Journal of General Virology (2004), **85**, 275–282 **DOI** 10.1099/vir.0.19650-0

Hepatitis B virus X antigen promotes transforming growth factor-β1 (TGF-β1) activity by up-regulation of TGF-β1 and down-regulation of α_2-macroglobulin

Jingbo Pan,[1] Marcy Clayton[1] and Mark A. Feitelson[1,2]

[1]Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Room 222 Alumni Hall, 1020 Locust Street, Philadelphia, PA 19107, USA

[2]Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA

Correspondence
Mark A. Feitelson
Mark.Feitelson@jefferson.edu

Received 12 September 2003
Accepted 10 October 2003

Hepatitis B virus (HBV) X antigen (HBxAg) may contribute to the development of hepatocellular carcinoma (HCC) by activation of signalling pathways such as NF-κB. To identify NF-κB target genes differentially expressed in HBxAg-positive compared to -negative cells, HepG2 cells consistently expressing HBxAg (HepG2X cells) were stably transfected with pZeoSV2 or pZeoSV2-IκBα. mRNA from each culture was isolated and compared by PCR select cDNA subtraction. The results showed lower levels of α_2-macroglobulin (α_2-M) in HepG2X-pZeoSV2 compared to HepG2X-pZeoSV2-IκBα cells. This was confirmed by Northern and Western blotting, and by measurement of extracellular α_2-M levels. Elevated transforming growth factor-β1 (TGF-β1) levels were also seen in HepG2X compared to control cells. Serum-free conditioned medium (SFCM) from HepG2X cells suppressed DNA synthesis in a TGF-β-sensitive cell line, Mv1Lu. The latter was reversed when the SFCM was pretreated with exogenous, activated α_2-M or with anti-TGF-β. Since elevated TGF-β1 promotes the development of many tumour types, these observations suggest that the HBxAg-mediated alteration in TGF-β1 and α_2-M production may contribute importantly to the pathogenesis of HCC.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection may result in the development of liver diseases, including hepatitis, cirrhosis and hepatocellular carcinoma (HCC). There is increasing evidence that the HBV-encoded X antigen, HBxAg, contributes importantly to the development of HCC, although the mechanisms whereby it does so are incompletely understood (Feitelson, 1999). HBxAg is a *trans*-activating protein that functions by binding to and altering the activity of transcription factors in the nuclei of infected cells and by constitutively activating a number of cytoplasmic signalling pathways (Henkler & Koshy, 1996; Feitelson, 1999). Among the latter, HBxAg activates phosphorylation dependent signal transduction pathways involving Ras/Raf/MAPK (Cross *et al.*, 1993; Benn & Schneider, 1994; Natoli *et al.*, 1995a), diacylglycerol (DAG)/protein kinase C (PKC) (Cross *et al.*, 1993; Kekule *et al.*, 1993), Jak1/STAT (Lee & Yun, 1998) and NF-κB (Su & Schneider, 1996). The findings that NF-κB blocks hepatocellular apoptosis (Beg *et al.*, 1995; Beg & Baltimore, 1996), that HBxAg protects liver cells from apoptosis by inducing NF-κB (Pan *et al.*, 2001), and that HCC is resistant to apoptosis (Natoli *et al.*, 1995b), suggest that HBxAg activation of NF-κB may play an important role in hepatocarcinogenesis.

Transforming growth factor-β1 (TGF-β1) is an important growth regulatory molecule that triggers apoptosis in hepatocytes (Gressner *et al.*, 1997). HCC is resistant to TGF-β1 even though the latter is transcriptionally up-regulated in tumour cells, and TGF-β1 levels are commonly elevated in the sera of HCC patients (Ito *et al.*, 1991; Song *et al.*, 2002). Under these circumstances, TGF-β1 may promote tumour growth, in part by killing or inhibiting the growth of surrounding hepatocytes. Some data suggest that the resistance of HCC to TGF-β1 is associated with mutation and loss of the receptor that mediates TGF-β1 signalling (Yamada *et al.*, 1997), although this has not been consistently observed (Wada *et al.*, 1999). Alternatively, HBxAg may suppress expression of the TGF-β1 type II receptor (Oshikawa *et al.*, 1996). Interestingly, the TGF-β1 gene is transcriptionally up-regulated by HBxAg (Yoo *et al.*, 1996), probably by the constitutive activation of transcriptional complexes containing Smad4 (Lee *et al.*, 2001), which mediates TGF-β1 signalling. TGF-β1 signalling also promotes the development of fibrosis and cirrhosis in patients with chronic liver disease (CLD) (Czaja *et al.*, 1989) and in TGF-β1 transgenic mice (Sanderson *et al.*, 1995). These mice also develop HCC (Factor *et al.*, 1997). In other tumour types, elevated TGF-β1 stimulates angiogenesis,

suppresses anti-tumour immune responses, promotes the development of drug resistance and enhances metastasis (Teicher, 2001), through activation of the Smad and/or other signalling pathways that override apoptosis (Akhurst, 2002). These observations suggest that the regulation of TGF-β1 activity may contribute importantly to the pathogenesis of HCC.

α_2-Macroglobulin (α_2-M) is a broad-spectrum proteinase inhibitor in serum (Hall & Roberts, 1978; Sottrup-Jensen, 1989) that binds to many cytokines (Borth, 1992), and in some cases, binding inactivates cytokine activity (Gonias *et al.*, 1994). In other cases, α_2-M–protease and α_2-M–cytokine complexes are rapidly cleared by endocytosis after binding to the LRP/α_2-M receptor on hepatocytes, fibroblasts and macrophages (Williams *et al.*, 1994). Studies with α_2-M gene knockout mice suggest that the primary function of α_2-M *in vivo* may be to regulate the activities of cytokines in the TGF-β family (Umans *et al.*, 1995; Webb *et al.*, 1996). In HCC, α_2-M expression is decreased (Paradis *et al.*, 2003), although it is not clear whether part of the mechanism whereby HBxAg enhances TGF-β1 activity is through decreased expression of α_2-M. Hence, experiments were designed to test the hypothesis that HBxAg up-regulated TGF-β1 activity was due not only to increased TGF-β1 production, but also to a decrease in α_2-M expression.

METHODS

Cell lines and culture. The HepG2X (encoding HBx) and HepG2CAT (encoding bacterial chloramphenicol acetyltransferase) cells were made and characterized previously (Lian *et al.*, 1999). G418-resistant cultures were passaged by standard trypsinization without selection of individual colonies, and the presence of HBxAg and CAT verified by Western blotting and standard CAT assay, respectively, as described (Lian *et al.*, 1999).

