The hepatitis B x antigen effector, URG7, blocks tumour necrosis factor α-mediated apoptosis by activation of phosphoinositol 3-kinase and β-catenin

Jingbo Pan,1 Zhaorui Lian,1 Sarah Wallet1,2 and Mark A. Feitelson1,3†

Hepatitis B x antigen (HBxAg) contributes significantly to the pathogenesis of chronic infection and development of hepatocellular carcinoma. To discern some of its operative pathways, HepG2 cells were stably transduced with HBx or the bacterial chloramphenicol acetyltransferase (CAT) gene. Differential gene expression has previously revealed an upregulated gene, clone 7 (URG7), that conferred resistance to anti-Fas killing on HepG2X cells. Given that tumour necrosis factor alpha (TNFα) is also an important mediator of chronic hepatitis, and partially shares signalling with Fas, experiments were designed to test whether URG7 blocks TNFα killing of HepG2X cells. HepG2X cells expressing URG7 and HepG2 cells overexpressing URG7 in the absence of HBxAg were resistant to TNFα killing compared with HepG2CAT cells. URG7 small interfering RNA restored the sensitivity of HepG2X cells to TNFα killing. Killing was associated with the activation of caspases 3 and 8, suggesting that URG7 blocked these caspases. This resistance was also associated with activation of phosphoinositol 3-kinase/Akt. Given that Akt and HBxAg also activate β-catenin, experiments were designed to determine whether URG7 blocked apoptosis via activation of β-catenin. Both HBxAg and URG7 activated fragments of the β-catenin promoter, and also promoted expression of β-catenin target genes. Hence, URG7 inhibits TNFα-mediated killing by blocking one or more caspases in the apoptotic pathway and by activating phosphoinositol 3-kinase and β-catenin, thereby overriding the apoptotic signalling of TNFα. This suggests that URG7 helps to protect virus-infected hepatocytes during chronic hepatitis B virus infection.

INTRODUCTION

Hepatitis B virus (HBV) establishes chronic infection in more than 350 million people worldwide (Tiollais et al., 1985). These people are at high risk for the development of hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (Beasley & Hwang, 1984). The pathogenesis of chronic infection is immune-mediated (Chisari & Ferrari, 1995), yet HBV is rarely cleared despite the persistence of cell-mediated and humoral immune responses against virus-infected cells. The findings that acute, resolving hepatitis is associated with strong, polyspecific and persistent immune responses, while chronic hepatitis is associated with weak, monospecific and transient immune responses (Chisari & Ferrari, 1995), suggest that the carrier state and chronic liver disease (CLD) depend upon the quality, timing and amplitude of immune responses that develop following acute infection.

HBV encodes a small trans-activating protein, referred to as hepatitis B x antigen (HBxAg), that contributes to the establishment of the carrier state and to the pathogenesis of CLD. For example, woodchucks are naturally infected with an HBV-like woodchuck hepatitis virus, and when an infectious molecular clone of the virus was used for experimental infection of neonatal animals, most became carriers, developed CLD, and then HCC (Popper et al., 1987). However, when the X gene was mutated in this clone, so that no corresponding protein was made, experimental infection consistently failed to give rise to carriers and no liver disease developed (Chen et al., 1993; Zoulim et al., 1994). This work suggested that X antigen
trans-activation of virus gene expression and replication was important for the development of the carrier state. Independent work showed a direct correlation between X antigen staining in woodchuck and human infections, and the intensity of CLD (Wang et al., 1991a, b; Feitelson et al., 1993; Jin et al., 2001), suggesting that X antigen may also protect infected hepatocytes from immunologically mediated killing. If so, X antigen would help preserve replication space for the virus despite ongoing immune responses aimed at the elimination of virus-infected hepatocytes.

Given that HBxAg is a trans-activating protein, it is possible that the upregulated expression of one or more host proteins contributes to the apparent resistance of infected cells to immune-mediated killing (Feitelson & Duan, 1997). To test this, cultures of HepG2 cells have been stably transduced with recombinant retrovirus encoding HBxAg or the bacterial chloramphenicol acetyltransferase (CAT) gene as a control (Lian et al., 1999). When the differential expression of cellular mRNAs was studied by PCR select cDNA subtraction, an uncharacterized gene, provisionally designated upregulated gene, clone 7 (URG7), was found to partially protect cells against anti-Fas-mediated killing (Lian et al., 2001). Given that Fas killing contributes to the pathogenesis of CLD (Mochizuki et al., 1996; Luo et al., 1997; Roskams et al., 2000), it is likely that the upregulated expression of URG7 in HBxAg-positive cells provides partial protection against immune clearance. Tumour necrosis factor alpha (TNFz) is also an important mediator of inflammation in CLD (Lau et al., 1991; Hussain et al., 1994; Marinos et al., 1995; Fang et al., 1996), and shares part of its signalling cascade with Fas [at the level of the Fas-associated death domain (FADD) and caspase 8], raising the possibility that URG7 may protect against TNFz killing as well. Hence, experiments were designed to test the hypothesis that HBxAg, through URG7, blocked TNFz killing, and to elucidate some of the mechanistic steps associated with resistance to these killing signals.

