 4pX-1 4pX-1-E2F1 pseudokd |
|---------------------------|
| E2F1                      | 1     | 1     |
| actin                     |       |       |

| 4pX-1 4pX-1- ATR pseudokd |
|--------------------------|
| ATR                       | 1     | 1     |
| actin                     |       |       |

| 4pX-1 4pX-1-p38MAPKα pseudokd |
|-----------------------------|
| p38MAPKα                    | 1     | 1     |
| actin                       |       |       |

D. 4pX-1 4pX-1-p38MAPKα pseudokd

| p17-α                 | 4pX-1 | 4pX-1-ATR pseudokd |
|-----------------------|-------|--------------------|
| GAPDH                 |       |                    |

| tet:  |
|-------|
| 4pX-1 |
| +     |
| -     |
| +     |
| +     |
| +     |
| +     |
| -     |
| +     |
| +     |
do-knockdown cell lines exhibit activation of caspase3 in response to pX expression (Fig. 7B). Similar results were obtained by monitoring caspase3 activation by immunofluorescence microscopy, employing the cleaved caspase3 antibody (data not shown).

Because our results show that activation of the p38MAPK pathway by pX is necessary both for the stabilization of p53 (Fig. 2) and the activation of ATR (Fig. 6), we conclude that the p38MAPK pathway couples the stabilization of p53 with the transcriptional activation of p53.

DISCUSSION

In this study, we determined the mechanism by which the weakly oncogenic HBV X protein activates p53, leading to apoptosis. The significance of our study is the determination of the mechanism by which a viral oncoprotein induces both the stabilization and transcriptional activation of p53; this mechanism involves the sustained activation of the p38MAPK pathway (21). Stabilization of p53 differs from p53 activation, the latter requiring multisite phosphorylations necessary for nuclear retention, p53 oligomerization, DNA binding, CBP recruitment, and pro-apoptotic gene transcription, leading to p53-mediated apoptosis (42). It is well established that Ser-18 and Ser-23 phosphorylations of murine p53, mediated by ATM/ATR, transcriptionally activate p53 (10, 41, 49). Viral oncoproteins including E1A, SV40 T-antigen, E7 of HPV 16 induce p53-mediated apoptosis by binding to Rb, releasing E2F, and inducing ARF expression, thereby stabilizing p53 (3). Likewise, overexpression of cellular oncogenes including c-myc (50) and H-RasV12 (51), induce p53-mediated apoptosis. Overexpression of oncopgenic c-myc promotes p53 apoptosis via ATM-mediated activation of p53 (52). However, how overexpression of these cellular and viral oncogenes mediate transcriptional activation of p53 has not been determined.

In this study we provide evidence that pX expression increases the half-life of p53 by activating the p38MAPK pathway which induces Cdk4/Cdk2 activation, Rb phosphorylation, E2F1 release, and E2F1-mediated ARF expression. This mechanism of pX-mediated p53 stabilization is in agreement with the frequent genetic deletions or mutations of Rb/E2F1/ARF. The mechanism of p53 activation was not determined. E2F1 also induces transcription of additional E2F1 genes, including ASPP2 and Chk2. The pX-mediated p38MAPK activation induces ATR activation, leading to p53 activation, p53 pro-apoptotic gene transcription, and apoptosis. Furthermore, ATR activation contributes to p53 stabilization upon serum withdrawal as shown in Fig. 6B.

ATR is the sensor of replication stress. The pX-dependent ATR activation we report agrees with our earlier studies (23), demonstrating that pX expression in 4pX-1 cells promotes robust cell cycle entry, unscheduled entry into the S-phase, and an S-phase pause. This activity of pX resembles the unscheduled S-phase entry of other viral oncoproteins including E1A, T-antigen, E7 of HPV 16, whose expression also induces p53-mediated apoptosis (3). However, in those studies (3), the mechanism of p53 activation was not determined.

FIGURE 7. E2F1, ATR and p38MAPKα are essential in p53 apoptosis in response to pX. A and B, Western blot assays of active caspase3 using WCE isolated from (A) apoptotic 4pX-1 and 4pX-1-p38MAPKα, 4pX-1-E2F1α, 4pX-1-ATRα, and 4pX-1-p38MAPKαα cells or (B) the respective pseudo-knockdown cell lines, grown with (+) or without (−) pX as indicated. Quantification is by the Scion software. A representative assay is shown from three independent experiments. C, Western blot assays of p53, E2F1, ATR, and p38MAPKα using WCE from 4pX-1 cells and the indicated pseudo-knockdown cell lines. D, agarose gel electrophoresis of PCR reactions employing RNA isolated from the indicated cell lines grown with (+) or without (−) tetracycline for 24 h. pT7-X is the positive control. GAPDH is the internal control.
mediated apoptosis in response to pX. We have previously shown that weakly oncogenic viral protein, the HBV pX, activating p53 and in the activation of ATR. We conclude that HBV X Protein Mediates p53 Apoptosis by ATR Activation