The TGF-β-sensitive cell line, Mv1Lu (mink lung epithelial cell line; ATCC, CCL-64), was cultured in the same growth medium as HepG2 cells.

Stable transfection of HepG2X cells with pZeoSV2-IκBα-HA. Separate cultures of HepG2X and HepG2CAT cells were stably transfected with pZeoSV2-IκBα-HA or pZeoSV2 vector, as described (Pan *et al.*, 2001). Cells were selected in zeocin for 4 weeks. Exogenous IκBα expression was detected by Western blotting using anti-HA as the primary antibody (Pan *et al.*, 2001). NF-κB activity was measured in a luciferase assay by transient transfection of cells with the reporter plasmid pGL2-HIV-LTR-1. In this plasmid, luciferase expression was under control of the HIV LTR-1 promoter, which contained an NF-κB binding site that is responsive to HBxAg (Twu *et al.*, 1989; Pan *et al.*, 2001).

PCR select cDNA subtraction, cloning and sequencing. mRNA was isolated from HepG2X-pZeoSV2 and HepG2X-pZeoSV2-IκBα cells (see below), reverse transcribed to cDNA fragments with random primers, and then subjected to PCR select cDNA subtraction (BD Biosciences/Clontech), as described (Lian *et al.*, 1999). The cDNA fragments obtained from subtraction were then individually cloned into pT7BlueT vector (Novagen). DNA sequencing was performed at the sequencing facility on campus. The sequences obtained were compared to those in GenBank by GCG software for homology to known genes.

Northern blot analysis. Total RNA from HepG2CAT-pZeoSV2, HepG2X-pZeoSV2 and HepG2X-pZeoSV2-IκBα cells was isolated using the RNeasy Mini Kit (Qiagen). Ten μg of total RNA from each culture was analysed by formaldehyde denaturing agarose gel electrophoresis. The α_2-M cDNA fragment identified from subtraction was labelled with [α-^{32}P]dCTP using the Prime-a-Gene Labelling system (Promega) for use as a probe. A G3PDH probe was used to normalize for RNA loading in each sample.

Western blot analysis. HepG2CAT-pZeoSV2, HepG2X-pZeoSV2 and HepG2X-pZeoSV2-IκBα cells were treated 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 Na_3VO_4), protease inhibitors (1 mM PMSF, 10 μg leupeptin ml^{-1}, 10 μg pepstatin ml^{-1}) and 1 % Triton X-100 for 15 min on ice. Forty μg of total protein in each sample was reduced in 10 % 2-mercaptoethanol, and analysed by SDS-PAGE on 4–20 % gradient gels. Proteins were then blotted onto PVDF membranes (Millipore) and then blocked overnight in PBS containing 5 % nonfat milk. For primary antibody, rabbit anti-human α^2-M polyclonal antibody (A749/R5H, Accurate Chemical Co., Westbury, NY, USA) was used at a 1 : 2000 dilution. Mouse anti-human β-actin monoclonal antibody (Clone AC-15, Sigma) was used at 1 : 5000 as an internal control. For secondary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (1 : 10 000 dilution) or HRP-conjugated goat anti-mouse Ig (1 : 3000 dilution) (Accurate) was used. The results were visualized using the ECL detection system (Amersham).

Immunoprecipitation of α_2-M in culture medium from metabolically labelled cells. Five-million HepG2CAT-pZeoSV2, HepG2X-pZeoSV2 or HepG2X-pZeoSV2-IκBα cells in 100 mm dishes were incubated for 16 h in complete medium. After each culture had been washed twice with PBS, the cells were incubated with methionine-free medium containing 5 % dialysed FCS for 1 h, which was then replaced with 4 ml of the same medium containing 70 μCi [^{35}S]methionine (NEN) ml^{-1} for 16 h. Cell supernatants were collected through a 0·45 μm filter to remove cell debris, and 1·2 ml of each sample was used for immunoprecipitation. All immunoprecipitation steps were done at 4 °C. Samples precleared with protein A–agarose beads were incubated with 15 μl of rabbit anti-human α_2-M for 3 h, and then with 20 μl of protein A–agarose beads overnight. Beads were collected and washed four times with NP-40 buffer (150 mM NaCl, 50 mM Tris/HCl, pH 6·8, 1 % NP40) by centrifuging at 4000 r.p.m. for 5 min each time. Finally, samples were analysed by SDS-PAGE on 7·5 % gels, and results visualized by autoradiography.

Preparation of serum-free conditioned medium (SFCM). Cells were seeded in 100 mm dishes (4×10^6 cells per dish) in complete medium. The next day, the cultures were washed twice with PBS, and then incubated overnight in serum-free Eagle's MEM containing 0·02 % BSA. Cells were then incubated in fresh serum-free medium for an additional 24 h. Cell culture supernatants were collected, filtered, aliquoted and stored at −80 °C until use.

DNA synthesis. To activate TGF-β in SFCM, the pH was adjusted to 1·5 with HCl. Samples were incubated at room temperature for 15 min, and then neutralized with NaOH/HEPES to pH 7·4–7·5. Activated SFCM was diluted to 75 % with fresh serum-free medium (to allow for addition of other reagents) for DNA synthesis assays. In some experiments, 75 % SFCM was incubated with 10 μg TGF-β antibody ml^{-1} (clone 1D11, which binds all TGF-β isoforms; R & D Systems) for 1 h at 37 °C prior to evaluation in the DNA synthesis assay. Mouse IgG (Sigma) was used as a control in place of TGF-β antibody. In other experiments, 75 % SFCM was incubated with methylamine-treated α_2-M (α_2-M-MA) (BioMac, Leipzig, Germany)

at 37 °C for 2 h. Methylamine treatment transforms (activates) nearly 100 % of α_2-M, which was confirmed by the faster migration of the activated form in Rate (native)-PAGE (data not shown).

The Mv1Lu cells were seeded in 96-well plates (1×10^4 cells per well) in complete medium for 24 h. Medium was changed to 75 % of activated SFCM with or without TGF-β neutralizing antibody, and with or without α_2-M-MA. After incubation for 18 h at 37 °C, [^{3}H]thymidine (1 µCi per well) was added for an additional 7 h. Cells in each well were harvested onto glass fibre filters using a cell harvester and the radioactivity incorporated in DNA was determined by scintillation counting. Each experiment was done in quadruplicate.