**METHODS**

**Cell lines and tissue culture conditions.** The human hepatoblastoma cell line, HepG2 (Aden et al., 1979) (ATCC), was cultured on tissue culture dishes or plates coated with type-I rat tail collagen (Becton Dickinson). Cells were grown in Earle's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM l-glutamine, 100 μM non-essential amino acids, 1 mM sodium pyruvate, and standard concentrations of penicillin plus streptomycin (all from Mediatech). HepG2X and HepG2CAT cells were prepared and characterized as described previously (Lian et al., 1999), and cultured at 37 °C in 5% CO₂. The human hepatocellular carcinoma cell line, Huh7 (Nakabayashi et al., 1982), was also used to create Huh7X and Huh7CAT cultures in parallel experiments.

**Plasmids and transient transfections.** The retroviral plasmid, pSLXCMVneo, was used to clone URG7 cDNA (Lian et al., 1999). This was done by PCR, amplifying the 652 bp fragment encoding full-length URG7, flanked by MluI and BglII restriction endonuclease sites, which facilitated cloning to the corresponding sites within the pSLXCMV polylinker. Correct cloning was verified by DNA sequencing in the appropriate facility at the Kimmel Cancer Institute of Thomas Jefferson University.

To measure NF-kB activity, the reporter plasmid pGL2-HIV-LTR-luciferase was used as described previously (Lian et al., 1999). To measure AP-1 activity, the plasmid p-AP-1-luciferase was purchased from Stratagene. To test for β-catenin promoter activity, two fragments of the β-catenin promoter cloned into pSEAP-basic (a gift from Dr Frans van Roy, University of Ghent) (Nollet et al., 1996) were used. The fragments were FRAG 2 (−298 to +139) and FRAG 3 (the 6 kb fragment adjacent to the 5’ end of the gene). Promoter activity was evaluated by measuring secreted human placental alkaline phosphatase (SEAP) at 24 and 48 h after transfection using a Phospho-Light chemiluminescent reporter gene assay (Soriano et al., 1991). To measure β-catenin activity on cognate promoters, cells were transiently transfected with the T-cell factor reporter plasmids p-TOPFLASH (which is β-catenin responsive) or p-TOPFLASH (a mutant that is β-catenin unresponsive) (both from Upstate Technology).

For transient transfections, cells were seeded in six-well plates (4 × 10⁵ cells per well) and incubated overnight at 37 °C in 5% CO₂. Reporter plasmids (0.5 μg per transfection) were transiently transfected by standard calcium phosphate precipitation. After overnight incubation with the DNA precipitates, cells were washed with PBS and incubated in fresh complete medium for another 24 h. Luciferase activity in 10 μg total protein lysate from each sample was measured by using a luciferase assay kit (Promega) according to the manufacturer’s instructions.

**Preparation of HepG2URG7 cells.** Recombinant retrovirus encoding URG7 was prepared as described previously (Lian et al., 1999), and then used to stably transduce HepG2 cells. Cells were selected in G418 (1 mg/ml) for 3 weeks, and all drug-resistant cells were passaged without selection of individual colonies. Lysates prepared from 5 × 10⁶ HepG2URG7 cells were assayed for URG7 by Western blot analysis with a mixture of peptide antibodies, as described previously (Lian et al., 2001). Some experiments were performed using peptide antibodies with the same specificities that were kindly provided by Dr Ling-Xun Duan (Aiviva Biosystems).