It has been suggested that E2F1 activation integrates the Rb/ARF pathway to the DNA damage response pathway (38). Functional E2F1 binding sites have been mapped in the promoters of ARF, ASPP2, ATM, and Chk2 genes, using E2F1 over-expression or overexpression of viral E1A or E7 proteins known to deregulate the Rb/E2F pathway (38, 56). In contrast to those studies, our studies monitored the endogenous E2F1 activity in the activation of AT by pX, the activation of ATM by pX is detectable only with doxorubicin co-treatment of apoptotic 4pX-1 cells.4

In summary, our studies show that (i) pX mediates apoptosis via p53; (ii) E2F1 is essential in pX-mediated apoptosis, stabilizing p53, and integrating with the DNA damage pathway; (iii) ATR is essential in pX-mediated apoptosis by transcriptionally activating p53; and (iv) p38MAPKα is essential both in the stabilization of p53 and in the activation of ATR. We conclude that the p38MAPK pathway coordinates Rb/E2F1/ARF and DNA damage response pathways by inducing both p53 stabilization and p53 transcriptional activation, respectively, leading to p53-mediated apoptosis in response to pX.

Acknowledgments—We thank Dr. S. Mendrysa and Leo Studach for critical review of this manuscript.

REFERENCES

1. Kinzler, K. W., and Vogelstein, B. (1997) Nature 386, 761–763
2. Giaccia, A. J., and Kastan, M. B. (1998) Genes Dev. 12, 2973–2983
3. Lowe, S. W., and Ruley, H. E. (1993) Genes Dev. 7, 535–545
4. de Stanchina, E., McCurrach, M. E., Zindy, F., Shieh, S.-Y., Ferbeyer, G., Samuelson, A. V., Prives, C., Roussel, M. F., Sherr, C. J., and Lowe, S. (1998) Genes Dev. 12, 2434–2442
5. Günsberg, D. (2002) FEBS Lett. 529, 122–125
6. Huett, Y., Maya, R., Kazar, A., and Oren, M. (1997) Nature 387, 296–299
7. Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., and Vousden, K. K. (1998) Nature 395, 124–125
8. Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, I., Chen, K., Orlow, I., Lee, C. H., Cordon-Cardo, C., and DePinho, R. A. (1998) Cell 92, 713–723
9. Tang, Y., Luo, J., Zhang, W., and Gu, W. (2006) Mol. Cell 24, 827–839
10. Cattaneo, S. C., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) Science 281, 1677–1679
11. Kastan, M. B., and Lim, D. S. (2000) Nat. Rev. Mol. Cell Biol. 1, 179–186
12. Hainaut, P., Hernandez, T., Robinson, A., Rodriguez-Tome, P., Flores, T., Hollstein, M., Harris, C. C., and Montesano, R. (1998) Nucleic Acids Res. 26, 205–213
13. Bulavin, D. V., Demidov, O. N., Saito, S., Kauraniemi, P., Phillips, C., Amundson, S. A., Ambrosino, C., Sauter, G., Nebreda, A. R., Anderson, C. W., Kallioniemi, A., Fornace, A. J., Jr., and Appella, E. (2002) Nat. Genet. 31, 210–215
14. Greenblatt, M. S., Bennett, W. P., Hollstein, M., and Harris, C. C. (1994) Cancer Res. 54, 4855–4870
15. Buendia, M. A. (2000) Semin Cancer Biol. 10, 185–200
16. Parkin, D. M., Bray, F. I., and Devesa, S. S. (2001) Eur. J. Cancer 37, 54–66
17. Andrisani, O. M., and Barnabas, S. (1999) Int. J. Oncol. 15, 373–379
18. Bouchard, M. J., and Schneider, R. J. (2004) J. Virol. 78, 12725–12734
19. Benn, J., and Schneider, R. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11125–11129
20. Lee, C., Hong, B., Choi, J. M., Kim, Y., Watanabe, S., Ishimi, Y., Enomoto, T., Tada, S., Kim, Y., and Cho, Y. (2004) Nature 430, 913–917
21. Wang, W. H., Gregori, G., Hullinger, R. L., and Andrisani, O. M. (2004) Mol. Cell Biol. 24, 10352–10365
22. Tarn, C., Bilodeau, M. L., Hullinger, R. L., and Andrisani, O. M. (1999) J. Biol. Chem. 274, 2327–2336