Determination of TGF-β1, -β2 and α_2-M levels in SFCM. TGF-β1, -β2 and α_2-M levels in SFCM were determined by ELISAs for each antigen. ELISA kits for human TGF-β1 (Quantikine kit) and TGF-β2 (Quantikine kit) were obtained from R&D Systems. α_2-M was determined by 'sandwich' ELISA. Rabbit anti-human α_2-M (IgG fraction) was used as capture antibody and affinity-purified HRP-conjugated goat anti-human α_2-M (Accurate) used as detection antibody. Purified human α_2-M (Sigma) was used to create a standard curve for each experiment. All samples were tested in duplicate.

Statistics. Student's *t*-test was used to evaluate (1) the mean levels of DNA synthesis in Mv1Lu cells treated with 75 % SFCM from different sources; (2) the significance of mean levels of NF-κB activity, as measured by luciferase levels, in transiently transfected cells; (3) the mean levels of α_2-M and TGF-β1 in SFCM; and (4) the relative levels of α_2-M mRNA and protein in differently treated cells. A significant result is when $P < 0{\cdot}05$.

RESULTS

Introduction of exogenous IκBα into HepG2X cells

The findings that HBxAg stimulates NF-κB (Su & Schneider, 1996), that NF-κB is hepatoprotective (Beg *et al.*, 1995), in part, by inhibiting apoptosis (Beg & Baltimore, 1996), that HBxAg confers resistance to apoptosis in a number of cell lines tested (Shih *et al.*, 2000; Pan *et al.*, 2001), and that an important characteristic of HCC is its resistance to apoptosis (Natoli *et al.*, 1995b), suggest that the constitutive activation of NF-κB in HBxAg-positive cells may contribute importantly to hepatocarcinogenesis. To examine this in more detail, HepG2X cells (Lian *et al.*, 1999) were stably transfected with pZeoSV2 or pZeoSV2-IκBα (Pan *et al.*, 2001). Following selection in zeocin, exogenous IκBα expression was demonstrated by Western blotting, and suppressed NF-κB activity in a transient luciferase reporter gene assay with HepG2X cells (Pan *et al.*, 2001).

Identification and verification of α_2-M as a gene down-regulated in HepG2X-pZeoSV compared to HepG2X-pZeoSV-IκBα cells

To discern differences in gene expression triggered by NF-κB in HepG2X cells, PCR select cDNA subtraction was performed with whole cell mRNA isolated from HepG2X-pZeoSV2 and HepG2X-pZeoSV2-IκBα cells. The results yielded three cDNA fragments that may represent differentially expressed genes. Among the two up-regulated genes, one consisted of a 792 bp fragment that had 99·4 % homology over 621 bp with the human fibronectin gene (GenBank accession no. A14133), which encodes an important extracellular matrix component of the fibrogenic response. The other up-regulated gene consisted of a 540 bp fragment that was 98·9 % homologous over a 444 bp overlap with a human fetal liver cDNA clone (GenBank accession no. H49417). Cloning and expression of the full-length cDNA from the latter, referred to as up-regulated gene 7 (URG7), is stimulated nearly 5-fold in HepG2X cells. HBxAg blocks anti-Fas-mediated apoptosis in HepG2 cells, and it was found that most of this was due to the NF-κB-dependent expression of URG7 (Lian *et al.*, 2001). Sequence analysis of the cDNA fragment that was strongly down-regulated in HepG2X-pZeoSV2 cells compared to HepG2X-pZeoSV2-IκBα cells showed 100 % homology over a 500 bp region with human α_2-M cDNA when compared to entries in GenBank (Benson *et al.*, 1997). This fragment, spanning nucleotides 3026–3525, based upon GenBank accession no. A21185, was used as a probe for further studies. To verify the results of subtractive hybridization, Northern blotting was performed with RNA isolated from the cells used for PCR select cDNA subtraction. The results show that the levels of α_2-M RNA in HepG2X-pZeoSV2-IκBα cells were 4·7-fold higher than in HepG2X-pZeoSV2 cells following normalization with G3PDH. In comparison, the levels of α_2-M mRNA in HepG2CAT-pZeoSV2 control cells were 6·9-fold higher than in HepG2X-pZeoSV2 cells (Fig. 1a). Western blotting for α_2-M yielded similar results following normalization to β-actin (Fig. 1*b*). Hence, HepG2X-pZeoSV2

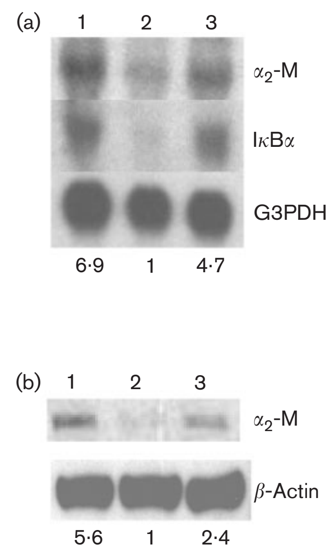

Fig. 1. Levels of α_2-M mRNA were determined by Northern blot analysis (a) while levels of α_2-M protein were determined by Western blot analysis (b) in lysates from HepG2CAT-pZeoSV2 (lane 1), HepG2X-pZeoSV2 (lane 2) or HepG2X-pZeoSV2-IκBα cells. The results shown in each panel are from one of three experiments.

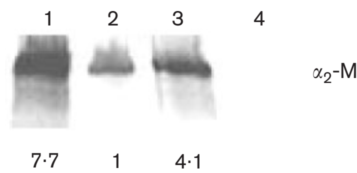

Fig. 2. Immunoprecipitation of α_2-M labelled with [^{35}S]methionine from cell-culture supernatants of HepG2CAT-pZeoSV2 (lane 1), HepG2X-pZeoSV2 (lane 2) and HepG2X-pZeoSV2-IκBα (lane 3). In lane 4, the immunoprecipitation in lane 1 was repeated with an equivalent amount of normal IgG. The results shown here are from one of three independent experiments.

cells had depressed levels of α_2-M expression relative to HepG2CAT-pZeoSV2 cells, and the introduction of exogenous IκBα to HepG2X cells restored from about half to two-thirds of the α_2-M levels in HepG2X-pZeoSV2 compared to control cells (HepG2CAT-pZeoSV2). These results suggest that HBxAg suppresses α_2-M gene expression. Although this suppression may be at the transcriptional level and/or effect α_2-M mRNA stability, it appears to be NF-κB dependent.