**Western blot analysis.** Cell lysates were prepared with lysis buffer containing 50 mM Tris/HCl (pH 7.4), 250 mM NaCl, 5 mM EDTA, phosphatase inhibitors (50 mM NaF, 0.1 mM Na₂VO₄), protease inhibitors (1 mM PMSF, 10 μg leupeptin ml⁻¹ and 10 μg pepstatin ml⁻¹) and 1% Triton X-100. Each sample was analysed on a 4–20% Tris/HCl Ready gel (Biorad Laboratories), and the proteins then transferred to PVDF membranes (Millipore). After blocking, Western blot analysis was performed with rabbit anti-URG7 or anti-URG11 peptide antibodies (Lian et al., 2001, 2006), with rabbit anti-phospho-AKT (ser473; Upstate Cell Signalling), with mouse monoclonal β-catenin antibody (E-5; Santa Cruz Biotechnology, which recognized β-catenin in HepG2 cells), with mouse anti-GSK3β (for total GSK3β levels; Santa Cruz), with mouse anti-phospho-GSK3β (at serine 6; Santa Cruz), or with a mouse monoclonal antibody recognizing only activated wild-type β-catenin (clone 8E7; Upstate Cell Signalling). All antibodies (except anti-URG7; 1:5000) were used at a 1:1000 dilution. The secondary antibodies were horseradish peroxidase-(HRP) conjugated goat anti-rabbit Ig (diluted 1:4000; Accurate), or HRP-conjugated goat anti-mouse Ig (diluted 1:3000; Accurate), and the results were visualized using enhanced chemiluminescence (ECL; Amersham). Mouse anti-α-tubulin monoclonal antibody (Clone AC-15; Sigma) was used as an internal control.

**Inhibition of phosphoinositol 3-kinase (PI3K).** To evaluate the effects of the PI3K inhibition on p-AKT levels, cells were pretreated
with 50 μM Ly294002 (Cell Signalling Technology) for 24 h, lysed, and then analysed for p-AKT by Western blotting, as described above.

**RNA isolation and Northern blot analysis.** Total cellular RNA was isolated using the RNA Mini kit (Qiagen). A 10 μg aliquot of RNA from each sample was analysed on 1% denaturing agarose gels using formaldehyde, and the integrity was assessed with rRNA. Samples were then blotted onto nitrocellulose membranes (Schleicher & Schuell). Northern blot analysis was carried out using a URG7 probe obtained from a pSLXCMV URG7 fragment insert that was radiolabelled with [α-32P]-labelled dCTP using the Prime-a-Gene labelling system (Promega). Following autoradiographic exposure, membranes were stripped and rehybridized with a radiolabelled glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe (Clontech). The G3PDH signal served to normalize the URG7 mRNA levels following gel scanning.

**TNFα treatment, signalling inhibitors and cell viability.** Approximately 18 000 cells, in 90 μl of complete culture medium, were seeded into each well of a 96-well plate and incubated overnight at 37 °C in 5% CO₂. To each well, 10 μl of 10 x solutions of TNFα and cycloheximide (CHX) were added to achieve final concentrations of 1 ng ml⁻¹ and 10 μg ml⁻¹, respectively. After 24 h, cells were analysed using the MTT assay [20 μl per well, CellTiter 96 AQueous One Solution cell proliferation assay (Promega)], and absorption was read 2–4 h later with an ELISA plate reader. Cells treated with only CHX were used as controls. The percentage cell survival was calculated as follows: (average OD₄₉₀ of wells treated with TNFα plus CHX/average OD₄₉₀ of wells treated with CHX only) x 100.

In some experiments, cells were pretreated with the caspase inhibitors Z-VAD-FMK or Z-IETD-FMK, or with the PI3K inhibitor Ly294002 (all from Cell Signalling Technology), for 1 h prior to the addition of TNFα and CHX. A 1 μl aliquot from 100 × stocks of each inhibitor was added to each well to give a final inhibitor concentration of 50 μM.

**Caspase-3 assay.** Cells were cultured in six-well plates (8 x 10⁵ per well) in complete medium overnight. Cultures were divided into five groups and treated with complete medium, TNFα, CHX, TNFα plus CHX, or TNFα and CHX plus Z-VAD-FMK. After 6 h incubation, cells were lysed and 30 μg of total protein from each sample was evaluated for caspase-3 activity using a commercially available colorimetric assay (Promega CaspACE assay system). The specific activity of caspase-3 [pmol p-nitroaniline (pNA) liberated h⁻¹ per μg protein] was calculated as per the manufacturer’s instructions.