23. Lee, S., Tarn, C., Wang, W. H., Chen, S., Hullinger, R. L., and Andrisani, O. M. (2002) J. Biol. Chem. 277, 8730–8740
24. Su, Q., Schroder, C. H., Hofmann, W. J., Otto, G., Pichlmayr, R., and Bannasch, P. (1998) Hepatology 27, 1109–1120
25. Thorgeirsson, S. S., and Grisham, J. W. (2002) Nat. Genet. 31, 339–346
26. Dirac, A. M., and Bernards, R. (2003) J. Biol. Chem. 278, 11731–11734
27. Benjamin, J. C., Paris, M., Wang, W.-H., Hong, S., Kim, K., Hullinger, R. L., and Andrisani, O. M. (2006) J. Biol. Chem. 281, 2969–2981
28. Paris, M., Wang, W.-H., Shin, M.-H., Franklin, D. S., and Andrisani, O. M. (2006) Mol. Cell Biol. 26, 8826–8839
29. Murphy, P. J., Galigniana, M. D., Morishima, Y., Harrell, J. M., Kwok, R. P., Ljungman, M., and Pratt, W. B. (2004) J. Biol. Chem. 279, 30195–30201
30. Chirillo, P., Pagano, S., Natoli, G., Puri, P. L., Burgio, V. L., Balsano, C., and Leverero, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8162–8167
31. Cerrato, J. A., Khan, T., Koul, D., Lang, F. G., Conard, C. A., Wung, K. H., and Liu, T. J. (2004) Int. J. Oncol. 24, 401–417
32. Moon, N. S., Frolov, M. V., Kwon, E. J., De Stefano, L., Dimova, D. K., Morris, E. J., Taylor-Harding, B., White, K., and Dyson, N. J. (2005) Dev. Cell 9, 465–475
33. Pediconi, N., Ianari, A., Costanzo, A., Belloni, L., Gallo, R., Cimino, L., Porcellini, A., Screpanti, I., Balsano, C., Alessi, E., Gulino, A., and Leverero, M. (2003) Nat. Cell Biol. 5, 552–558
34. Wells, J., Gravel, C. R., Barthel, S. M., Madore, S. J., and Farnham, P. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 3890–3895
35. Hunter, T. (1995) Cell 80, 225–236
36. Morgan, D. O. (1995) Nature 374, 131–134
37. Fogal, V., Kartasheva, N. N., Trigiante, G., Llanos, S., Yap, D., Voussden, K. H., and Lu, X. (2005) Cell Death Differ. 12, 369–376
38. Hershko, T., Chauussied, M., Oren, M., and Ginsberg, D. (2005) Cell Death Differ. 12, 377–383
39. Zhang, Y., and Xiong, Y. (2001) Science 292, 1910–1915
40. Gu, W., and Roeder, R. G. (1997) Cell 90, 595–606
41. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998) Science 281, 1674–1677
42. Meek, D. W. (1999) Oncogene 18, 7666–7675
43. Deleted in proof
44. Deleted in proof
45. Deleted in proof
HBV X Protein Mediates p53 Apoptosis by ATR Activation

Brown, E. J., and Baltimore, D. (2000) *Genes Dev.* **14**, 397–402

Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999) *Genes Dev.* **13**, 152–157

Hermeking, H., and Eick, D. (1994) *Science* **265**, 2091–2093

Seger, Y. R., Garcia-Cao, M., Piccinin, S., Cunsolo, C. L., Doglioni, C., Blasco, M. A., Hannon, G. J., and Maestro, R. (2002) *Cancer Cell* **2**, 401–413

Pusapati, R. V., Rounbehler, R. J., Hong, S., Powers, J. T., Yan, M., Kiguchi, K., McArthur, M. J., Wong, P. K., and Johnson, D. C. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 1446–1451

Bulavin, D. V., Higashimoto, Y., Popoff, I. J., Gaarde, W. A., Basrur, V., Potapova, O., Appella, E., and Fornace, A. J., Jr. (2001) *Nature* **411**, 102–107

Tibbetts, R. S., Cortez, D., Brumbaugh, K. M., Scully, R., Livingston, D., Elledge, S. J., and Abraham, R. T. (2000) *Genes Dev.* **14**, 2989–3002

Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., Donehower, L. A., and Elledge, S. J. (2000) *Genes Dev.* **14**, 1448–1459

Rogoff, H. A., Pickering, M. T., Frame, F. M., Debatis, M. E., Sanchez, Y., Jones, S., and Kowalski, T. F. (2004) *Mol. Cell Biol.* **24**, 2968–2977