HBxAg suppresses accumulation of extracellular α_2-M

Since extracellular α_2-M binds to and inactivates TGF-β, it was important to see whether the suppression of α_2-M expression in HBxAg-positive HepG2 cells also resulted in decreased accumulation of α_2-M in the tissue culture supernatant. Accordingly, HepG2CAT-pZeoSV2, HepG2X-pZeoSV2 and HepG2X-pZeoSV2-IκBα cells were radiolabelled with [^{35}S]methionine. Equal volumes of tissue culture supernatants were immunoprecipitated with anti-human α_2-M, and then analysed by SDS-PAGE and autoradiography. The results showed that there was 7·7-fold more α_2-M in the supernatant of HepG2CAT-pZeoSV2 cells (Fig. 2, lane 1) compared to that of HepG2X-pZeoSV2 cells ($P<0·001$) (Fig. 2, lane 2). In comparison, the amount of α_2-M recovered from the supernatants of HepG2X-pZeoSV2-IκBα cells (Fig. 2, lane 3) was approximately 4-fold higher than the levels observed in HepG2X-pZeoSV2 supernatants ($P<0·003$). Immunoprecipitation of the supernatant from HepG2CAT-pZeoSV2 cells with normal IgG resulted in no discernable band (Fig. 2, lane 4), demonstrating that the immunoprecipitations were specific. These results suggest that the decreased levels of α_2-M within HepG2X cells were also decreased in the supernatant from the same cultures, and that a significant fraction of the α_2-M could be recovered when HepG2X cells were stably transfected with pZeoSV2-IκBα.

HBxAg up-regulates TGF-β1 in HepG2 cells

Given that TGF-β1 is transcriptionally activated by HBxAg (Yoo *et al.*, 1996; Lee *et al.*, 2001), the levels of TGF-β1 were assayed by quantitative ELISA in SFCM from HepG2X-pZeoSV2, HepG2X-pZeoSV2-IκBα and HepG2CAT-pZeoSV2 cells. In SFCM from HepG2CAT-pZeoSV2 cells, the levels of TGFβ1 were some 7- to 8-fold lower than the SFCM from HepG2X-pZeoSV2 ($P<0·001$) and HepG2X-pZeoSV2-IκBα cells (Fig. 3). As expected, when extracellular α_2-M was assayed by ELISA, the levels were highest in SFCM of HepG2CAT-pZeoSV2 cells, about 7-fold lower in the SFCM from HepG2X-pZeoSV2 cells ($P<0·001$), but less than 2-fold reduced in the SFCM from HepG2X-pZeoSV2-IκBα cells (Fig. 3). TGF-β2, also assayed by ELISA, was not detectable in any of the SFCM, suggesting HBxAg alters only TGF-β1 but not TGF-β2 expression. Hence, TGF-β1 expression is up-regulated in the supernatants of HBxAg-positive cells, but its increased production, unlike that of α_2-M, is not NF-κB dependent.

When the α_2-M : TGF-β1 ratio was determined on a molar basis in the SFCM of HepG2CAT-pZeoSV2 cells (Fig. 3), it was about 1440, while for HepG2X-pZeoSV2 cells it was 29. The ratio of these numbers showed that the introduction of HBxAg into HepG2 cells resulted in an almost 50-fold change in the relative amounts of α_2-M and TGF-β1 in the SFCM. The question now became, what were the functional consequences of this altered α_2-M : TGF-β1 ratio?

Decreased α_2-M : TGF-β1 ratio was associated with increased TGF-β activity

To determine whether there is a net difference in TGF-β activity in HBxAg-positive compared to -negative HepG2 cells, SFCM from HepG2CAT-pZeoSV2 or HepG2X-pZeoSV2

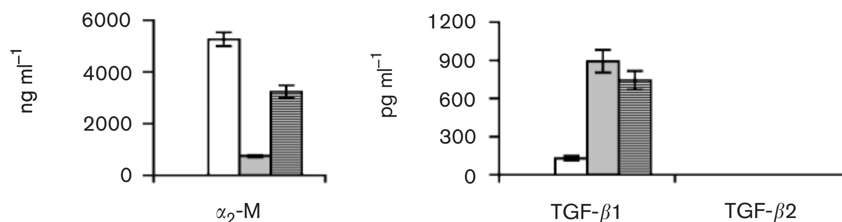

Fig. 3. α_2-M, TGF-β1 and TGF-β2 were each detected by specific ELISAs in serum-free medium (SFM) alone (black bar), or in the supernatants of HepG2CAT-pZeoSV2 cells (white bar), HepG2X-pZeoSV2 cells (grey bar) or HepG2X-pZeoSV2-IκBα cells (striped bar). The data shown are from one of three experiments, each done in duplicate.

cells was added to cultures of Mv1Lu cells. DNA synthesis ([^{3}H]thymidine uptake) in this cell line is very sensitive to inhibition by TGF-β. In this assay, acidification followed by neutralization of the SFCM was performed in order to activate TGF-β. Accordingly, when this experiment was done with serum-free medium, which contained no TGF-β, high levels of DNA synthesis were observed (Fig. 4, group 1). When unacidified SFCM from HepG2CAT-pZeoSV2 or HepG2X-pZeoSV2 was tested, no inhibition of DNA synthesis was observed (Fig. 4, group 1), since all of the TGF-β in unacidified SFCM was inactive. In group 2, in which TGF-β was activated, serum-free medium again showed no inhibition of DNA synthesis. In contrast, there was about a 3·5-fold inhibition of [^{3}H]thymidine incorporation when SFCM from HepG2CAT-pZeoSV2 cells was tested (white bar), while a 15- to 18-fold decrease in proliferation was observed when the assay was repeated with SFCM from HepG2X-pZeoSV2 cells. Each of these differences was highly significant compared to the values for serum-free medium alone ($P<0{\cdot}001$). Comparison of the inhibitory activities of SFCM from HepG2CAT-pZeoSV2 and HepG2X-pZeoSV2 showed that the latter inhibited 5- to 6-fold more than the former ($P<0{\cdot}001$) (Fig. 4, group 2). To determine whether inhibition of DNA synthesis was related to TGF-β activity, the SFCM samples from group 2 were preincubated with normal IgG, which had no effect upon the inhibition of DNA synthesis (Fig. 4, group 3), or with anti-human TGF-β (Fig. 4, group 4), which completely reversed the inhibitory effect of the SFCM samples. These results suggest that the HBxAg-mediated increase in expression of TGFβ (and suppression of α_2-M expression) resulted in a stronger inhibition of DNA synthesis on Mv1Lu reporter cells compared to the same experiment done with SFCM isolated from HBxAg-negative HepG2 cells, and that all of the inhibition observed in this assay was due to TGF-β.