**URG7 and URG11 small interfering RNAs (siRNAs).** To verify the contribution of upregulated URG7 to cell survival, cells were pretreated with URG7-specific or control siRNA. Accordingly, cells were seeded in 96-well plates (1 x 10⁴ per well) in antibiotic-free complete medium and cultured overnight. URG7 siRNA (sense sequence: CAAAGGCAAGGUGACACUdTdT) was transfected into the cells with DharmaFECT1 (DF1, Dharmacon) according to the manufacturer's instructions. In some experiments, URG7 siRNAs (residues 420–438: CAGACGGAUUGCUGUACUU and residues 1385–1403: ACACAGACUUACCUACAA) were used. For transfection, 100 nM siRNA and 0.2 μl DF1 were added to each well. Parallel wells were transfected with siControl Non-Targeting #1 siRNA (Dharmacon), or with transfection reagent only. After 48 h incubation, the medium was replaced with CHX with or without TNFα. Cell viability was measured by using the MTT assay 24 h later.

To verify that URG7 siRNA suppressed URG7 or p-AKT, Western blots analyses were performed after the transfection of cells with URG7 siRNA. Briefly, 3.5 x 10⁵ cells per well were cultured overnight in six-well plates, and then transfected with 100 nM siRNA and 6 μl DF1. Cell lysates were prepared 48 h later and 40 μg total protein was analysed by Western blotting. Transfection with siControl #1 or with transfection reagent alone provided additional controls.

**Statistical analysis.** Comparisons of HepG2X, HepG2CAT and HepG2URG7 cells with regard to sensitivity to TNFα killing, relative caspase 3 or 8 activities, relative phospho-Akt levels, relative levels of β-catenin promoter activity, and of β-catenin effector genes, were made using the Student’s t-test. A significant difference was scored when P<0.05.

**RESULTS**

**URG7 provides resistance to TNFα-mediated apoptosis by blocking caspases 3 and 8**

To study the properties of URG7 in the absence of HBxAg, URG7 cDNA was cloned into pSLXCMVneo. HepG2 cells were then transduced with recombinant retroviruses, selected in G418 for 3 weeks, and the entire culture was expanded without selection of individual colonies. The stable upregulated expression of URG7 was then verified by Northern blot analysis, which showed that HepG2URG7 cells had 5.1 ± 0.3-fold more URG7 mRNA than HepG2CAT cells (Fig. 1a). By comparison, HepG2X cells had 4.2 ± 0.44-fold more URG7 mRNA than HepG2CAT cells (Fig. 1a), indicating that the levels of URG7 in HepG2URG7 and HepG2X were similar, and that both were significantly elevated compared with HepG2CAT cells (P<0.001). Similar observations were obtained by Western blot analysis, which showed that URG7 protein levels in HepG2URG7 and HepG2X cells were 4.8 ± 0.3-fold and 4.6 ± 0.4-fold higher, respectively, than in HepG2CAT cells (P<0.001) (Fig. 1b).

To determine whether the differences in URG7 expression in these culture systems also correspond to differences in the biological response of these cells to TNFα, these cultures were treated with TNFα plus CHX for 24 h. Live cells were measured by MTT assay in TNFα plus CHX-treated cultures and compared to the same cultures treated with CHX alone. The results showed that the survival of HepG2URG7 (72%) and HepG2X cells (82%) was significantly greater than that of HepG2CAT cells (49%, P<0.005, Fig. 1c), suggesting that HBxAg, perhaps mostly through URG7, blocks the ability of TNFα to trigger killing of HepG2 cells. Pretreatment of these cells with the broad spectrum caspase inhibitor, Z-VAD-FMK, completely protected these cells from TNFα killing. Pretreatment of these cells with the caspase 8 inhibitor, Z-IETD-FMK, also offered protection against TNFα killing, suggesting that URG7 blocked TNFα signalling by inhibiting caspase 8 (Fig. 1c). Given that HBxAg blocks caspase 3 (Gottlob et al., 1998), and that this effector caspase is downstream of caspase 8 in TNFα signalling, it was expected that this would also be the case for URG7. The results showed that caspase 3 was not activated by medium, by TNFα alone, or by CHX alone, but required the combination of TNFα plus CHX, and that caspase 3 activity was suppressed 2.6 ± 0.19-fold in HepG2X and 2.1 ± 0.23-fold in HepG2URG7...
compared with HepG2CAT cells (P<0.005; Fig. 2). Hence, it appears that HBxAg, through URG7, provides protection against TNF-α killing by blocking the action of one or more caspases in the TNF-α signalling pathway that triggers apoptosis.

**URG7 provides resistance to TNF-α-mediated apoptosis by activation of PI3K/Akt signalling**

PI3K has been shown previously to protect cells from apoptosis in a caspase-dependent manner (Berra et al., 1998; Chen et al., 1998; Gibbs & Grabbe, 1999). Since PI3K is also activated by HBxAg (Lee et al., 2001), experiments were designed to ask whether URG7 overexpression activated PI3K. Accordingly, HepG2X, HepG2URG7 and HepG2CAT cells were analysed for the phosphorylated (activated) form of Akt, a substrate for PI3K. HepG2X and HepG2URG7 cells had 4.6 ± 0.52- and 4.3 ± 0.48-fold more phosphorylated Akt than HepG2CAT cells (P<0.001, Fig. 3a). When these cells were treated with the PI3K inhibitor, Ly294002, no phosphorylated Akt was detected (Fig. 3a). When these cells were incubated with Ly294002 for 1 h, before the addition of TNF-α plus CHX, significant reductions in viability were observed for HepG2X (P<0.005) and HepG2URG7 (P<0.01), while the decrease in viability among HepG2CAT cells did not reach statistical significance (P>0.1, Fig. 3b), suggesting that Akt activation in HepG2URG7 and HepG2X cells was associated with increased resistance to TNF-α killing.

**Effect of URG7-specific siRNA upon Akt signalling and resistance to TNF-α killing**

To verify that URG7 expression is important for Akt activation and resistance to TNF-α-mediated killing, HepG2X and HepG2URG7 cells were transiently transfected with URG7-specific or control siRNA. The results showed that the introduction of URG7-specific siRNA suppressed URG7 expression several fold in the cultures tested (Fig. 4a, lane 2), compared with mock-transfected cells (lane 1) or cells transfected with an irrelevant siRNA (lane 3), demonstrating that it was active in down-regulating expression of URG7 (P<0.001). In parallel...
experiments, treatment with URG7-specific siRNA significantly depressed the levels of phosphorylated (activated) Akt roughly sixfold in HepG2URG7 (P<0.001) and roughly fivefold in HepG2X (P<0.001) compared with the same cells treated with an irrelevant siRNA (Fig. 4b). When the experiments were repeated and cell viability was monitored after transient transfection, only cells transfected with URG7-specific siRNA were highly sensitive to TNFα killing (Fig. 4c). Given that this work was performed in HepG2 cells, parallel experiments were conducted with the human hepatoma cell line, Huh7, stably transduced with the X or CAT genes. Transient transfection of URG7-specific but not control siRNA into Huh7X and Huh7CAT cells blocked endogenous URG7 polypeptide expression and Akt phosphorylation (data not shown but similar to Fig. 4a and b, respectively). Treatment of Huh7X and Huh7CAT with TNFα plus CHX along with URG7-specific siRNA blocked the ability of HBxAg to protect cells from TNFα killing, while control siRNA-transfected cells did not block the ability of HBxAg to protect cells from TNFα (Fig. 4c), suggesting that this activity was not restricted to HepG2 cells. In addition, the fact the cultures tested were not of clonal origin, underscores the generality of these findings. These results confirm that elevated URG7 is associated with activation of Akt, and that the latter is also associated with resistance to TNFα.

URG7 activates β-catenin signalling

Glycogen synthase 3β (GSK3β) is inactivated after phosphorylation by Akt, resulting in the stabilization of β-catenin (Morin, 1999). Given that HBxAg activates β-catenin by inactivation of GSK3β and by trans-activating the β-catenin promoter (Lian et al., 2006), experiments were designed to test whether the URG7-activation of Akt did the same. The results showed that GSK3β was strongly phosphorylated in HepG2URG7 and HepG2X cells compared with HepG2CAT cells (P<0.001, Fig. 5a), suggesting that HBxAg inactivated GSK3β through URG7. To determine whether HBxAg trans-activated the β-catenin promoter through URG7, cells were transiently transfected with reporter plasmids expressing luciferase under the control of two overlapping fragments of the β-catenin promoter (Nollet et al., 1996). The results showed that both HBxAg and URG7 stimulated the β-catenin promoter by 3–4-fold (P<0.005, Fig. 5b). Co-transfection of URG7-specific or control siRNAs into HepG2URG7 cells showed that upregulated β-catenin promoter activity was a function of URG7 expression (Fig. 5b). Given that both HBxAg and URG7 stimulate PI3K/Akt (Figs 3a and 4b), that HBxAg activates NF-κB (Sliva, 2004; Amiri & Richmond, 2005), and that there are NF-κB-binding sites in the β-catenin promoter (Nollet et al., 1996; Li et al., 2004), these results suggest that β-catenin may be transcriptionally targeted by these mechanisms. This was supported by Western blot analysis using an antibody that specifically bound activated β-catenin, which showed a 4–5-fold increase in activated β-catenin in HepG2URG7 and HepG2X cells relative to HepG2CAT (Fig. 5c). To see whether the accumulation of β-catenin resulted in the activation of its downstream target genes, the β-catenin-responsive TOPFLASH reporter and β-catenin-non-responsive FOPFLASH reporter constructs were transiently transfected into HepG2URG7, HepG2X and HepG2CAT cells. The results showed very strong activation of β-catenin-responsive genes in HepG2URG7 (more than 50-fold) and HepG2X cells (more than 80-fold) compared to these same cultures transfected with the control FOPFLASH vector (P<0.001) (Fig. 5d). In contrast, HepG2CAT cells showed only baseline activation of TOPFLASH (Fig. 5d). Co-transfection of URG7-specific or control siRNAs into HepG2URG7 cells showed that pTOPFLASH activity was a function of URG7 expression.