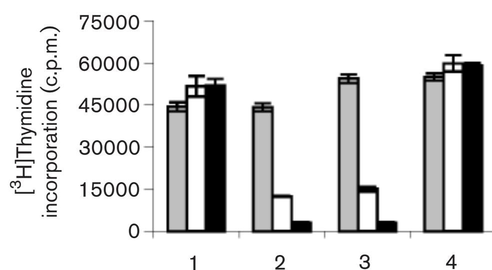

Fig. 4. Effect of SFCM on DNA synthesis in Mv1Lu cells. MV1LU cells were incubated with serum-free medium (grey bars), SFCM from HepG2CAT-pZeoSV2 cells (white bars) or SFCM from HepG2X-pSV2zeo cells (black bars). Test medium was added to the reporter cell line unacidified (group 1), acidified and neutralized (group 2), acidified, neutralized and preincubated with normal IgG (10 µg ml^{-1}) (group 3), or acidified, neutralized and preincubated with anti-TGF-β (10 µg ml^{-1}) (group 4). The data presented are means of three experiments, each done in triplicate.

Exogenous α_2-M blocks the TGF-β1 inhibition of DNA synthesis in SFCM

If in natural HBV infections the levels of α_2-M in serum regulate the activity of TGF-β1, then addition of exogenous activated (methylamine-reated) α_2-M or α_2-M-MA to SFCM from HepG2X-pZeoSV2 cells should inactivate the TGF-β1 and relieve the inhibition of DNA synthesis in Mv1Lu. When this experiment was done, the SFCM from HepG2X-pZeoSV2 cells significantly inhibited proliferation relative to the SFCM from HepG2CAT-pZeoSV2 cells ($P<0{\cdot}001$) (Fig. 5), as expected. However, when the amount of exogenous α_2-M-MA added to the SFCM from HepG2X-pZeoSV2 cells was increased to levels similar to that found in normal human serum (which is in the mg % range) (LaMarre *et al.*, 1991), TGF-β1 activity was blocked, and control levels of DNA synthesis were observed (Fig. 5). These observations show that the inhibitory effects of TGF-β1 on DNA synthesis are reversible in the presence of activated α_2-M.

DISCUSSION

The observations that HBxAg activates NF-κB (Henkler & Koshy, 1996; Su & Schneider, 1996), that NF-κB is hepatoprotective (Beg *et al.*, 1995), in part, by blocking apoptosis (Beg & Baltimore, 1996), and that HBxAg inhibits both Fas and TGF-β1 triggered apoptosis (Shih *et al.*, 2000; Pan *et al.*, 2001), suggest that the constitutive activation of NF-κB by HBxAg may contribute importantly to the development of preneoplastic foci and HCC. Using PCR select cDNA subtraction, this study revealed that HBxAg suppressed the expression of α_2-M, which is known to bind to and inactivate TGF-β. In fact, α_2-M is a major negative

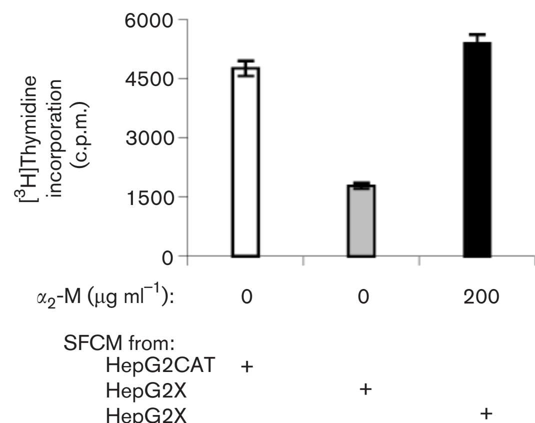

Fig. 5. Rescue of DNA synthesis in Mv1Lu cells by addition of exogenous activated α_2-M to SFCM from HepG2X-pZeoSV2 cells. Mv1Lu cells seeded into 96-well plates (1×10^4 cells per well) were incubated with 75 % of acidified SFCM from HepG2X-pZeoSV2 cells with or without α_2-M-MA. SFCM from HepG2CAT cells served as a positive control.

regulatory factor for the TGF-β family of proteins (LaMarre *et al.*, 1991; Gonias *et al.*, 1994; Webb *et al.*, 1996, 1998). α_2-M expression was suppressed 5- to 6-fold in HepG2X-pZeoSV2 compared to HepG2CAT-pZeoSV2 cells (Figs 1 and 2). Similar differences were observed in SFCM from these cells (Fig. 3), while TGF-β1 expression increased 7- to 8-fold (Fig. 3). This resulted in a significant increase in the TGF-β1 DNA synthesis inhibitory activity in a reporter cell line (Fig. 4). Therefore, HBxAg increases TGF-β1 signalling through *trans*-activating the TGF-β1 gene (Yoo *et al.*, 1996), by stimulating Smad4 (which mediates TGF-β1) signalling (Lee *et al.*, 2001) and by reducing expression of the major TGF-β1 inhibitor, α_2-M (this study). The latter is likely to be another mechanism whereby HBxAg promotes TGF-β1 activity during CLD.

TGF-β1 is elevated in the blood of HBV carriers with HCC (Castilla *et al.*, 1991; Ito *et al.*, 1991; Song *et al.*, 2002), while a recent microarray-based study demonstrated a consistent suppression of α_2-M gene expression in HCC (Paradis *et al.*, 2003). α_2-M gene expression is also reportedly reduced or absent in several human hepatoma cell lines (Kondo *et al.*, 1998). Hence, the observations made here are consistent with those of increased TGF-β1 and decreased α_2-M made in clinical studies. Collectively, these observations suggest that the increase in TGF-β1 and decrease in α_2-M by HBxAg parallels the shift in expression of these proteins *in vivo*. Moreover, α_2-M gene expression has been reported to be depressed in adenocarcinomas and squamous cell carcinomas of the lung (McDoniels-Silvers *et al.*, 2002), suggesting that the phenomenon observed here may occur in other tumour types, although by other mechanisms.

The impact of α_2-M expression levels in HepG2 cells upon TGF-β1 activity was measured using a [^{3}H]thymidine incorporation assay in a mink lung epithelial cell line that is highly sensitive to the inhibitory effects of TGF-β1 upon DNA synthesis. The results in Fig. 4 show that the TGF-β1 levels in the SFCM from HepG2CAT-pZeoSV2 cells clearly inhibited DNA synthesis compared to serum-free medium, but that when SFCM from HepG2X-pZeoSV2 cells was used, the inhibition was significantly greater, suggesting that altered α_2-M : TGF-β1 ratios in the supernatants of HepG2X-pZeoSV2 compared to HepG2CAT-pZeoSV2 cells (Fig. 3) also had functional consequences. In this context, it is proposed that HBxAg-positive hepatocytes, being relatively TGF-β1 resistant, would acquire a survival advantage over HBxAg-negative hepatocytes during chronic infection. This would promote the persistence of virus-infected cells in the face of ongoing immune responses, which would be important for the maintenance of the carrier state despite multiple bouts of CLD. This would also promote the accumulation of HBxAg-positive cells in carriers with CLD, which is an important early step in tumorigenesis (Koike *et al.*, 1994; Ueda *et al.*, 1995). These observations would predict a strong correlation between HBxAg staining in the liver and severity of CLD, which has already been reported (Feitelson, 1999; Jin *et al.*, 2001).

There are additional ramifications of increased TGF-β1 activity in HBxAg-positive liver cells. For example, TGF-β represents an important mediator of liver fibrosis (Czaja *et al.*, 1989; Gressner & Bachem, 1995) by stimulating the synthesis of collagen, fibronectin (Ignotz & Massague, 1986), hyaluronan and proteoglycan (Bachem *et al.*, 1989) in hepatic stellate cells. TGF-β also inhibits matrix degradation, by reducing collagenase gene expression and plasmin synthesis (Rieder *et al.*, 1993), and by increasing the synthesis of metalloproteinase inhibitors (Edwards *et al.*, 1987). TGF-β staining in hepatocytes also correlates with the degree of liver fibrosis in man (Castilla *et al.*, 1991; Nagy *et al.*, 1991) and rats (Czaja *et al.*, 1989; Armendariz-Borunda *et al.*, 1990). In addition, α_2-M inhibits TGF-β-induced collagen synthesis in human liver myofibroblasts (Tiggelman *et al.*, 1997), and reduces elevated matrix synthesis of cultured rat hepatic stellate cells (Schuftan & Bachem, 1999). This suggests α_2-M may have an antifibrogenic effect *in vivo*, which is blocked in chronic HBV infection by HBxAg.

Elevated TGF-β1 activity may be immunosuppressive (Teicher, 2001). For example, *in vitro* experiments with peripheral blood mononuclear cells isolated from HBV carriers have shown that TGF-β1 treatment significantly inhibited HBV core antigen-stimulated proliferation, interferon-γ production and core antigen-specific cytotoxic T-cell activity (Kamura *et al.*, 1993). Although TGF-β1 also suppresses anti-tumour immunity in other tumour types (Teicher, 2001), it is not clear whether this occurs with HCC.

Although TGF-β1 signalling negatively regulates cell growth, the constitutive activation of other signalling pathways, such as those involving NF-κB, Akt/PI3K, c-Jun and Ras/Raf/MAPK, may block Smad-mediated growth inhibition and apoptosis, and instead promote cell survival, motility, migration and other features commonly associated with tumour cells (Akhurst, 2002). In this context, HBxAg is known to stimulate most of these signalling pathways (Henkler & Koshy, 1996; Feitelson, 1999), so that increased TGF-β1 expression in HBxAg-positive cells may result in autocrine and/or paracrine growth and survival signals to HBxAg-positive hepatocytes at the expense of uninfected cells. Akt/PI3K, for example, is stimulated by HBxAg, and is known to block TGF-β1 apoptosis in HBxAg-positive cells, but it also known to block α_2-M transcription (Kortylewski *et al.*, 2003). NF-κB also blocks α_2-M gene expression by blocking STAT3 binding (which stimulates α_2-M expression) to the α_2-M promoter (Bode *et al.*, 2001). Under these conditions, increased TGF-β1 production by HBxAg-positive compared to -negative HepG2 cells will only serve to further promote the growth and survival of HepG2X (but not HepG2CAT) cells, which is observed in this study. Hence, the shift in α_2-M and TGF-β1 expression levels by HBxAg during chronic infection may be another pathway whereby HBxAg contributes to hepatocarcinogenesis on the molecular level.

ACKNOWLEDGEMENTS

This work was supported by NIH grants CA48656 and CA66971 to M. F.

REFERENCES

Akhurst, R. J. (2002). TGF-β antagonists: why suppress a tumor suppressor? *J Clin Invest* **109**, 1533–1536.

Armendariz-Borunda, J., Seyer, J. M., Kang, A. H. & Raghow, R. (1990). Regulation of TGF-β gene expression in rat liver intoxicated with carbon tetrachloride. *FASEB J* **4**, 215–221.

Bachem, M. G., Riess, U., Melchior, R., Sell, K. M. & Gressner, A. M. (1989). Transforming growth factor (TGF alpha and TGF beta 1) stimulate chondroitin sulfate and hyaluronate synthesis in cultured rat liver fat storing cells. *FEBS Lett* **257**, 134–137.

Beg, A. A. & Baltimore, D. (1996). An essential role for NF-κB in preventing TNF-α-induced cell death. *Science* **274**, 782–784.

Beg, A., Sha, W., Bronson, R., Ghosh, S. & Baltimore, D. (1995). Embryonic lethality and liver regeneration in mice lacking the RelA component of NF-κB. *Nature* **376**, 167–170.

Benn, J. & Schneider, R. J. (1994). Hepatitis B virus HBX protein activates Ras–GTP complex formation and establishes a Ras, Raf, MAP kinase signaling cascade. *Proc Natl Acad Sci U S A* **91**, 10350–10354.

Benson, D. A., Boguski, M. S., Lipman, D. J. & Ostell, J. (1997). GenBank. *Nucleic Acids Res* **25**, 1–6.

Bode, J. G., Fischer, R., Haussinger, D., Graeve, L., Heinrich, P. C. & Schaper, F. (2001). The inhibitory effect of IL-1 beta on IL-6 induced alpha 2-macroglobulin expression is due to activation of NF-kappa B. *J Immunol* **167**, 1469–1481.