**Fig. 3.** Relationship between p-Akt levels and survival of HepG2 cultures following TNFα treatment. (a) p-Akt levels in the indicated cultures were assayed by Western blotting with or without the PI3K inhibitor, Ly294002, after 24 h of treatment. The results shown are representative of three experiments. (b) Corresponding survival of HepG2URG7 (grey bars), HepG2X (black bars) and HepG2CAT (white bars) cells following TNFα treatment with or without Ly294002. The results shown are the mean of three experiments, each performed in duplicate.
Fig. 4. Effect of siRNA on the expression of URG7 and survival of HepG2 and Huh7 cultures to TNFα. Western blotting results for (a) URG7 and (b) p-Akt levels in HepG2URG7 and HepG2X cells mock transfected (lane 1), transfected with URG7-specific siRNA (lane 2) or transfected with non-specific siRNA (lane 3). (c) Survival of the indicated cultures left untreated (grey bars), mock transfected (black bars), transfected with URG7-specific siRNA (white bars) or with control siRNA (hatched bars). These results are the mean of two experiments, each performed in duplicate.

Fig. 5. β-Catenin activation and signalling. (a) Western blot analysis of phosphorylated GSK3β. (b) Fragments 2 and 3 of the β-catenin promoter were transiently transfected into HepG2URG7 (grey bars), HepG2X (black bars) and HepG2CAT (white bars) cells. In some experiments, promoter activity was assayed in HepG2URG7 cells co-transfected with URG7-specific siRNA (bars with horizontal lines) or control siRNA (bars with diamonds). SEAP was measured by ELISA 24 h later. (c) Western blot analysis of activated β-catenin. For panels (a) and (c), the ratios following normalization with β-actin are indicated below the blot. (d) Activity of β-catenin–effector genes was assayed by transient transfection of HepG2URG7, HepG2X and HepG2CAT cells with pTOPFLASH (recognizing wild-type β-catenin) or pFOPFLASH (control). In some experiments, luciferase activity was assayed in HepG2URG7 cells co-transfected with URG7-specific siRNA (bars with horizontal lines) or control siRNA (bars with diamonds) after 24 h. The experiment was performed twice, each time in duplicate. RLU, Relative luminescence units.
This suggested that URG7 significantly stimulated β-catenin signalling and turned on β-catenin-responsive genes.

The finding that HepG2 cells make both wild-type and a constitutively active truncated mutant of β-catenin (Carruba et al., 1999), that both HBxAg and URG7 inactivate GSK3β (Fig. 5a) and that both stimulate wild-type β-catenin (Fig. 5c), raises the question as to the contribution of mutant β-catenin under these circumstances. To address this, GSK3β was targeted by transfection of specific siRNA into HepG2CAT, HepG2X and HepG2URG7 cells. The results showed that GSK3β-specific siRNA was effective in blocking the expression of GSK3β in all the cultures tested (Fig. 6a, lanes 2, 4 and 6), while control siRNA was ineffective (Fig. 6a, lanes 1, 3 and 5). The levels of phosphorylated GSK3β decreased in parallel (Fig. 6b). Western blot analysis for total β-catenin showed a modest decrease in wild-type β-catenin, but no change in mutant β-catenin, upon treatment of cells with GSK3β-specific siRNA (Fig. 6d). This profile was similar to that obtained when Western blot analysis was conducted with another antibody that only detected active β-catenin (Fig. 6e). Given that the truncated mutant of β-catenin lacks the GSK3β-binding site (Carruba et al., 1999), it is not surprising that there is no change in mutant β-catenin levels in Fig. 6(d). Hence, it is likely that the functional changes observed in Fig. 5(d) are due to changes in the levels and activities of wild-type β-catenin.