Borth, W. (1992). Alpha-2-macroglobulin, a multifunctional binding protein with targeting characteristics. *FASEB J* **6**, 3345–3353.

Castilla, A., Prieto, J. & Fausto, N. (1991). Transforming growth factors β1 and α in chronic liver disease. Effects of interferon alpha therapy. *N Engl J Med* **324**, 933–940.

Cross, J. C., Wen, P. & Rutter, W. J. (1993). Transactivation by hepatitis B virus X protein is promiscuous and dependent on mitogen-activated cellular serine/threonine kinases. *Proc Natl Acad Sci U S A* **90**, 8078–8082.

Czaja, M. J., Weiner, F. R., Flanders, K. C., Giambrone, M. A., Wind, R., Biempica, L. & Zern, M. A. (1989). *In vitro* and *in vivo* association of transforming growth factor-β1 with hepatic fibrosis. *J Cell Biol* **108**, 2477–2482.

Edwards, D. R., Murphy, G. & Reynolds, J. J. (1987). Transforming growth factor beta modulates the expression of metalloproteinase inhibitor. *EMBO J* **6**, 1899–1904.

Factor, V. M., Kao, C. Y., Santoni-Rugiu, E., Woitach, J. T., Jensen, M. R. & Thorgeirsson, S. S. (1997). Constitutive expression of mature transforming growth factor β1 in the liver accelerates hepatocarcinogenesis in transgenic mice. *Cancer Res* **57**, 2089–2095.

Feitelson, M. A. (1999). Hepatitis B virus in hepatocarcinogenesis. *J Cell Physiol* **181**, 188–202.

Gonias, S. L., LaMarre, J., Crookston, K. P., Webb, D. J., Wolf, B. B., Lopes, M. B., Moses, H. L. & Hayes, M. A. (1994). α2-macroglobulin and the α2-macroglobulin receptor/LRP: a growth regulatory axis. *Ann N Y Acad Sci* **737**, 273–290.

Gressner, A. M. & Bachem, M. G. (1995). Molecular mechanisms of liver fibrogenesis – a homage to the role of activated fat-storing cells. *Digestion* **56**, 335–346.

Gressner, A. M., Lahme, B., Mannherz, H. G. & Polzar, B. (1997). TGF-β-mediated hepatocellular apoptosis by rat and human hepatoma cells and primary rat hepatocytes. *J Hepatol* **26**, 1079–1092.

Hall, P. K. & Roberts, R. C. (1978). Physical and chemical properties of human plasma α2-macroglobulin. *Biochem J* **171**, 27–38.

Henkler, F. & Koshy, R. (1996). Hepatitis B virus transcriptional activators: mechanisms and possible role in oncogenesis. *J Viral Hepat* **3**, 109–121.

Ignotz, R. A. & Massague, J. (1986). Transforming growth factor beta stimulates the expression of fibronectin and collagen and their incorporation into extracellular matrix. *J Biol Chem* **261**, 4337–4345.

Ito, N., Kawata, S., Tamura, S. & 7 other authors (1991). Elevated levels of transforming growth factor-β messenger RNA and its polypeptide in human hepatocellular carcinoma. *Cancer Res* **51**, 4080–4083.

Jin, Y. M., Yun, C., Park, C., Wang, H. J. & Cho, H. (2001). Expression of hepatitis B virus X protein is closely correlated with the high periportal inflammatory activity of liver diseases. *J Viral Hepat* **8**, 322–330.

Kakumu, S., Ito, Y., Takayanagi, M., Yoshioka, K., Wakita, T., Ishikawa, T., Higashi, Y. & Yang, Z. Q. (1993). Effect of recombinant human transforming growth factor β1 on immune responses in patients with chronic hepatitis B. *Liver* **13**, 62–68.

Kekule, A. S., Lauer, U., Weiss, L., Luber, B. & Hofschneider, P. H. (1993). Hepatitis B virus transactivator HBx uses a tumor promoter signalling pathway. *Nature* **361**, 742–745.

Koike, K., Moriya, K., Iino, S., Yotsuyanagi, H., Endo, Y., Miyamura, T. & Kurokawa, K. (1994). High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. *Hepatology* **19**, 810–819.

Kondo, T., Sakaguchi, M., Yamada, H. & Namba, M. (1998). Two dimensional gel electrophoretic analysis of the changes after immortalization of human cells: decrease of intracellular α2-macroglobulin fragment. *Electrophoresis* **19**, 1836–1840.

Kortylewski, M., Feld, F., Kruger, K. D., Bahrenberg, G., Roth, R. A., Joost, H. G., Heinrich, P. C., Behrmann, I. & Barthel, A. (2003). Akt modulates STAT3-mediated gene expression through a FKHR (FOXO1a)-dependent mechanism. *J Biol Chem* **278**, 5242–5249.

LaMarre, J., Wollenberg, G. K., Gonias, S. L. & Hayes, M. A. (1991). Biology of disease. Cytokine binding and clearance properties of proteinase-activated α2-macroglobulins. *Lab Invest* **65**, 3–14.

Lee, Y. H. & Yun, Y. (1998). HBx protein of hepatitis B virus activates Jak1-STAT signaling. *J Biol Chem* **273**, 25510–25515.

Lee, D. K., Park, S. H., Yi, Y. & 8 other authors (2001). The hepatitis B virus encoded oncoprotein pX amplifies TGF-β family signaling through direct interaction with Smad4: potential mechanism of hepatitis B virus-induced liver fibrosis. *Genes Dev* **15**, 455–466.

Lian, Z., Pan, J., Liu, J., Zhang, S., Zhu, M., Arbuthnot, P., Kew, M. & Feitelson, M. A. (1999). The translation initiation factor, hu-Sui1 may be a target of hepatitis B X antigen in hepatocarcinogenesis. *Oncogene* **18**, 1677–1687.

Lian, Z., Liu, J., Pan, J., Tufan, N. L. S., Zhu, M., Arbuthnot, P., Kew, M., Clayton, M. M. & Feitelson, M. A. (2001). A cellular gene up-regulated by hepatitis B virus encoded X antigen promotes hepatocellular growth and survival. *Hepatology* **34**, 146–157.

McDoniels-Silvers, A. L., Nimri, C. F., Stoner, G. D., Lubet, R. A. & You, M. (2002). Differential gene expression in human lung adenocarcinomas and squamous cell carcinomas. *Clin Cancer Res* **8**, 1127–1138.