**URG11 contributes to the observed resistance to TNFα killing**

The finding that URG7 and another HBxAg-upregulated protein, URG11, both stimulate β-catenin activity (Fig. 5; Lian et al., 2006), suggests that URG11 may contribute to the TNFα resistance observed in HBxAg-expressing or URG7-overexpressing cells. Accordingly, when the levels of URG11 were assessed in HepG2X, HepG2URG7 and HepG2CAT cells, they were observed to be elevated 4.9 ± 0.4-fold in HepG2X cells and 2.1 ± 0.25-fold in HepG2CAT cells compared with HepG2URG7 cells (Fig. 7a). This confirms that HBxAg promotes the stability of β-catenin, which is associated with the upregulated expression of URG11 (Lian et al., 2006). In contrast, there seems to be an inverse relationship between intracellular levels of URG7 and URG11 in HepG2URG7 compared with HepG2CAT cells. When each of these cultures were treated with TNFα along with URG11-specific siRNA, URG11 expression was blocked (data not shown), and the viability of HepG2URG7 cells was significantly less than that of HepG2CAT or HepG2X cultures, suggesting that URG11 also contributes to the resistance of liver cells to apoptosis (P<0.001, Fig. 7b).

**Effect of URG7 upon NF-κB and AP-1 signalling**

TNFα signalling, through RIP and TRAF-2, may stimulate the anti-apoptotic AP-1 and NF-κB pathways. HBxAg has also been observed to stimulate AP-1 and NF-κB signalling (Kekule et al., 1993; Natoli et al., 1994). To see if either or both of these pathways were activated by URG7; HepG2X, HepG2URG7 and HepG2CAT cells were transiently transfected with pGL2-HIV-LTR-luciferase to measure NF-κB activity, or with p-AP-1-luciferase to measure AP-1 activity. The results show that only HepG2X cells stimulate these reporter plasmids (Fig. 8), indicating that URG7 does not stimulate these signalling pathways.

**DISCUSSION**

There is considerable evidence that the pathogenesis of CLD and HCC is immune-mediated (Chisari & Ferrari, 1995), and that both TNFα- and Fas-mediated apoptotic pathways play central roles. For example, strong Fas/FasL and TNFα/TNF receptor 1 (TNFR) expression are often observed in hepatocytes around areas of intense inflammation (Mochizuki et al., 1996; Luo et al., 1997) adjacent to HCC nodules (Roskams et al., 2000), and generally correlate with the severity of CLD (Lau et al., 1991; Hussain et al., 1994; Marinos et al., 1995; Fang et al., 1996). Importantly, the distribution of HBxAg is similar (Wang et al., 1991a, b; Feitelson et al., 1993; Jin et al., 2001), suggesting that HBxAg may modulate Fas and TNFα
expression and/or signalling. Interestingly, HBxAg transactivates TNFα expression in cell culture (Lara-Pezzi et al., 1998). Although this suggests that HBxAg may promote apoptosis, the accumulation of HBxAg-positive hepatocytes during CLD (Wang et al., 1991a; Jin et al., 2001) suggests that HBxAg is protecting infected cells from TNFα killing at the expense of uninfected hepatocytes. HBxAg also upregulates the expression of FasL in liver and in hepatoma cell lines (Shin et al., 1999), which may kill cytotoxic T cells, and permit the escape of HBxAg-positive cells from immune-mediated killing. In addition, HBxAg activates NF-κB (Pan et al., 2001), SAPK/JNK (Diao et al., 2001) and PI3K (Suzuki et al., 2000; Lee et al., 2001), which protect HBxAg-positive cells from Fas-mediated killing. The finding in this report, that HBxAg protects cells from TNFα killing through the upregulated expression of URG7 and URG11, identifies key cellular genes that are likely to contribute to the survival of virus-infected cells during chronic infection.

In addition to direct killing through the Fas/FasL pathway, which is often mediated by activated T cells, hepatocytes, Kupffer cells, other inflammatory cells, and peripheral blood mononuclear cells produce TNFα (and other cytokines) at elevated levels in the serum of HBV carriers (Sheron et al., 1991; Gonzalez-Amaro et al., 1994). A general characteristic of these inflammatory cytokines is that they trigger the generation of reactive oxygen intermediates (ROI) during CLD. ROI stimulate selected signalling pathways, such as AP-1 and NF-κB. The findings that HBxAg also stimulates these pathways (Kekule et al., 1993; Natoli et al., 1994), and that URG7 is a target gene for NF-κB (Lian et al., 2001), imply that in the presence of HBxAg, the levels of NF-κB activation pass a threshold whereby the URG7 gene becomes upregulated. URG7 would then stimulate PI3K/Akt signalling (Figs 3 and 4), resulting in the inactivation of GSK3β, and the stabilization of wild-type β-catenin (Figs 5 and 6). While these, and perhaps other pathways, may override the apoptotic signals triggered by TNFα, the finding that URG7 blocks caspase 8 and downstream caspase 3 activities (Figs 1 and 2) provides a mechanism whereby apoptotic pathways are shut off at the same time that survival-related pathways are turned on. The blockage of caspase 8 may also contribute importantly to the mechanism of how HBxAg-positive cells become resistant to anti-Fas-mediated killing (Fig. 1), since TNFα, through the TNFR-associated death domain, and anti-Fas, through FADD, both converge and share caspase 8 as an important link whereby these mechanisms trigger apoptosis.

It is intriguing that overexpression of URG7 does not promote the growth of HepG2 (or Hep3B or Huh7) cells in soft agar or accelerate tumourigenesis in nude mice (Lian et al., 2001) even though high levels of URG7 expression are associated with the activation of β-catenin (Figs 5 and 6). In this context, there is an increasing role for upregulated β-catenin in blocking apoptosis, independent of its role in promoting tumourigenesis (Mikami et al., 2005; Ormestad et al., 2006; Yang et al., 2006), and it is proposed here that one of the ways this could happen is by the stimulated expression of URG7. Importantly, HBxAg upregulates another novel cellular gene, URG11, that also stabilizes/upregulates β-catenin, but in the latter case, the upregulated expression of β-catenin was shown to strongly stimulate growth in soft agar and tumour formation in nude mice (Lian et al., 2003, 2006). Evidence presented here suggests that URG11 also contributes to the resistance of URG7 overexpressing cells to apoptosis (Fig. 7), suggesting it may have a dual role in chronic HBV infection. Hence, HBxAg upregulates the expression of two cellular genes that help to protect cells from immune-mediated killing, which may promote virus persistence in the context of CLD.
The model whereby URG7 acts as outlined above may be only part of the picture. For example, caspase 8 catalyses the cleavage of the anti-apoptotic Bid to the proapoptotic tBid (Li et al., 1998), which translocates to the mitochondria, where it triggers the release of cytochrome c, leading to the activation of caspase 9, and then caspase 3 (Roy & Nicholson, 2000). If caspase 8 activity is inhibited by URG7, it would not only block extrinsic (receptor-mediated) apoptosis, but also endogenous (mitochondrial-based) apoptosis. In fact, activation of β-catenin is known to block mitochondria-mediated apoptosis (Yang et al., 2006). In addition, the expression of Bid is lower in HCC than in the surrounding non-tumour liver (Chen et al., 2001a), and a significant decrease in Bid has also been observed in hepatoma cells transfected with HBxAg (Chen et al., 2001b). PI3K/Akt is also known to phosphorylate the proapoptotic molecule BAD converting it to the anti-apoptotic molecule pBAD, so again, the question becomes whether URG7 could mediate the phosphorylation of BAD. Furthermore, the finding that the combined activation of PI3K and NF-κB in several tumour types is associated with the upregulation of BCL-2 and cell survival (Catz & Johnson, 2003) raises the question as to whether the combined actions of HBxAg and URG7 upregulate BCL-X<sub>L</sub> in the liver during chronic infection. In other systems, activation of NF-κB by PI3K is associated with highly invasive cancers (Silva, 2004; Amiri & Richmond, 2005). The fact that URG7 does not activate NF-κB (or AP-1; Fig. 8) may indicate that activation of PI3K/Akt and β-catenin under these circumstances promotes cell survival by blocking apoptosis, while the additional activation of NF-κB by the combination of ROI, HBxAg and PI3K promotes tumourigenesis as well.

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expression of Bid in human hepatocellular carcinoma is related to hepatitis B virus X protein. 