Nagy, P., Schaff, Z. & Lapis, K. (1991). Immunohistochemical detection of transforming growth factor-β_1 in fibrotic liver disease. *Hepatology* **14**, 269–273.

Natoli, G., Avantaggiati, M. L., Chirillo, P., DeMarzio, E., Collepardo, D., Falco, M., Balsano, C. & Levrero, M. (1995a).

Modulation of intracellular signal transduction pathways by the hepatitis B virus transactivator pX. *J Hepatol* **22**, 14–20.

Natoli, G., Ianni, A., Costanzo, A., De Petrillo, G., Ilari, I., Chirillo, P., Balsono, C. & Levrero, M. (1995b). Resistance to Fas-mediated apoptosis in human hepatoma cells. *Oncogene* **11**, 1157–1164.

Oshikawa, O., Tamura, S., Kawata, S. & 7 other authors (1996). The effect of hepatitis B virus X gene expression on response to growth inhibition by transforming growth factor-β1. *Biochem Biophys Res Commun* **222**, 770–773.

Pan, J., Duan, L. X., Sun, B. S. & Feitelson, M. A. (2001). Hepatitis B virus X protein protects against anti-Fas-mediated apoptosis in human liver cells by inducing NF-κB. *J Gen Virol* **82**, 171–182.

Paradis, V., Bieche, I., Dargere, D. & 7 other authors (2003). Molecular profiling on hepatocellular carcinomas (HCC) using a large-scale real-time RT-PCR approach. Determination of a molecular diagnostic index. *Am J Pathol* **163**, 733–741.

Rieder, H., Armbrust, T., Meyer-zum-Buschenfelde, K. H. & Ramadori, G. (1993). Contribution of sinusoidal endothelial liver cells to liver fibrosis: expression of transforming growth factor beta 1 receptors and modulation of plasmin-generating enzymes by transforming growth factor beta 1. *Hepatology* **18**, 937–944.

Sanderson, N., Factor, V., Nagy, P., Kopp, J., Kondaiah, P., Wakefield, L., Roberts, A. B., Sporn, M. B. & Thorgeirsson, S. S. (1995). Hepatic expression of mature transforming growth factor β1 in transgenic mice results in multiple tissue lesions. *Proc Natl Acad Sci U S A* **95**, 2572–2576.

Schuftan, G. G. & Bachem, M. G. (1999). α2-Macroglobulin reduces paracrine- and autocrine-stimulated matrix synthesis of cultured rat hepatic stellate cells. *Eur J Clin Invest* **29**, 519–528.

Shih, W. L., Kuo, M. L., Chuang, S. E., Cheng, A. L. & Doong, S. L. (2000). Hepatitis B virus X protein inhibits transforming growth factor-β-induced apoptosis through the activation of phosphatidylinositol 3-kinase pathway. *J Biol Chem* **275**, 25858–25864.

Song, B. C., Chung, Y. H., Kim, J. A. & 7 other authors (2002). Transforming growth factor-β1 as a useful serologic marker of small hepatocellular carcinoma. *Cancer* **94**, 175–180.

Sottrup-Jensen, L. (1989). Alpha-2 macroglobulins: structure, shape and mechanism of proteinase complex formation. *J Biol Chem* **264**, 11539–11542.

Su, F. & Schneider, R. J. (1996). Hepatitis B virus HBx protein activates transcription factor NF-κB by acting on multiple cytoplasmic inhibitors of *rel*-related proteins. *J Virol* **70**, 4558–4566.

Teicher, B. A. (2001). Malignant cells, directors of the malignant process: role of transforming growth factor-beta. *Cancer Metastasis Res* **20**, 133–143.

Tiggelman, A. M., Linthorst, C., Boers, W., Brand, H. S. & Chamuleau, R. A. (1997). Transforming growth factor-β-induced collagen synthesis by human liver myofibroblasts is inhibited by α2-macroglobulin. *J Hepatol* **26**, 1220–1228.

Twu, J. S., Rosen, C. A., Haseltine, W. A. & Robinson, W. S. (1989). Identification of a region within the human immunodeficiency virus type 1 long terminal repeat that is essential for transactivation by the hepatitis B virus gene X. *J Virol* **63**, 2857–2860.

Ueda, H., Ullrich, S. J., Gangemi, J. D., Kappel, C. A., Ngo, L., Feitelson, M. A. & Jay, G. (1995). Functional inactivation but not structural mutation of p53 causes liver cancer. *Nat Genet* **9**, 41–47.

Umans, L., Serneels, L., Overbaugh, L., Lorent, K., Van Leuven, F. & Van den Berghe, H. (1995). Targeted inactivation of the mouse α2-macroglobulin gene. *J Biol Chem* **270**, 19778–19785.

Wada, I., Kanada, H., Nomura, K., Kato, Y., Machinami, R. & Kitagawa, T. (1999). Failure to detect genetic alteration of the mannose-6-phosphate/insulin-like growth factor 2 receptor. *Hepatology* **29**, 1718–1721.

Webb, D. J., Wen, J., Lysiak, J. J., Umans, L., Van Leuver, F. & Gonias, S. L. (1996). Murine α-macroglobulins demonstrate divergent activities as neutralizers of transforming growth factor β and as inducers of nitric oxide synthesis. *J Biol Chem* **271**, 24982–24988.

Webb, D. J., Wen, J., Karns, L. R., Kurilla, M. G. & Gonias, S. L. (1998). Localization of binding site for transforming growth factor-β in human α2-macroglobulin to a 20-KDa peptide that also contains the bait region. *J Biol Chem* **273**, 13339–13346.

Williams, S. E., Kounnas, M. Z., Argraves, K. M., Argraves, W. S. & Strickland, D. K. (1994). The α2-macroglobulin receptor/low density lipoprotein receptor protein and the receptor-associated protein: an overview. *Ann N Y Acad Sci* **737**, 1–13.

Yamada, T., De Souza, A. T., Finkelstein, S. & Jirtle, R. L. (1997). Loss of the gene encoding mannose 6-phosphate/insulin-like growth factor II receptor is an early event in liver carcinogenesis. *Proc Natl Acad Sci U S A* **94**, 10351–10355.

Yoo, Y. D., Ueda, H., Park, K., Flanders, K. C., Lee, Y. I., Jay, G. & Kim, S. J. (1996). Regulation of transforming growth factor-β1 expression by the hepatitis B virus (HBV) X transactivator. *J Clin Invest* **97**, 388–395.